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INHIBITION OF *IN VITRO* RUMEN METHANE PRODUCTION BY THREE STATINS*

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Abstract

The aim of this study was to evaluate the effects of increasing concentrations of three pure statins on *in vitro* methane production and rumen fermentation. The effects of atorvastatin, rosuvastatin and simvastatin at three concentrations (1, 10, and 100 mg/L of culture fluid) were evaluated using *in vitro* 24 h batch incubation of buffered rumen fluid with a 70:30 forage:concentrate substrate. All statins tested demonstrated the ability to reduce methanogenesis. Methane inhibition potential was decreasing in the following order: simvastatin>atorvastatin>rosuvastatin. Methane production was reduced (P<0.05) by simvastatin at 10 mg/L (by 9.3%) and by atorvastatin at 100 mg/L (by 13.2%) without compromising fermentation and feed digestibility. Simvastatin at 100 mg/L decreased methane production by 26.2%, however, net production of volatile fatty acids (nVFA) was also reduced (P<0.05). The only effect of rosuvastatin was a slight reduction (P<0.05) of methane proportion at 10 and 100 mg/L. Simvastatin and atorvastatin at 100 mg/L increased (P<0.05) relative proportion of propionate at the expense of acetate and butyrate. Ammonia-N concentrations were not affected (P>0.05) by statins. The current study demonstrated that selected statins could selectively decrease methane production. The effects of statins on methanogenesis and overall rumen fermentation vary depending on statin type and concentration. Hydrophobic statins, such as simvastatin and atorvastatin, seem to be more effective compared to the hydrophilic statins, such as rosuvastatin.

Key words: rumen fermentation, methane, digestibility, statin, *in vitro*

Ruminants produce a considerable amount of methane (e.g., each cow can produce 150–420 L of methane per day). This ruminant-derived methane contributes to

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greenhouse gas production, also accounting for a loss of dietary energy, which might otherwise be available for animal maintenance or food production (Janssen, 2010). Methane is a natural by-product of microbial fermentation of consumed feed in the rumen. More specifically, methanogenic archaea (methanogens) convert hydrogen, formed by bacteria, fungi, and protozoa to methane (Henderson et al., 2018). Currently, there is a great effort to decrease rumen methanogenesis without affecting fermentation and digestion of feeds. In this regard, low levels of statins showed promising results as specific inhibitors of methanogens and methanogenesis (Miller and Wolin, 2001).

Statins are critical lipid-lowering agents of fungal origin commonly used to decrease cholesterolemia in humans. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the cholesterol production pathway in humans (Sirtori, 2014). Unlike bacteria, archaea use an HMG-CoA reductase in their specific way of membrane lipid synthesis. Therefore, it is generally believed that statins can selectively inhibit rumen methanogens through inhibition of HMG-CoA reductase (Wolin and Miller, 2006; Gottlieb et al., 2016). In recent years, several *in vitro* and *in vivo* studies have examined the potential of statins to reduce rumen methane production. In these studies, only two statins, lovastatin and mevastatin, were evaluated, but results were inconclusive (Patra et al., 2017; Henderson et al., 2018). Data for other statins is completely lacking. Furthermore, in previous studies, the concentrations of statins that decreased methane production without negative effects on rumen fermentation spanned a wide range (Soliva et al., 2011; Morgavi et al., 2013).

This study aimed to investigate the effects of increasing concentrations (1, 10, and 100 mg/L) of three statins (atorvastatin, rosuvastatin, and simvastatin) on methane production and in general on rumen fermentation using *in vitro* 24 h batch incubation of buffered rumen fluid. We hypothesized that selected statins at low levels would inhibit rumen methane production without adverse effects on VFA production and feed digestibility.

Material and methods

Procedures with animals were conducted in accordance with the Czech legislation (Act No 246/1992 Coll., on the protection of animals against cruelty) and applicable European guidelines and regulations (Directive 2010/63/EU, on the protection of animals used for scientific purposes) for experimentation with animals.

Experimental design and treatments

Effects of increasing concentrations (1, 10, and 100 mg/L of culture fluid) of atorvastatin (atorvastatin calcium, purity ≥98%; Sigma-Aldrich, Prague, Czech Republic), rosuvastatin (rosuvastatin calcium, ≥98%; Sigma-Aldrich, Prague, Czech Republic), and simvastatin (≥97%, Sigma-Aldrich, Prague, Czech Republic) on rumen methane production were evaluated using *in vitro* 24 h batch incubation of

buffered rumen fluid. Statins were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Prague, Czech Republic) to generate a stock solution of 10 mg/mL. Just prior to incubation, individual solutions were prepared by diluting the stock solutions so that the addition of 200 µL gave the desired concentration in the culture fluid. Each concentration of statins was tested in triplicate (analytical replicates) within the incubation, and incubations were repeated on three separate days (runs). Three blanks (no substrate, no additive) were always included with each run. The values from triplicate within incubation were averaged. These averaged values from each run were used as statistical replicates in statistical analysis.

Donor animals and rumen fluid

Fresh rumen content (solid + liquid fraction) was manually collected from various locations within the rumen of three permanently rumen-fistulated lactating Holstein cows (averaging 632 ± 47 kg body weight, 183 ± 15 days in milk, and 30 ± 2 kg of milk/d), at 3 h post-morning feeding. The donor cows were fed a total mixed ratio (TMR) *ad libitum* (offered fresh two times per day) composed of (DM basis) alfalfa silage (284 g/kg), corn silage (252 g/kg), concentrate mixture (358 g/kg), wet brewers grain (95 g/kg), and wheat straw (11 g/kg).

Rumen content from each cow was placed into a pre-warmed 500 mL CO_2-flushed insulated flask. The flasks were completely filled, leaving no headspace and were closed with a screw cap with a valve in open position. The open valve should prevent the accumulation of CO_2 in the flask with subsequent decrease of pH of rumen content and impede potential shifts in microbial community composition. Immediately after sampling, the insulated flasks with rumen content were placed into CO_2-flushed cooler (Icetime® 26 L, Campingaz, France) with ambient temperature (approximately 23°C) and transported to the laboratory. Rumen fluid was obtained by straining rumen content through a sieve with a pore size of 250 µm under continuous CO_2 flushing. Then, the rumen fluid was pooled in equal proportions and used as a source of inoculum. The time required from rumen content collection to inoculation of bottles was less than 45 min.

***In vitro* incubation**

The *in vitro* batch incubations were conducted as described by Joch et al. (2019) with modifications. These modifications (reducing the amount of substrate and volume of culture fluid) were made to ensure headspace pressure in bottles to be not more than 483 hPa as recommended by Theodorou et al. (1994). On the day before incubation, the dried milled (to pass through 1 mm screen) substrate (200 mg) was weighed into a 120 mL capacity gastight CO_2-flushed serum bottles. The experimental substrate consisted (DM basis) of alfalfa silage (350 g/kg), corn silage (350 g/kg), and concentrate mixture (300 g/kg). These three ingredients were the same as fed to donor cows. The chemical composition (per kg DM) of the substrate was 930 g organic matter, 157 g crude protein, 19 g ether extract, 465 g NDF, and 293 g ADF.

The culture fluid was prepared by mixing (at a ratio 1:4, v/v) the rumen fluid with a phosphate buffer (per liter of distilled water: 28.8 g $Na_2HPO_4 \cdot 12H_2O$; 6.9 g $NaH_2PO_4 \cdot 2H_2O$; 0.7 g NH_4Cl; flushed with CO_2 and adjusted to pH 6.9 ± 0.1 with

5M NaOH). The culture fluid was immediately gassed with CO_2 while maintained at 39°C for 10 min before starting to fill up the bottles. A sample (1 mL) of culture fluid was acidified with 20 µl of 9M H_2SO_4 and stored at −18°C until volatile fatty acids (VFA) analysis. Before adding the culture fluid, statin dissolved in DMSO (200 µL) was pipetted to each bottle. The equivalent amount of DMSO was pipetted to the respective blank and control bottles. The final concentration of DMSO in the culture fluid was always less than 140 mmol/L (<1%, v/v). Then, 20 mL of the culture fluid was dispensed using bottle top dispenser (Calibrex 530 salutae, Socorex, Switzerland) into the serum bottles. The headspace of each bottle was flushed with CO_2. Finally, the bottles were sealed with butyl rubber stoppers and aluminum crimp caps, and placed in a water bath (SW 22, Julabo, Germany) at 39°C for 24 h with shaking (frequency 90 rpm).

Sampling and analysis

To determine total gas production (TGP), the headspace pressure of accumulated gas in the bottles was measured using a manometer (Traceable, Fisher Scientific, Pittsburgh, USA) after 24 h of incubation. This was achieved by inserting a 23 gauge (0.6 mm) needle attached to the manometer through the stopper. Then, 5 mL of fermentation gas was transferred to a tube filled with distilled water by displacement. Methane concentration in fermentation gas was determined by gas chromatography (Joch et al., 2019), calibration and calculations were performed as described previously (López and Newbold, 2007).

After gas sampling, fermentation was terminated by cooling bottles in an ice-water bath. The samples (1 mL; acidified with 20 µL of 9M H_2SO_4 and stored at −18°C) of culture fluid were analyzed using a gas chromatograph (Joch et al., 2019) to determine VFA concentrations. Concentrations of VFA are reported as net amounts produced (nVFA) and were calculated as the difference between concentrations in the fluid sample after 24 h of incubation and initial concentrations. Ammonia-N concentrations were assayed by the phenol-hypochlorite method with NH_4Cl as a standard (Weatherburn, 1967).

The apparent dry matter disappearance (aDMd) was determined gravimetrically. The entire content of each fermentation bottle (except the 1 mL sample used for VFA and ammonia-N analysis) was transferred into a pre-weighed 50 mL centrifuge tube, rinsed, and centrifuged (507 × g, 10 min, 4°C, two times). Then, the supernatant was removed, and the pellet was dried for 48 h at 50°C. The difference between the weight of the incubated substrate and the dry weight of the fermentation residue normalized to the residue weight in the blank was regarded as weight of disappeared dry matter.

Statistical analysis

To compare the effect of statins with the control, the data were subjected to ANOVA using the PROC MIXED procedure in SAS (SAS Enterprise Guide 6.1, SAS Institute Inc., Cary, NC, USA) according to the model:

$$Y_{ij} = \mu + T_i + R_j + e_{ij}$$

Where Y_{ij} is the dependent variable, μ is the overall mean, T_i is the fixed effect of treatment (i = 10 levels, 3 statins × 3 concentrations + control), R_j is the random effect of run (j = 1, 2 and 3), and e_{ij} is the residual error. Treatment means were compared with the control using the Dunnett's post-hoc test; differences between each treatment and the control were declared significant at $P<0.05$.

To test for statin by concentration interactions and to test for linear and quadratic effects of statin concentrations, a second analysis was performed after removing the control treatment (as only one control for all treatment was used). The data were analyzed using the PROC MIXED according to the model:

$$Y_{ijk} = \mu + T_i + C_k + (T \times C)_{ik} + R_j + e_{ijk}$$

Where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the fixed effect of treatment (i = 3 statins), C_k is the fixed effect of concentration (k = 3 concentrations), $(T \times C)_{ik}$ is the fixed effect of interaction between ith treatment and kth concentration, R_j is the random effect of run (j = 1, 2 and 3), and e_{ijk} is the residual error. Orthogonal polynomials were fitted to test for linear and quadratic effects of increasing concentrations of statins using CONTRAST option (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). The IML procedure (SAS version 9.4) was used to correct the contrast coefficients of orthogonal polynomial because of unequal intervals of concentrations.

Results

Methane production

All statins tested demonstrated the ability to decrease at least one of the methane production parameters, i.e. methane per dry matter incubated (DMi), TGP, aDMd, or VFA (Table 1). The magnitude of inhibition of methane production was type- and concentration-dependent. The most pronounced inhibition was noted for simvastatin. At the two highest concentrations, 10 and 100 mg/L, it decreased ($P<0.05$) methane production (mL/g DMi) by 9.3% and 26.2%, respectively, compared to the control. Atorvastatin (100 mg/L) decreased by 13.2% ($P<0.05$) and rosuvastatin did not affect methane production expressed per g DMi. The relative proportion of methane (mmol/mol TGP) was decreased ($P<0.05$) by two highest concentrations (10 and 100 mg/L) of simvastatin and rosuvastatin and the highest concentration of atorvastatin (100 mg/L). Methane production per aDMd (mL/g aDMd) and per nVFA (mol/mol nVFA) was reduced ($P<0.05$) compared to the control only by the highest concentration of simvastatin (100 mg/L).

Atorvastatin with increasing concentrations linearly decreased ($P<0.05$) methane production expressed per DMi, TGP and nVFA. Similarly, simvastatin linearly decreased ($P<0.05$) methane production per TGP and aDMd, and quadratically affected ($P<0.05$) methane production per DMi and nVFA.

Table 1. Effects of statins on *in vitro* gas and methane production, apparent dry matter disappearance (aDMd), ammonia-N concentration, and pH

Treatment Concentration (mg/L)	TGP[1] (mL/g DMi[3])	Methane				aDMd[2] (g/g)	Ammonia-N (mg/100 mL)	pH
		(mL/g DMi)	(mmol/mol TGP)	(mL/g aDMd)	(mol/mol nVFA[4])			
Control								
0	224.0	30.2	134.8	53.2	0.192	0.580	35.5	6.39
Atorvastatin								
1	220.4	28.2	128.0	50.0	0.186	0.566	35.3	6.41
10	226.7	29.2	129.0	52.6	0.188	0.559	36.7	6.40
100	215.7	26.2*	121.8*	48.8	0.175	0.539	36.5	6.41
contrast	Q	L	L	NS	L	NS	Q	NS
Rosuvastatin								
1	222.3	28.5	128.1	48.6	0.192	0.591	36.5	6.40
10	221.5	27.6	124.5*	48.2	0.185	0.579	36.8	6.39
100	223.8	28.0	125.1*	53.3	0.187	0.532	37.2	6.39
contrast	NS	NS	NS	NS	NS	L	NS	NS
Simvastatin								
1	228.3	30.4	133.0	56.7	0.211	0.540	37.7	6.38
10	218.5	27.4*	125.4*	49.4	0.183	0.561	37.4	6.40
100	198.6*	22.3*	112.1*	41.8*	0.165*	0.536	35.5	6.41
contrast	L	Q	L	L	Q	NS	NS	NS
SEM	1.47	0.33	1.04	0.84	0.0021	0.0053	0.35	0.004
P-value								
statin	0.003	0.036	0.207	0.814	0.567	0.242	0.443	0.039
concentration	<0.001	<0.001	<0.001	0.134	<0.001	0.026	0.590	0.650
statin × concentration	<0.001	<0.001	<0.001	<0.001	0.002	0.495	0.151	0.126

[1]Total gas production.
[2]Apparent dry matter disappearance.
[3]Dry matter incubated.
[4]Net production of volatile fatty acids.
*Means within a column differ significantly ($P<0.05$) from corresponding control (0 mg/L). SEM, standard error of the mean.
L, linear contrast ($P<0.05$); Q, quadratic contrast ($P<0.05$); NS, not significant.

Table 2. Effects of statins on *in vitro* volatile fatty acids production and proportion

Treatment Concentration (mg/L)	nVFA[1] (mmol/L)	Molar proportion of VFA (mol/100 mol)						A:P[2]
		acetate	propionate	butyrate	*iso*-butyrate	valerate	*iso*-valerate	
Control								
0	64.8	56.7	23.3	13.5	1.45	2.14	2.90	2.4
Atorvastatin								
1	62.5	57.1	23.3	13.2	1.37	2.22	2.82	2.5
10	63.8	56.6	23.3	13.3	1.61	2.27	2.87	2.4
100	61.6	55.9	24.9*	12.7*	1.33	2.27	2.87	2.2*
contrast	NS	L	L	L	NS	NS	NS	L
Rosuvastatin								
1	61.0	56.7	23.5	13.3	1.41	2.21	2.87	2.4
10	61.5	56.1	23.8	13.5	1.47	2.24	2.84	2.4
100	62.1	56.4	24.0	13.3	1.26	2.27	2.79	2.3
contrast	NS	NS	NS	NS	NS	NS	NS	NS
Simvastatin								
1	60.7	56.0	23.6	13.8	1.51	2.28	2.93	2.4
10	61.6	56.1	23.8	13.5	1.61	2.25	2.84	2.4
100	55.6*	54.2*	27.0*	12.1*	1.50	2.33	2.74	2.0*
contrast	L	L	L	L	NS	NS	L	L
SEM	0.56	0.15	0.14	0.09	0.043	0.102	0.028	0.02
P-value								
statin	0.011	<0.001	<0.001	0.055	0.245	0.388	0.849	<0.001
concentration	0.069	<0.001	<0.001	<0.001	0.112	0.256	0.115	<0.001
statin × concentration	0.109	0.007	<0.001	<0.001	0.899	0.854	0.099	<0.001

[1]Net production of volatile fatty acids.
[2]Acetate:propionate.
*Means within a column differ significantly (P<0.05) from corresponding control (0 mg/L). SEM, standard error of the mean.
L, linear contrast (P<0.05); Q, quadratic contrast (P<0.05); NS, not significant.

Total gas production, dry matter disappearance, pH, and ammonia-N concentrations

Total gas production was reduced ($P<0.05$) by simvastatin at 100 mg/L when compared to the control (Table 1). The linear and quadratic effects on TGP were noted ($P<0.05$) for simvastatin and atorvastatin, respectively. Neither aDMd nor pH and ammonia-N concentrations were affected by the addition of statins when compared to the control. Ammonia-N concentrations were quadratically affected ($P<0.05$) by atorvastatin, and aDMd was linearly decreased ($P<0.05$) by rosuvastatin.

Volatile fatty acid concentrations and proportions

Inclusion of simvastatin (100 mg/L) reduced nVFA by 14.2% when compared to the control (Table 2). The molar proportion of acetate was also reduced by the simvastatin at 100 mg/L. Conversely, neither atorvastatin nor rosuvastatin affected ($P>0.05$) nVFA or the molar proportion of acetate. The molar proportion of propionate increased ($P<0.05$), and the molar proportion of butyrate decreased ($P<0.05$) with the inclusion of the highest concentration (100 mg/L) of simvastatin and atorvastatin. The molar proportions of *iso*-butyrate, valerate and *iso*-valerate were not affected ($P>0.05$) by the supplementation of statins. The highest concentration (100 mg/L) of simvastatin and atorvastatin lowered ($P<0.05$) the acetate-to-propionate ratio.

Atorvastatin and simvastatin with increasing concentrations linearly decreased ($P<0.05$) the molar proportions of acetate and butyrate, and the acetate-to-propionate ratio. In addition, simvastatin linearly ($P<0.05$) decreased nVFA and the molar proportion of iso-valerate. Conversely, with increasing concentrations atorvastatin and simvastatin linearly increased ($P<0.05$) the molar proportion of propionate.

Discussion

In the presented study, the three most popular (Lippi et al., 2019) and most widely prescribed statins in human medicine (Wang et al., 2019) were evaluated for their effects on *in vitro* fermentation with a mixed culture of ruminal microorganisms. In particular, this work aims to determine: (i) potential of pure atorvastatin, rosuvastatin, and simvastatin at three concentrations to selectively inhibit methane production and (ii) overall effects of these statins on rumen fermentation efficiency, especially on VFA production, feed digestibility, and N metabolism.

The significant interactions between statins and concentrations in our study suggest that the effect of statins on rumen methanogenesis as well as fermentation efficiency is type- and concentration-dependent. Inhibition magnitude (mL methane/g DMi) was decreasing in the following order: simvastatin>atorvastatin>rosuvastatin. The minimum concentration that decreased the methane production under our conditions was 10 mg/L for simvastatin. Similarly, Morgavi et al. (2013) showed that as little as 5 mg/L of monacolin K (naturally produced lovastatin) can reduce rumen methane *in vitro*. The lower effective concentration of monacolin K may be a result of higher bioavailability of naturally produced statins compared to purified

statins (Xiong et al., 2019). In our study, simvastatin (10 mg/L) and atorvastatin (100 mg/L) reduced methane production without affecting TGP, nVFA production, and aDMd, which suggests their selective inhibitory potential on methane production. These findings are in agreement with the earliest reported selective inhibition of methanogens using lovastatin and mevastatin (Miller and Wolin, 2001). Previous studies showed that effective *in vitro* concentration of statins varies considerably. For example, lovastatin can reduce methane without negative effects on rumen fermentation in concentrations of 5–150 mg/L (Soliva et al., 2011; Morgavi et al., 2013). In our study, the maximal methane reduction was observed for simvastatin (100 mg/L). However, this concentration also decreased significantly TGP and nVFA and numerically aDMd, suggesting that reduced methane was partly due to inhibition of overall fermentation. Rosuvastatin slightly reduced the relative proportion of methane in fermentation gas. The reduction was similar at 10 and 100 mg/L, which indicates no further anti-methanogenic potential. Different statin efficiency reported herein could be related to the differences in mode of action.

Although simvastatin and atorvastatin significantly reduced methane production in our study, taken together, the effects of three selected statins on methanogenesis seem to be limited and less promising compared to originally reported effects of lovastatin and mevastatin (Miller and Wolin, 2001). Besides the inherent potential of individual statins, a limited effect on methanogenesis and rumen fermentation reported here might be explained by at least two factors, a form of supplemented statins and technique used for evaluation. First, statins exist in two forms, lactone and hydroxy acid, which can be converted one to the other (Yang and Hwang, 2006). It has been shown that methanogenesis was preferentially inhibited by more hydrophobic lactone form compared to hydroxy acid form (Gottlieb et al., 2016). In keeping with this, the most effective statin in our study, simvastatin, was supplemented as lactone whereas atorvastatin and rosuvastatin as the calcium salt of the hydroxy acid. Second, the efficacy of statins in our study could be compromised due to the use of mixed ruminal culture. It is possible that statins were degraded or metabolized to a less active form by some members of rumen microbiota (Aura et al., 2011). For example, at least part of the simvastatin lactone (semisynthetic analog of lovastatin) might be converted to hydroxy acid form by anaerobic bacteria (Nkamga et al., 2017). This may be an explanation for the higher potential of statins to inhibit methanogenesis in pure cultures of methanogens (Miller and Wolin, 2001). Further research is warranted to evaluate the effects of lactone and hydroxy acid forms of statins on the pure culture of methanogens and to determine the ability of rumen microbiota to metabolize statins.

The mode of anti-methanogenic action may be related to several mechanisms. Statins may directly suppress methane production through inhibiting cell membrane biosynthesis of methanogens, leaving bacteria unaffected (Miller and Wolin, 2001). Methane production may also be selectively inhibited by a decrease of F420-dependent NADP oxidoreductase enzyme activity that catalyzes a critical electron transfer step in the methanogenesis (Sharma et al., 2011). Alternatively, statins may specifically inhibit bacteria that produce hydrogen. Lowered hydrogen availability can limit methane production as hydrogen is the primary substrate for methanogenesis in the

rumen (Liu and Whitman, 2008). Decreased methane production without inhibition of nVFA production in our study indicates that lower concentrations of statins, such as simvastatin (10 mg/L), selectively inhibit methanogens. By contrast, inhibition of nVFA production at higher concentrations (100 mg/L) suggests that bacteria were also suppressed. This data is supported by previous results that reported minimal inhibitory concentrations of simvastatin against bacteria about 26 µg/mL (26 mg/L) (Masadeh et al., 2012).

The reduction of nVFA with simvastatin (100 mg/L) suggested that this concentration is too high for the positive modulation of rumen fermentation. Soliva et al. (2011), by contrast, found that 150 mg/L of lovastatin did not affect total VFA concentration in *in vitro* rumen. Likely, this discrepancy reflects the different antibacterial potential of particular statins, with simvastatin being one of the most potent (Bergman et al., 2011; Masadeh et al., 2012). Because VFA are the principal source of energy for ruminants, lowered VFA production *in vivo* would impair ruminant performance.

The profile of VFA was significantly affected by the highest concentration (100 mg/L) of simvastatin and atorvastatin. These two statins increased the proportion of propionate at the expense of acetate and butyrate. Our results are consistent with the higher proportion of propionate with supplementation of naturally produced lovastatin reported *in vitro* (Morgavi et al., 2013) and *in vivo* (Candyrine et al., 2018). However, generally, reported effects of lovastatin on the proportion of VFA are inconsistent (Soliva et al., 2011; Faseleh Jahromi et al., 2013; Ramírez-Restrepo et al., 2014). The effects of simvastatin and atorvastatin on VFA proportions in our study may be explained by increased H_2 partial pressure because of reduced methanogenesis. Then, the higher H_2 pressure might modify the fermentation pathway toward more propionate production as an alternative H_2 sink (Janssen, 2010). Alternatively, simvastatin and atorvastatin might selectively inhibit acetate and/or butyrate-producing bacteria. This hypothesis is supported by stronger effects of hydrophobic statins, simvastatin, and atorvastatin, on VFA production and proportion, compared to hydrophilic rosuvastatin. Bergman et al. (2011) suggested that hydrophobic statins could perturb bacterial membranes resulting in bacterial death.

Levels of ammonia N remained unaffected, which indicates the low ability of three tested statins to affect N metabolism in the rumen. This finding was further supported by unchanged proportions of branched-chain VFA (*iso*-butyrate and *iso*-valerate) as these VFA are primarily products of deamination of amino acids (Patra and Yu, 2014).

The eventual use of pure statins as anti-methanogenic feed additives in ruminants nutrition would be primarily limited by the high cost of statins as suggested by our results with effective concentrations of simvastatin and atorvastatin at 10 mg/L and 100 mg/L, respectively. However, extrapolation of *in vitr*o results to *in vivo* conditions may be unrepresentative and sometimes controversial. Thus, further research is required to determine effective *in vivo* concentrations of these hydrophobic statins. Beside of the ruminant nutrition sector, our results may help to reveal the therapeutic importance of statins in human constipation-related disorders, because methane

was suggested as a gasotransmitter with inhibitory activity on the colonic and ileal smooth muscle (Gottlieb et al., 2016).

Conclusion

In summary, this *in vitro* study showed that selected statins could selectively decrease methane production. The effects of statins vary depending on statin type and concentration. Simvastatin and atorvastatin at 10 and 100 mg/L, respectively, reduced methane production without adverse effects on fermentation and feed digestion. Therefore, these hydrophobic statins may offer the opportunity to modify rumen fermentation in a beneficial way.

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