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ORIGINAL ARTICLE

Effects of fumaric acid on rumen fermentation, milk composition and metabolic parameters in lactating cows

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Summary

The aim of this study was to determine the influence of fumaric acid (FA) on ruminal fermentation and its effects on the acid-base balance of seven ruminally and duodenally fistulated multiparous German Holstein cows. The experiment was conducted in a change-over design with three periods in which the animals were randomly arranged in one of three treatments: Control (C; without FA), 300 or 600 g FA per day. The diets consisted of 7.4 kg DM grass silage, 4.2 kg concentrate mixture and 0, 300 or 600 g FA or wheat starch as isocaloric compensation per day and cow. FA supplementation decreased the rumen pH, acetic acid and butyric acid and increased propionic acid in rumen fluid. The results of the single-strand conformation polymorphism analysis (SSCP) did not show an influence of FA on the microbial population in the rumen. The beta-hydroxybutyrate (BHB) concentration in blood and the pH of the urine decreased, while the blood gases were unaffected by supplementation of the acid. The microbial protein per MJ ME decreased in the duodenum with FA supplementation. The milk fat concentration decreased after addition of FA. We conclude that in this study feeding of up to 600 g FA per day did not result in an acidosis. It seems that up to 600 g FA per day did not have a significant influence on the acid-base balance of dairy cows.

Keywords fumaric acid, rumen fermentation, lactating cows, grass silage, digestibility acid-base balance

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Introduction

In the past, the propionic acid precursor fumaric acid (FA) has often been discussed as a potential methane (CH_4) reducer (e.g. Bayaru et al., 2001). A FA-mediated reduction of ruminal CH_4 production would have an influence on energy available for animal maintenance and productivity because CH_4 itself implies a gross energy loss of 6–8% for the animal (Flachowsky and Brade, 2007) due to its inherent energy content. However, there are only few studies addressing the effect of this organic acid on dairy cows and ruminal fermentation. Moreover, for animal health and productivity, it is important to estimate the influence of feed additives on the whole organism and not only regarding the reduction of CH_4. Because of its acidity [pk_a = 3.03; (Engel et al., 2008)], especially the effect of FA on ruminal fermentation and the acid-base balance has to be considered. It was presumed that supplementation of FA results in a ruminal acidosis (Wood et al., 2009) with ultimate consequences for ruminal microbial communities and nutrient fermentation. Hence, the aim of the study was to investigate the consequences for the animals if they were fed with up to 600 g FA. Literature research showed that with this FA concentration, reactions and effects of the animals could be expected.

Therefore, besides effects of FA on ruminal fermentation and microbial communities possible acidotic effects were studied in this experiment. As *in vitro* experiments suggested that FA effects strongly depend on its dose, we developed a dose–response design for the present. Moreover, in the view that high fibre diets are associated with higher levels of CH_4 production we used exclusively grass silage as roughage to induce ruminal conditions favourable for CH_4 production. Possible acidotic effects were studied in this experiment especially.

Materials and methods

Experimental design

Treatments and experiments were conducted according to the German regulations concerning animal welfare (Anonymous, 2006). All experiments were approved by the Regional Council of Braunschweig, Niedersachsen, Germany (File Number 33.11.42502-04-057/07). The study was conducted at the experimental station of the institute of Animal Nutrition (FLI) in Braunschweig with a total of seven pluriparous lactating German Holstein cows. The experiments comprised three periods in which the animals were fed one of the three diets with 0, 300, or 600 g FA per day and animal (Table 1) in accordance with a cross-over design. In each period, two cows per treatment were included. Another animal was allocated in the first period in treatment C, in the second period in treatment 300 and in the third period in treatment 600.

Each cow received 7.4 kg dry matter (DM) grass silage as roughage and 4.2 kg concentrate mixture per day. The concentrate was composed of 28.4% rapeseed meal, 23% barley, 23% dried sugar beet pulp, 23% maize, 0.6% Calcium-carbonate and 2% mineral and vitamin mix. FA was added to the concentrate up to 600 g per day per cow. Wheat starch was fed at the expense of FA for isocaloric compensation. The group fed wheat starch and no FA was used as control group (C).

At the beginning, mean body weight of the cows was 506 kg (SD ± 39 kg). They were lactating for 110 (SD ± 49 days) days. All animals were equipped with two cannulas: a large rubber cannula in the dorsal sac of the rumen (inner diameter 10 cm), and a t-shaped cannula at the proximal duodenum (close to the pylorus with an inner diameter of 2 cm). The cows were housed in a tethered stable with neck straps and individual troughs with free access to water and a salt block containing sodium chloride. The cows were milked at 5:00 and 16:00 hour.

The DM of the silage was analysed twice a week to ensure a constant ratio of silage to concentrate. Forage and concentrate (with FA or starch on top) were offered twice a day in two equal portions at 05:30 and 15:30 hour.

Table 1 Composition of the experimental diets of the three different treatments with increasing FA supplementation

Treatment (FA)	C*	300†	600‡
Animals per group	7	6§	7
Feed [kg/animal/day]			
Concentrate	4.2	4.2	4.2
Fumaric acid	0	0.3	0.6
Wheat starch	0.6	0.3	0
Grass silage (DM)	7.4	7.4	7.4

DM, dry matter.
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.
§One animal had to be excluded because of losing its cannula.

Sampling

Each period lasted 5 weeks: 3 weeks of adaptation to the appropriate diet and 2 weeks for sample collection. Milk yield was recorded daily during the two sampling weeks. In the first sampling week, samples of milk and ruminal fluid were taken. On the first and fourth day, 50 ml milk of successive morning and evening milking were preserved with bronopol (2-bromo-2-nitropropane-1,3-diol) and kept at 8 °C until analysis. On the third day of the first sampling week, ruminal fluid was taken from the ventral sac of the rumen through the rumen cannula using a hand-operated vacuum pump. Seven samples, each of them 100 ml, were taken per cow per period: directly before morning feeding, 30, 60, 90, 120, 180 and 360 min after feeding. A total of 100 ml of rumen fluid was taken 360 min after feeding and immediately frozen at −18 °C for single-strand conformation polymorphism analysis (SSCP) of microbial collective.

In the second sampling week, on five consecutive days, duodenal digesta were collected every two hours. At each sampling time, four samples of approximately 100 ml were taken via duodenal cannula from each cow. Immediately, pH values of each sample were measured with a glass electrode (digital pH measurement devise, pH 525; WTW, Weilheim, Germany). The sample with the lowest pH was added to the daily pool sample for each cow to prevent contaminations by endogenous secretion (Rohr et al., 1984) and stored at −18 °C. Chromium oxide (Cr_2O_3) was used as marker to calculate daily digesta flow. Cr_2O_3 was mixed with wheat flour (ratio 1:4). Portions of 50 g were distributed in the rumen via the rumen cannula every 12 h, beginning ten days before duodenal sampling week. One day before and throughout the sampling week, 25 g was given into the rumen every 6 h.

On 2 days in the week of duodenal chymus sampling, 100 ml samples of spontaneous urine were collected per cow to estimate net acid-base excretion (NABE). Feed samples were taken daily in the

duodenal chymus sampling week. Feed samples and eventually occurring feed refusals were pooled over the week for the analysis of nutrient content. Two days after duodenal chymus sampling, blood samples (serum, heparin, EDTA and blood gas tubes) were taken from *Vena jugularis externa* 3 h after feeding to monitor animal health status (Kraft and Dürr, 2005). The blood samples were analysed for red blood cell count, BHB, non-esterified fatty acids (NEFA) and glucose. Blood gas status was determined to estimate the acid-base balance of the animals (Brown et al., 2000). The blood gas tubes were stored on ice immediately. At once, rectal temperatures from cows were measured and used as an input parameter for the blood gas analytics.

Analyses Crude nutrients in dried and ground feedstuffs and in freeze-dried and ground duodenal chymus were analysed according to the methods of the VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten) (Naumann and Bassler, 1993). Amylase was applied for the analysis of NDFom in the sample of wheat starch because of its high starch content. All the other samples were analysed without amylase.

An infrared milk analyser (Milkoscan FT 6000 combined with a Fossomatic 5000, Foss Electric, Hillerød, Denmark) was used for analysing the contents of milk fat, protein, lactose and urea.

A gas chromatograph equipped with a flame ionization detector (Geissler et al., 1976) served for analysing the short-chain fatty acids (SCFA) in the ruminal fluid.

Ammonia-N (NH_3–N) in rumen fluid and freshly thawed duodenal chyme was analysed according to DIN38406-E5-2 (1998). Total N was analysed in thawed duodenal chyme samples according to Kjeldahl according to VDLUFA method No. 4.1.1 (Naumann and Bassler, 1993).

To estimate daily duodenal DM flow (DMF), Cr-concentrations in duodenal samples were determined using an inductively coupled plasma optical emission spectrometry (ICP-OES; GBC Scientific Equipment Pty Ltd., Braeside, Vic., Australia) after dissociation as described by Williams et al. (1962) .

According to (Lebzien and Paul, 1997), near infrared spectroscopy (NIRS) was applied to determine the proportion of microbial-N of non-ammonia-N (NAN) in the duodenal samples.

According to the DMF, one aliquot pooled sample of the chyme per cow per week was generated. In the pooled samples of the freeze-dried duodenal chyme, proximate nutrients were quantified applying the same methods as for the feedstuff.

Red blood cell count was determined by an automated haematology analyser (Celltac alpha MEK-6450, Nihon Kohden Corporation, Tokyo, Japan) and blood gases using rapidlab 348 system (Siemens Healthcare Diagnostics Deerfield, IL, USA).

Glucose, BHB and NEFA in blood serum were determined photometrically (Eurolyser CCA 180 VET; Greiner Diagnostik GmbH, Bahlingen, Germany).

Urine was examined immediately after sampling. The net acid-base excretion was analysed according to the titrimetic method by Kutas (1965). Concentrations of base, acid and NH_4^+ were determined with titration of HCl and after fixation of NH_4^+ with formaldehyde with titration of NaOH (Lachmann, 1981).

Single-strand conformation polymorphism analysis (SSCP) analysis

Sample preparation

Liquid-associated micro-organisms were isolated by means of differential centrifugation according to Brandt and Rohr (1981). Pellets of microbes were resuspended in ice-cold physiological saline, frozen in liquid N_2 and stored at −80 °C until extraction of genomic DNA (gDNA).

DNA extraction

Microbial cells (100 mg) were mechanical lysed by bead beating (Ribolyser Cell Disrupter, Hybaid GmbH, Heidelberg, Germany) in the presence of 1x TEN buffer [Tris(hydroxymethyl)-aminomethane-HCl (10 mM; pH 8.0), EDTA (10 mM; pH 8.0), NaCl (150 mM)] in two steps (speed 6.0, 4.5) for 40 s each. Additionally, chemical cell lysis was performed. Supernatant was treated with lysozym (100 mg/ml) and RNase A (10 mg/ml) for 30 min at 37 °C, followed by incubation with proteinase K (20 mg/ml) and 20% SDS (sodium dodecyl sulphate) for 1 h at 37 °C. An additional incubation step in 10% CTAB (cetyltrimethylammoniumbromide in 0.7 M NaCl) and 4 M NaCl was conducted at 65 °C for 10 min. Proteins were removed with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). Precipitation of gDNA was carried out using 100% isopropanol and centrifugation at 13.000 g for 30 min, followed by a washing step with 80% ethanol. DNA pellet was resuspended in TE buffer [Tris (hydroxymethyl)/aminomethane/HCl (10 mM; pH 8.0), EDTA (10 mM; pH 8.0)].

Polymerase chain reaction

The primers used for the amplification of 16S rRNA gene fragments of archaea and bacteria are given in

Table 2. Two subsequent PCR were performed with each 1 µl of gDNA or PCR product as template. First PCR served for choosing the domain to be analysed using domain-specific primers for archaea or bacteria. Total reaction volume was 25 µl with a final concentration of 1x PCR buffer; each dNTP 0.2 mmol/µl; forward and reverse primer 0.05 µmol/µl; HotStarTaq DNA polymerase, 0.025 U/µl (Qiagen, Hilden, Germany). Amplification was carried out in a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) with an initial denaturation of 15 min at 95 °C, followed by thirty cycles of denaturation at 94 °C for 60 s, annealing at 50 °C (bacteria: F27 *fw*/R1493 *rv*) or 52 °C (archaea: A109 f *fw*/A934b *rv*) for 60 s and elongation at 72 °C for 70 s. Final elongation was at 72 °C for 5 min. Diluted PCR products (25 ng/µl) served as templates for the nested PCR using Com primers amplifying thus a shorter fragment of 408 bp. Composition of the reaction mix was as described previously, but total reaction volume comprised 50 µl. Cycling conditions were as follows: initial denaturation 15 min at 95 °C, twenty-five cycles of denaturation at 94 °C for 45 s, annealing at 50 °C (bacteria: Com1 *fw*/Com2-Ph *rv*) or 56 °C (archaea: Com1 m *fw*/Com2 m-Ph *rv*) for 45 s, elongation at 72 °C for 60 s. Size of PCR products were verified on 1% agarose gel. PCR products were purified using QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Single-strand digestion

Purified PCR products were diluted to obtain 1000 ng of double-strand DNA (dsDNA) in a volume of 26 µl. Single-strand DNA (ssDNA) was achieved from dsDNA by λ-exonuclease digestion of the 5′-phosphorylated strand. Phosphorylation occurred during PCR by Com2-Ph/Com2 m-Ph primers. About 1000 ng of dsDNA was incubated with 1x λ-exonuclease buffer, 2.5 U λ-exonuclease (New England Biolabs GmbH, Frankfurt, Germany) in a total reaction volume of 40 µl at 37 °C for 45 min. ssDNA was purified by means of MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

Gel electrophoresis and staining

The principle of separating ssDNA fragments of equal size is based on the differences in secondary structure (conformation) depending on primary structure (nucleotide sequence) affecting thus migrating behaviour in a polyacrylamide gel. The polyacrylamide gel consisted of MDE®-Gel Solution (0.625%; Biozym, Hessisch Oldendorf, Germany), 1x TBE buffer (Tris base, 89 mM; boric acid, 89 mM; EDTA, 20 mM; pH 8.0), TEMED (tetramethylethylendiamine, 0.45 nmol/ml), APS (ammoniumperoxodisulphate, 0.04%). Prior to loading the gel, samples were denatured in loading buffer (95% formamide, 10 mM NaOH, 0.025% bromophenol blue) at 95 °C for 2 min and afterwards cooled on ice for 3 min. Electrophoresis was conducted at 300 V and 20 °C for 22.5 h. Silver staining of polyacrylamide gels were performed according to Dohrmann and Tebbe (2004) to visualize SSCP profiles.

Calculations and statistics

Energy content of grass silage was calculated according to the German Society of Nutrition Physiology (GfE 2001) based on the results of balance experiments with wethers conducted according to the recommendation of the GfE (1991) as described by Remling et al. (2011). For the concentrate, energy content was calculated using table values (DLG 1997). The net energy contents for lactation (NEL) of FA and wheat starch were obtained from the Swiss Feed Database (Schweizerische-Futtermitteldatenbank, 2011). The energy content of the silage was 10.8 MJ ME/kg

Table 2 Primers for the amplification of 16S rRNA gene fragments of archaea and bacteria

Target	Primer name	Position	Primer sequence (5′-3′)	References
Domain-specific PCR				
Bacteria	F27 *fw*	8–27	AGA GTT TGA TC(A/C) TGG CTC AG	Lane (1991)
	R1492 *rv*	1492–1513	TAC GG(C/T) TAC CTT GTT ACG ACT T	Weisburg et al. (1991)
Archaea	A109f *fw*	109–125	AC(G/T) GCT CAG TAA CAC GT	Grosskopf et al. (1998)
	A934b *rv*	915–934	GTG CTC CCC CGC CAA TTC CT	Stahl and Amann (1991)
Nested PCR				
Bacteria	Com1 *fw*	519–536	CAG CAG CCG CGG TAA TAC	Schwieger and Tebbe (1998)
	Com2-Ph *rv*	907–926	CCG TCA ATT CCT TTG AGT TT	
Archaea	Com1 m *fw*	519–536	CAG C(A/C)G CCG CGG TAA (C/T)AC	Boguhn et al. (2010)
	Com2 m-Ph *rv* Co	907–926	CCG CCA ATT CCT TTA AGT TT	

DM and 6.6 MJ NEL/kg DM. Calculated energy content of the concentrate was 12.7 MJ ME/kg DM and 8.2 MJ NEL/kg DM. Energy content of FA is supposed to be 13.6 MJ ME/kg DM (Mach et al., 2009) and 6.6 MJ NEL/kg DM (Schweizerische-Futtermitteldatenbank, 2011). For starch, an energy content of 13.7 MJ ME/kg DM and 8.8 MJ NEL/kg DM was assumed (Schweizerische-Futtermitteldatenbank, 2011).

Netto-acid-base excretion (NABE) was calculated according to the formula:

NABE = Base − Acid − NH_4^+ (Lachmann, 1981).

The dry matter flow (DMF) at the duodenum was calculated using the following equation:

$$\text{DMF (kgday)} = \frac{\text{chromium application [mg/d]}}{\text{duodenal chromium concentration [mg/g DM]}} / 1000.$$

To estimate the duodenal flows of organic matter and nutrients, their concentrations in chyme DM were multiplied by the DMF.

Non-ammonia-N (NAN) was calculated by subtracting the amount of NH_3–N from the total N at the duodenum. The amount of NAN was multiplied with the proportion of the microbial protein obtaining the amount of microbial protein.

The ruminal fermented organic matter (FOM) was calculated using the following equation:

$$\begin{aligned}\text{FOM [kg/d]} = {} & \text{OM intake [kg/d]} \\ & - (\text{Duodenal OM flow [kg/d]} \\ & - \text{Microbial OM [kg/d]}).\end{aligned}$$

According to Schafft (1983) microbial OM was determined by the following equation:

$$\text{Microbial OM [kg/d]} = 11.8 * \text{Microbial} - \text{N [kg/d]}.$$

The utilizable crude protein (uCP) was calculated according to Lebzien and Voigt (1999):

$$\begin{aligned}\text{uCP [g/d]} = {} & (\text{NAN flow at the duodenum [g/d]}) * 6.25 \\ & - \text{endogenosus CP [g/d]}.\end{aligned}$$

The endogenous CP was calculated according to Brandt and Rohr (1981):

Endogenous CP [g/day] = (3.6 * Duodenal DM flow [kg]) * 6.25.

The ruminally degraded CP and the undegraded feed CP (UDP) were calculated by the following equations:

$$\begin{aligned}\text{Ruminally degraded CP [g/d]} = {} & \text{CP intake [g/d]} - \text{UDP [g/d]}\end{aligned}$$

$$\begin{aligned}\text{UDP [g/d]} = {} & 6.25 * (\text{NAN at the duodenum [g]} \\ & - \text{Microbial-N[g]}) - \text{Endogenous CP [g]}.\end{aligned}$$

The fat-corrected milk (FCM) was estimated by:

FCM [kg/day] = ((Milk fat [%] * 0.15) + 0.4) * Milk yield [kg] (Gaines, 1928).

Statistical analysis was carried out using the mixed procedure of SAS (9.1.3 Service Pack 4; SAS Institute, Cary, NC, USA). Treatment and experimental period were taken as fixed effects. The individual animal effect was taken into account by using the random statement. Sampling time (0, 30, 60, 90, 120, 180 and 300 min after feeding) was added as a covariable for the rumen fluid parameters. As *post hoc* test, the Tukey's test was applied. Differences were regarded as statistically significant at $p < 0.05$ and as trends at $p < 0.1$.

For statistics of the SSCP analysis, air-dried gels were scanned (ScanMaker i800, Mikrotek, Willich, Germany), and SSCP band patterns of digitalized images were compared using the software GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analyses with dendrograms based on similarity matrices calculated using the Pearson product-moment correlation coefficient were performed. Clustering algorithm was unweighted pair group method using arithmetic averages (UPGMA). Statistical analysis was carried out using dissimilarity matrices following the method of Anderson (2001). Differences between treatments were regarded as significant at Monte Carlo $p_{MC} < 0.05$ (Anderson and Robinson, 2003).

Results

In general, significant effects of the period were found for several parameters because of the total experimental duration of 5 months. Therefore, it could not be excluded that the animals were in different physiological states and that the progression in lactation interfered in some way with the examined parameters.

In the second period, one animal of treatment 300 had to be excluded from the study because of losing its cannula. Therefore, for this treatment, only data from the remaining six cows could be evaluated.

The mean feed composition is shown in Table 3. All animals, except of one of treatment 600 in the first period (average daily refusal of 0.5 kg DM), consumed the complete diets.

Rumen parameters

Table 4 shows the LSMEANS for pH values, NH_3-N and total SCFA concentrations as well as proportions of each SCFA in the rumen fluid. The pH values in the rumen fluid decreased significantly from 6.08 to 5.93 in response to FA supplementation. The molar proportions of acetic acid and butyric acid decreased, and the molar proportion of propionic acid increased. Consequently, the ratio of acetic acid to propionic acid decreased from 4.1 to 3.2 with increasing amounts of FA supplementation. The means of valeric acid, iso-butyric acid and NH_3-N concentrations were significantly influenced by the treatment showing the highest value in treatment 300. The concentration of total SCFA in rumen fluid was not affected by FA. Experimental period had a significant influence on each rumen parameter with the exception of acetic acid. Time after feeding influenced rumen measurements significantly. An interaction of FA × time of sampling was significant for all SCFA except for iso-valeric acid and the pH values.

SSCP

Archaea

SSCP profile of archaea was very homogeneous and resulted in three dominant bands per lane. Dendrograms showed high similarity values of >80% among

Table 3 Mean dry matter, nutrient and fibre content of the silage (n = 3), concentrate (n = 2) and starch (n = 1) and calculated for the whole diets of the different treatments over the three experimental periods (Means)

Composition of feeds	Grass silage	Concentrate	Starch	C*	300†	600‡
Dry matter [g/kg]	396	882	877	580	557	535
Crude nutrients [g/kg DM]						
Crude ash	101	67	0	84	84	84
Crude protein	117	208	0	141	141	141
Ether extract	29	25	0	26	26	26
Crude fibre	286	85	0	205	205	205
Acid detergent fibre	313	108	0	230	230	230
Neutral detergent fibre	535	246	0	413	413	413
Starch	2	382	986	177	151	126

*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.

Table 4 Effects of FA supplementation on fermentation parameters in rumen fluid of dairy cows (LSMEANS ± SEM)

Treatment (FA)	C*	300†	600‡	ANOVA probabilities			
n	7	6	7	FA	Period	Time	FA × Time
pH	6.08^a ± 0.05	6.07^a ± 0.06	5.93^b ± 0.06	0.008	<0.001	<0.001	0.207
NH_3-N [mM]	7.6^b ± 0.6	8.2^a ± 0.6	7.4^b ± 0.6	<0.001	<0.001	<0.001	<0.001
Acetic acid [mol%]	65.8^a ± 0.5	64.0^b ± 0.5	63.7^b ± 0.5	0.001	0.621	<0.001	0.003
Propionic acid [mol%]	16.5^b ± 0.8	19.7^a ± 0.8	20.2^a ± 0.8	<0.001	<0.001	<0.001	0.001
Acetic/propionic	4.1^a ± 0.2	3.3^b ± 0.2	3.2^b ± 0.2	<0.001	<0.001	<0.001	<0.001
iso-Butyric acid [mol%]	0.7^b ± 0.0	0.8^a ± 0.0	0.7^b ± 0.0	<0.001	<0.001	0.001	<0.001
Butyric acid [mol%]	14.4^a ± 0.6	12.9^b ± 0.6	12.9^b ± 0.6	<0.001	<0.001	<0.001	0.006
iso-Valeric acid [mol%]	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.172	<0.001	<0.001	0.062
Valeric acid [mol%]	1.3^b ± 0.1	1.7^a ± 0.1	1.4^b ± 0.1	0.001	<0.001	<0.001	<0.001
Total of SCFA [mM]	125.3 ± 4.6	125.7 ± 4.8	124.1 ± 4.7	0.896	<0.001	<0.001	<0.001

SCFA, short-chain fatty acids.
Values with different superscripts within a row are significantly different ($p < 0.05$).
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.

and between treatments (Fig. 1). There were no significant changes of the archaeal community due to treatment with FA (p_{MC} = 0.98).

Bacteria

SSCP profile of bacteria showed a high magnitude of bands per lane and a clear heterogeneity of population structure of bacterial community between individuals with similarity values between lanes being at least 47.6% (Fig. 2). Moreover, the obtained dendrogram revealed no treatment dependent clusters (Fig. 2). Supporting this result, there was no statistical evidence for an influence of FA on diversity of community of bacteria (p_{MC} = 0.19).

Blood analysis

Table 5 illustrates the results of the red blood cell count and the number of leucocytes, as well as the means of glucose, BHB and NEFA concentrations. The number of erythrocytes increased and concentration of blood BHB decreased significantly with FA supplementation. Erythrocytes, packed cell volume (PCV), concentration of thrombocytes (PLT), glucose and BHB concentration were significantly influenced by period.

The supplementation of FA tended to decrease the blood pH from 7.447 to 7.422 but did not have a significant influence on any of the other blood gas parameters (Table 6). The different periods of the experiment had a significant influence on blood pH, pCO_2, standard and acute concentration of bicarbonate as well as base excess.

Acid-base status in urine

Table 7 shows the results of urine analysis for acid-base balance and pH. FA supplementation decreased the pH value of urine significantly from 8.00 to 7.51. The base-acid quotients (BAQ), as well as acid and base concentrations were not influenced by FA. Concentrations of NABE decreased (from 71.2 to 48.0 mM) and NH_4^+ increased (from 4.03 to 7.21 mM) numerically with FA supplementation though not significant ($p > 0.1$). The period influenced BAQ, NABE, base concentration and pH of the urine.

Passage of nutrients at the duodenum

As shown in Table 8, the duodenal flow of nutrients was not influenced by FA supplementation. Apart from organic matter (OM) and starch, period

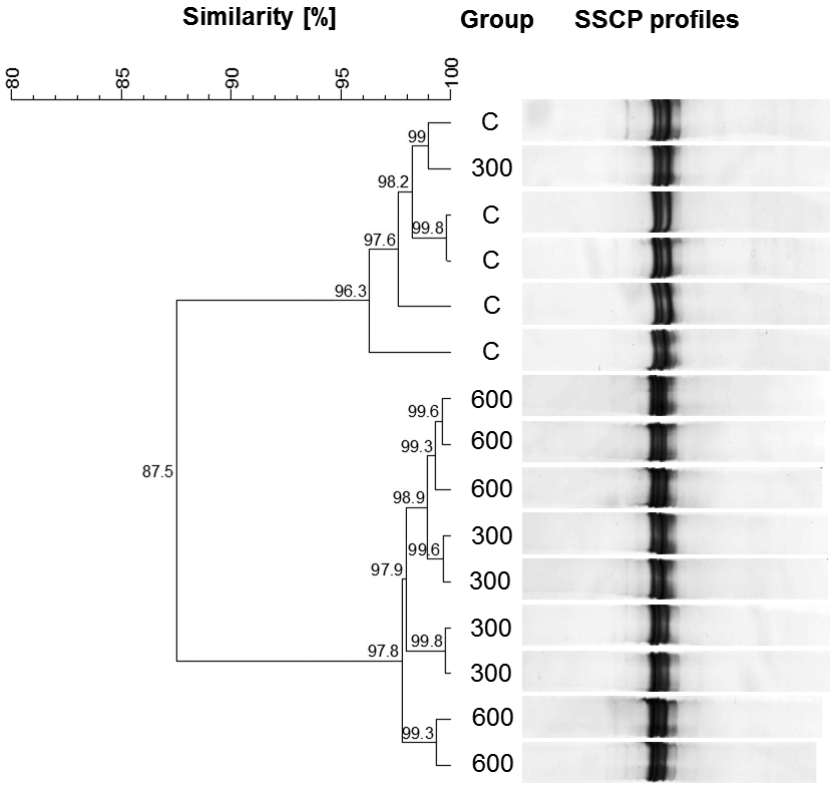

Fig. 1 Dendrogram for the results of SSCP analysis of the community of archaea from treatments C, 300 and 600. Given are the similarities between the individual animals.

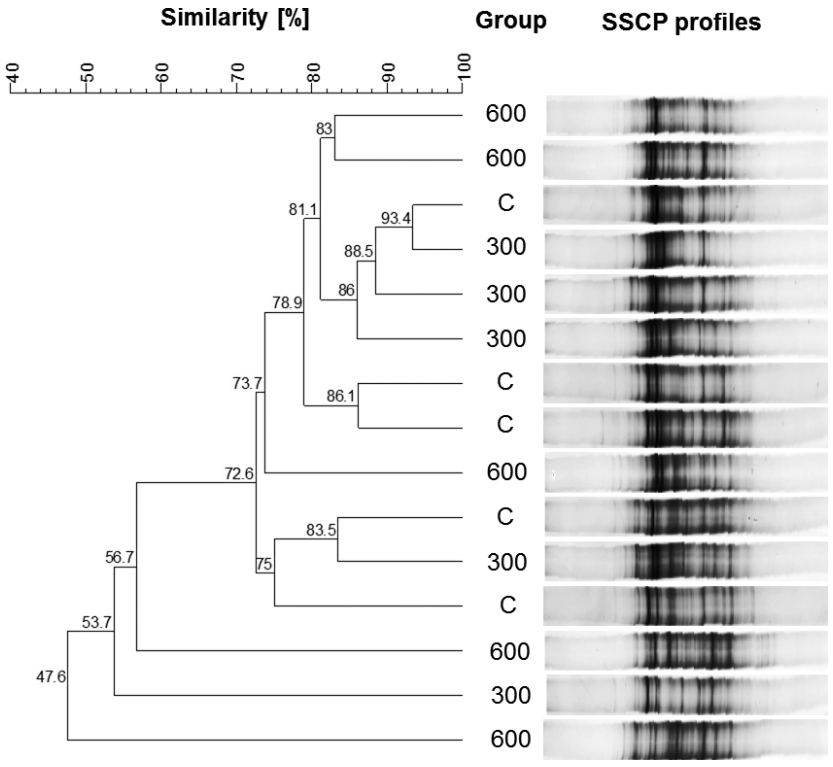

Fig. 2 Dendrogram for the results of SSCP analysis of the bacterial communities from treatments C, 300 and 600. Given are the similarities between the individual animals.

Table 5 Effects of FA supplementation on various blood parameters of dairy cows (LSMEANS ± SEM)

Treatment (FA)	Reference	C*	300†	600‡	ANOVA probabilities	
n	Range§	6	6	7	FA	Period
Erythrocytes [T/l]	6.0–8.0	6.3^{b} ± 0.3	6.4^{ab} ± 0.3	6.8^{a} ± 0.3	0.017	0.002
Haemoglobin [g/dl]	8.0–14.0	8.4 ± 0.3	8.7 ± 0.3	8.9 ± 0.3	0.288	0.087
PCV [%]	25.0–35.0	25.3 ± 0.8	26.0 ± 0.9	27.2 ± 0.8	0.145	0.040
MCV [μm^3]	40.0–60.0	40.4 ± 1.7	40.6 ± 1.7	40.5 ± 1.7	0.972	0.069
MCH [pg]	14.0–22.0	13.5 ± 0.5	13.5 ± 0.5	13.3 ± 0.5	0.545	0.071
MCHC [g/dl]	26.0–34.0	33.4 ± 0.2	33.4 ± 0.3	32.9 ± 0.2	0.235	0.096
PLT [G/l]	200–800	392 ± 36	368 ± 40	367 ± 34	0.863	0.017
Glucose¶ [mg/dl]		55.3 ± 2.7	52.8 ± 2.6	55.5 ± 2.5	0.379	0.001
NEFA¶ [mM]		0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.454	0.658
BHB¶ [mM]		1.50^{a} ± 0.10	1.11^{b} ± 0.10	0.89^{b} ± 0.10	0.003	<0.001

PCV, Packed cell volume (haematocrit); MCV, Mean corpusculat volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; PLT, Platelets (thrombocytes); NEFA, Non-esterified fatty acids; BHB, beta-hydroxybutyrate.
Values with different superscripts within a row are significantly different ($p < 0.05$).
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.
§Reference range given by Clinic for Cattle, University of Veterinary, Foundation Hannover.
¶No reference ranges available.

influenced duodenal flow of all nutrients. The apparent ruminal digestibility (ARD) of the nutrients was not influenced by FA supplementation. Period significantly effected ARD of DM, OM and ADF.

As shown in Table 9, FA reduced the MP production per day and MP per MJ ME intake. N flow was not influenced by FA supplementation but as well as the MP by experimental period.

Table 6 Effects of FA supplementation on blood gas analysis and rectal temperature of dairy cows (LSMEANS ± SEM)

Treatment (FA)	C*	300†	600‡	ANOVA probabilities	
n	7	6	7	FA	Period
RT [°C]	38.0 ± 0.2	38.1 ± 0.2	38.4 ± 0.1	0.274	0.161
pH	7.447 ± 0.008	7.446 ± 0.009	7.422 ± 0.007	0.080	<0.001
pCO_2 [mm Hg]	48.4 ± 0.7	46.0 ± 0.8	47.6 ± 0.7	0.100	<0.001
pO_2 [mm Hg]	37.8 ± 4.7	37.1 ± 5.3	33.7 ± 4.3	0.681	0.683
HCO_3a [mM]	32.4 ± 0.8	30.7 ± 0.8	30.1 ± 0.7	0.105	<0.001
HCO_3s [mM]	31.1 ± 0.8	29.7 ± 0.9	28.7 ± 0.7	0.125	0.003
BE [mM]	7.8 ± 0.8	6.3 ± 0.9	5.4 ± 0.7	0.126	0.002
O_2-Sat. [%]	63.6 ± 6.4	66.9 ± 7.4	59.7 ± 5.8	0.679	0.634

RT, rectal temperature; pCO_2, partial pressure of CO_2; pO_2, partial pressure of O_2; HCO_3a, current HCO_3; HCO_3s, standard HCO_3; BE, base excess; O_2-Sat., O_2 saturation.
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.

Table 7 Effects of FA supplementation on acid-base balance in the urine of dairy cows (LSMEANS ± SEM)

Treatment (FA)	Reference	C*	300†	600‡	ANOVA probabilities	
n	Range§	7	6	7	FA	Period
BAQ	2.5–4.8	1.86 ± 0.18	1.94 ± 0.21	1.60 ± 0.18	0.298	0.002
NABE [mM]	80–220	71.2 ± 9.8	48.0 ± 11.2	49.1 ± 9.7	0.123	<0.001
Acid [mM]	50–100	79.0 ± 6.6	76.5 ± 7.5	77.6 ± 6.5	0.960	0.219
Base [mM]	150–250	158.4 ± 11.3	150.7 ± 11.9	157.8 ± 11.3	0.771	0.004
NH_4^+ [mM]	<10	4.03 ± 1.64	4.02 ± 1.84	7.21 ± 1.63	0.133	0.140
pH		8.00^{a} ± 0.17	7.70^{ab} ± 0.19	7.51^{b} ± 0.17	0.015	<0.001

BAQ, base-acid quotient; NABE, net acid-base excess.
Values with different superscripts within a row are significantly different ($p < 0.05$).
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.
§Kraft and Dürr (2005).

Milk yield and composition

The milk yield was not influenced by feeding FA (see Table 10). On average, milk yield was between 17.7 and 19.9 kg per day and was unaffected by treatment. Due to the significant influence of FA on milk fat content, FCM decreased with 600 g FA supplementation per day. The milk fat yield tended to decrease with FA supplementation. Protein and lactose content were unaffected by the treatment. Period had a significant effect on all measured parameters with exception of fat content.

Discussion

In 2008, Molano et al. (2008) found depressed DMI in wether lambs when FA addition exceeded 4% of DM in the diet. Although in this study the FA percentage was up to 4.9% of the DM, the complete ration was consumed by the animals (except one animal in the first period). However, FA effects on voluntary feed intake cannot be evaluated in the present study because of the restrictive feeding regimen.

The results of the blood analysis from our study (see Table 5 and 6) suggested that the fistulated cows did not have any overt health problems due to the FA supplementation. This supports the conclusion of Buntenkötter (1979), who categorized FA as a harmless feed additive. All values of the red blood cell count (except the MCH) were within the reference ranges (Sahoo et al., 2009). A reason for the increasing level in erythrocytes in the dairy cows of the present experiment in response to FA feeding could not be found, but values were still in the reference range. In contrast to the present results, Remling et al. (2011) did not find significant effects of FA supplementation

Table 8 Effects of FA supplementation on duodenal flow and apparent ruminal digestibility of dairy cows (LSMEANS ± SEM)

Treatment (FA)	C*	300†	600‡	ANOVA (probabilities)	
n	6	6	7	FA	Period
Duodenal flow [kg/day]					
Organic matter (OM)	5.53 ± 0.27	5.68 ± 0.26	5.45 ± 0.25	0.628	0.083
NDF	1.65 ± 0.12	1.71 ± 0.11	1.66 ± 0.10	0.857	0.047
ADF	0.85 ± 0.06	0.90 ± 0.06	0.86 ± 0.05	0.661	0.022
Starch	0.58 ± 0.08	0.51 ± 0.08	0.49 ± 0.07	0.406	0.432
FOM [kg/day]	6.11 ± 0.21	6.06 ± 0.20	6.11 ± 0.19	0.967	0.003
FOM of OM intake [%]	61.0 ± 2.17	60.8 ± 2.07	61.7 ± 1.96	0.908	0.021
Apparent ruminal digestibility [% of intake]					
DM	34.1 ± 2.97	33.8 ± 2.87	36.7 ± 2.76	0.442	0.034
OM	43.4 ± 2.65	43.1 ± 2.54	45.1 ± 2.42	0.679	0.012
NDF	64.9 ± 2.37	64.3 ± 2.26	63.6 ± 2.14	0.873	0.132
ADF	68.0 ± 2.13	66.1 ± 2.06	67.2 ± 1.98	0.646	0.024
Crude fibre	72.9 ± 2.22	71.0 ± 2.13	72.3 ± 2.04	0.692	0.180
Starch	71.7 ± 4.56	71.6 ± 4.41	66.3 ± 4.27	0.274	0.821

NDF, neutral detergent fibre; ADF, acid detergent fibre; FOM, fermented organic matter.
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.

Table 9 Effects of FA supplementation on flow of nitrogen and microbial crude protein at the duodenum of dairy cows (LSMEANS ± SEM)

Treatment (FA)	C*	300†	600‡	ANOVA (probabilities)	
n	6	6	7	FA	Period
MP [g/day]	930 ± 33.8	931 ± 32.9	869 ± 32.1	0.043	0.003
MP [g/kg FOM]	160 ± 10.8	155 ± 10.5	145 ± 10.2	0.209	0.002
MP [g/MJ ME]	7.4^{a} ± 0.25	7.3^{a} ± 0.25	6.8^{b} ± 0.24	0.019	0.002
MP [g/g RDP]	0.77 ± 0.02	0.78 ± 0.02	0.74 ± 0.02	0.183	0.006
N [g/day]	255 ± 5.8	259 ± 5.5	247 ± 5.2	0.182	0.003
NAN [g/day]	242 ± 5.9	246 ± 5.6	234 ± 5.2	0.187	0.003
uCP [g/day]	1352 ± 29.9	1374 ± 28.2	1311 ± 26.3	0.190	0.003
UDP per CP [g/day]	423 ± 20.4	439 ± 19.8	439 ± 19.1	0.618	<0.001
UDP per CP[%]	25.9 ± 1.25	26.8 ± 1.22	27.0 ± 1.18	0.540	0.002

MP, microbial protein; FOM, fermented organic matter; ME, metabolizable energy; RDP, ruminally degraded protein; NAN, non-ammonia nitrogen; uCP, utilizable crude protein; UDP, ruminally undegraded feed protein; CP, crude protein.
Values with different superscripts within a row are significantly different ($p < 0.05$).
*Treatment C: 0 g FA/day/animal.
†Treatment 300: 300 g FA/day/animal.
‡Treatment 600: 600 g FA/day/animal.

regarding the number of erythrocytes. However, this study was a long-term experiment, conducted with growing bulls receiving 300 g FA daily (Remling et al., 2011).

The decreasing values of BHB content in plasma after FA supplementation might be explained by less butyric acid produced in the rumen due to FA supplementation. Increasing levels of butyrate in rumen fluid might result in higher BHB levels in blood (Kraft and Dürr, 2005) as a consequence of conversion of butyrate by rumen epithelia cells to BHB (Huhtanen et al., 1993). In this study, results from the analysis of the rumen fluid and blood support this assumption (see Tables 4 and 5).

The blood values of BHB, NEFA and glucose underlie postprandial variations (Miettinen and Huhtanen, 1989). In the present study, all blood samples were collected 3 h after feeding. This time interval between feeding and blood collection indicates a stadium of digestion and absorption whereby blood levels are supposed to be influenced by dietary treatments.

Bowden (1971) found strong effects of blood sampling and handling of animals on NEFA

Table 10 Effects of FA supplementation on milk yield and composition of dairy cows (LSMEANS ± SEM)

Treatment (FA)	C*	300†	600‡	ANOVA probabilities	
n	7	6	7	FA	Period
Milk [kg/day]	17.9 ± 1.0	19.6 ± 1.1	17.7 ± 1.0	0.208	0.013
Fat-corrected milk [kg/day]	19.7^{ab} ± 0.9	20.3^{a} ± 0.9	18.3^{b} ± 0.9	0.049	<0.001
Milk composition [%]					
Fat	4.7^{a} ± 0.1	4.2^{b} ± 0.1	4.2^{b} ± 0.1	0.018	0.868
Protein	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	0.735	0.008
Lactose	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	0.583	0.003
Yield [g/day]					
Fat	835 ± 41	825 ± 43	747 ± 41	0.058	0.005
Protein	532 ± 22	577 ± 23	535 ± 22	0.215	0.024
Lactose	845 ± 50	935 ± 53	837 ± 50	0.200	0.009
Urea [mM]	158 ± 11	151 ± 12	158 ± 11	0.771	0.004

Values with different superscripts within a line are significantly different ($p < 0.05$).

*Treatment C: 0 g FA/day/animal.

†Treatment 300: 300 g FA/day/animal.

‡Treatment 600: 600 g FA/day/animal.

concentrations in blood. However, all animals were handled the same way and fed at the same time. Therefore, this could be excluded as a reason for missing treatment effects.

Blood pH values and further blood gas results for all treatments were comparable with the results of Gianesella et al. (2010). Gianesella et al. (2010) found significantly lower blood pH values for cows with rumen pH values <5.5. In the present study, the lowest ruminal pH values were found for treatment 600 90 min after feeding with a value of 5.63 while the rumen fluid for the treatments C and 300 did not show values lower than 5.77 (data not shown). Therefore, the results of the pH of rumen fluid cannot explain the decreased blood pH. However, these values are spot samples, and ruminal pH values <5.5 could have occurred. Lacking effect of FA supplementation on BE, the decreasing effect on urine pH and the decreasing trend on blood pH demonstrates – in accordance with Pehrson et al. (1999) and Remling et al. (2011) – that an metabolic acidosis did not occur. Therefore, our results contradict the statement of Wood et al. (2009) that feeding of the free acid results in an acute acidosis.

FA decreased the pH of urine significantly, but concentrations of NABE and BAQ were not affected (Table 7). All observed urine values except acid are on a low level compared with the reference values (Table 7, Kraft and Dürr, 2005). Schlerka and Filar (1981) found that the pH of urine corresponds to ketone bodies in blood. Very high concentrations of ketone bodies lead to acidification of urine. In contrast, in this study, the urine pH values decreased with FA supplementation although the BHB concentration in the blood decreased as well. This could be a result of the acidifying potential of the free acid. Kutas (1967) found that the acid-base status of the urine reflects the rumen acid-base balance. In the present study, urine pH decreased together with rumen pH and blood pH (see Tables 4, 6 and 7). Therefore, the results of our experiment underline the statement of Kutas (1967).

The milk fat content decreased with increasing levels of FA supplementation due to the fact that milk fat is mainly generated from acetic acid and BHB (Huhtanen et al., 1993). In 1993, Huhtanen et al. (1993) found a relation between milk fat concentration and acetate plus butyrate/propionate ratio. This observation is in line with the results of this study (see Tables 4 and 10). Decreased amounts of FCM after supplementation with 600 g FA were a result of reduced fat concentration in the milk, as the milk yield was unaffected. The higher milk fat values for treatment C are in line with the theory that the animals in this treatment had a possible subclinical ketosis with enhanced BHB values.

Previous studies (*in vivo* as well as *in vitro*) reported increasing rumen pH after feeding FA or other organic acids (Isobe and Shibata, 1993; Callaway and Martin, 1996; Molano et al., 2008). These observations contradict the results of the present study. In addition, the assumption that organic acids like FA operate as buffer (Castillo et al., 2004) could not be supported. The decreased ruminal pH after feeding FA could be a result of the acidity [pKa = 3.03; (Engel et al., 2008)].

Moreover, the production of MP could be decreased for the same reason (see Table 9). The values of MP in

the duodenal chymus decreased with decreasing pH values in rumen fluid. Rohr (1986) found that MP per FOM is around 180 g/kg. The values of this study are on a low level, but still within the specified range. An other reason for the decreased production of MP could be that the fermentation of FA yield less energy for the micro-organisms than the fermentation of starch. This could be a result of different fermentation rates from FA and starch.

The unaffected sum of SCFA affirms the theory that FA and starch were fermented to SFCA to the same amount. As expected, the proportion of propionic acid increased with FA supplementation (see Table 4). The amounts of butyric and acetic acid decreased to the same amount. The results of this study show that starch and FA have different fermentation pattern.

Bayaru et al. (2001) found decreased levels of NH_3-N in a study with Holstein steers fed up to 2% FA. The same observations were made *in vitro* studies with up to 8 mM disodium fumarate (Mao et al., 2010). In the present study, this finding could not be demonstrated. The NH_3-N concentrations decreased slightly for treatment 600, but not significantly to treatment C (see Table 4). The highest values were found for treatment 300. The NH_3-N values for treatment 600 are not in line with the decreased MP production (Table 9). In consequence of the decreased pH values in the rumen fluid (Table 4), N of the ration could not effectively be used by the micro-organism (Wegner et al., 1940). This confirms the increased levels of NH_4^+ in urea (see Table 7). The organism may have tried to detoxify the ammonia, which accretes from the unused N in the rumen, by excretion via urea.

Although ruminal fermentation was influenced by feeding FA, an effect of FA on population structure of ruminal microbial community was not detected. It was not expected, that the changes in SCFA profile, NH_3-N content or MP in rumen fluid did not have an influence on the microbial community in the rumen. Quantitative changes are conceivable, but this was not included in this study. Therefore, it seems reasonable to investigate not only numbers of bacteria/archaea but also conduct SSCP analysis on a lower taxonomic level as bacterial or archaeal orders. High amounts of propionate in response to application of FA indicate that it was used as a substrate for bacteria. Regarding bacterial population structure, it seemed that rather an adaptation of bacterial metabolism might have occurred instead of qualitative changes of microbial community. Although there were no results of methane production available, SSCP profile of archaea leads to the conclusion that fumaric acid does not seem to limit H_2-concentrations in this study as Leibo et al. (2006) and Hansel et al. (2008) were able to show a clear dependence of methanogenic population structure in response to restricted H_2 concentrations.

Conclusions

This study shows that feeding FA, a potential methane reducer, up to 600 g per day to dairy cows does not lead to detectible health problems during the time of the experiment. The results of the blood samples and milk analyses showed tendencies of subclinical ketosis after feeding only the starch supplement. No influence of feeding FA on the acid-base balance could be found. A supposed acidosis resulting from the supplemented free acid could be compensated. Nevertheless, FA decreased urine as well as ruminal pH values. The dairy cows showed lower milk fat concentrations after FA supplementation. The ruminal digestibility and the duodenal flow of the nutrients were not affected by FA. Based on the presented results may be assumed, that up to 600 g per day FA can be supplemented for dairy cows without any consequences for the estimated parameters of animal welfare.

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