Influence of fumaric acid supplementation on animal health, acid-base balance, ruminal fermentation and energy benefit of growing bulls and fistulated dairy cows

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Abstract

Greenhouse gases like carbon dioxide, methane, and nitrous oxide are known to be responsible for the global warming effect. Methane is about 23 times as detrimental to the climate as carbon dioxide. In agriculture, ruminants produce about 33% of the annual methane production. Therefore, lots of mitigating strategies have been applied over the last decades.

One approach to reduce the ruminal methane production is the reduction of one of the main substrates of the methanogenesis: H_2. Fermentation pathways which produce propionate use H_2. Therefore, propionic acid precursors are potential methane reducers.

In *in vitro* studies, fumaric acid (FA) was the most potential propionic acid precursor. However, studies which estimate its effects on animal health and performance are rare. Therefore, the aim of the present thesis is to determine the side-effects of the potential methane reducer FA on animal health and rumen fermentation *in vivo*.

In the present thesis, a long-term study with 62 fattening bulls (German Holstein breed) was conducted over about 280 days. The animals (initial weight 266 ± 42 kg) were randomized into eight different feeding groups with four levels of FA supplementation (0 g, 100 g, 200 g, or 300 g FA per day) in the concentrates and maize or grass silage as roughage *ad libitum*. The daily feed and water intake and the weight gain were measured. Every 70 days, blood samples were taken for blood cell count and analysis of blood gases as parameters for acid-base status. The energy balance of growing bulls was reviewed with the blood parameter beta-hydroxybutyrate (BHB), non esterified fatty acids (NEFA) and glucose in plasma. The apparent total tract digestibility of different nutrients was calculated with feed and feces samples and acid insoluble ash as marker. At about 580 kg body weight, five animals of each group without and with 300 g FA per day were slaughtered and their organs were weighed. Samples of rumen fluid were analyzed for pH, short chain fatty acids (SCFA), NH_3-N and microbial community, and the rumen wall was examined histopathologically. The rumen villi were counted and their dimensions were measured.

No effects of FA on daily weight gain (Mean daily weight gain for all bulls: 1277 ± 24 g/d) or feed intake (Mean daily feed intake of all bulls: 8.81 ± 0.07 kg/d) could be demonstrated.

BHB in blood decreased with FA. The silage type affected the apparent digestibility of the different nutrients of the whole diet. The carcass dressing tended to increase with FA supplementation. Butyric acid concentration in rumen fluid decreased and the microbial community was not influenced by FA. The parameter center of inflammation (COI) of rumen mucosa showed an interaction of FA and silage type. The same results could be found for the dimension of the rumen villi. FA supplementation decreased the COI when maize silage was fed as roughage, while the rumen villi were less per cm^2 rumen wall and had greater dimensions. The opposite effects of FA could be demonstrated when the animals were fed with grass silage as roughage. Therefore, the present study with growing bulls shows a correlation between COI of rumen mucosa and ruminal villi dimensions. In addition, it could be demonstrated, that the concentrations of total SCFA in rumen fluid correlate with the villi surface per cm^2 of rumen mucosa.

The acid-base balance of the growing bulls was not influenced by FA. Neither the blood gases nor the pH values of blood and rumen fluid were affected by FA. None of the SCFA, except for butyric acid, showed an influence of the FA supplementation.

In the second experiment, seven lactating dairy cows fistulated in the rumen and the duodenum were fed with 7.4 kg dry matter grass silage, 4.2 kg concentrate mix either with or without 300 g or 600 g FA per day in a short-term study. Rumen fluid, duodenal chymus, urine, and blood plasma were collected.

The ruminal pH, as well as acetic acid and butyric acid concentrations decreased, the propionic acid concentration increased with FA, whereas the microbial community was unaffected. The percentage of propionic acid as a function of time after feeding suggested that the ruminal fermentation pattern of FA differs from that of starch, which was fed as isocaloric compensation. The apparent ruminal digestibility of the different nutrients was not influenced by FA. The blood gases were also not influenced. BHB in blood and pH in urine decreased when the feed was supplemented with FA. Correlations of NABE and urinal pH with BHB in blood could be established.

The milk composition showed decreased milk fat concentrations with FA supplementation and lower yields of fat corrected milk for cows fed with 600 g FA per day. All other measured parameters of milk were unaffected by FA.

In both experiments, no acceptance problems or any overt health problems could be observed. This suggests that the supplementation of FA as free acid in the current studies did not result in an acidosis, although the acid-base balance of the dairy cows was affected. The lack of effects of FA on blood gases indicate, that no acute acidosis occurred. The unaffected dry matter intakes of all animals suggest that no subacute ruminal acidosis appeared.

No dependence of FA on silage type could be shown in the present experiment with growing bulls, except its influence on rumen mucosa. The dose-depending effects were more obvious in the short-term study. Therefore, microbial adaptive mechanisms cannot be excluded. Hence, the microbial communities in both experiments were not affected by FA.

Based on the results of the present thesis, FA can be used with dosage of up to 300 g per day without any problems. However, no measurable energy benefit caused by FA supplementation could be shown in the present thesis. Further studies to estimate the long-term effects for dairy cows and 600 g acid per day are needed. Furthermore, further long-term *in vivo* studies which estimate the methane reduction as consequence of FA supplementations are needed.

Keywords: Fumaric acid, ruminal fermentation, acid-base balance

Zusammenfassung

Treibhausgase wie Kohlendioxid, Methan oder Lachgas sind dafür bekannt, dass sie zur Erderwärmung beitragen können. Dabei ist Methan etwa 23mal so klimaschädlich wie Kohlendioxid. In der Landwirtschaft werden etwa 33% des jährlichen Methanaufkommens durch Wiederkäuer, genauer deren Verdauung, produziert. Daher wurden in den letzten Jahrzehnten viele Reduzierungsansätze in diesem Bereich erforscht.

Ein Ansatz ist die Entfernung eines der Substrate, die die methanbildenden Bakterien zur Methanogenese benötigen: H_2. Fermentationswege im Pansen, welche Propionsäure als Endprodukt haben, verbrauchen H_2. Daher sind sogenannte Propionsäurevorstufen mögliche Methanreduzierer.

In *in vitro* Studien zeigte sich Fumarsäure (FS) als eine der erfolgreichsten Propionsäurevorstufen zur Methanreduzierung. Jedoch wurden bisher nur in wenigen Studien die Effekte des Fumarsäureeinsatzes auf die Tiergesundheit und deren Leistung untersucht. Das Ziel dieser Dissertation ist daher, die Nebeneffekte der FS auf Tiergesundheit und Pansenverdauung *in vivo* zu untersuchen.

Im Rahmen dieser Arbeit wurde eine Langzeitstudie mit 62 Mastbullen (Deutsche Holstein) über ca. 280 Tage durchgeführt. Die Tiere (Startmasse 266 ± 42 kg) wurden zufällig auf acht verschiedene Gruppen mit vier verschiedenen Zulagen FS (0g, 100g, 200g oder 300g FS pro Tier und Tag) im Kraftfutter und mit Grassilage oder Maissilage *ad libitum* als Grundfutter verteilt. Die täglichen Futter- und Wasseraufnahmen sowie die täglichen Gewichtszunahmen der einzelnen Tiere wurden gemessen. Alle 70 Tage wurden Blutproben gezogen, um rote Blutbilder zu analysieren. Die Blutgase wurden als Indikator für den Säure-Base-Haushalt der Tiere ermittelt. Als Parameter für den Energiehaushalt wurden β-Hydroxybutyrat (BHB), freie, unveresterte Fettsäuren (NEFA) und Plasmaglukose analysiert. Mit Hilfe der Kotproben und säureunlöslicher Asche (AIA) wurde die scheinbare Verdaulichkeit der Nährstoffe bestimmt. Mit etwa 580 kg Lebendmasse wurden aus den Gruppen ohne bzw. mit 300g FS jeweils fünf Tiere geschlachtet und ihre Organe gewogen. Pansensaftproben wurden gezogen, um den pH-Wert zu messen, sowie kurzkettige Fettsäuren, NH_3-N und die mikrobielle

Gemeinschaft zu analysieren. Proben der Pansenwände wurden histopathologisch untersucht. Die Pansenzotten wurden vermessen und ihre Oberflächengrößen berechnet.

Die FS hatte in dieser Studie keinen Einfluss auf die tägliche Gewichtszunahme der Tiere (mittlere tägliche Gewichtszunahme aller Tiere: 1277 ± 24 g/d) oder ihre Futteraufnahme (mittlere tägliche Futteraufnahme aller Tiere: 8,81 ± 0,07 kg/d). Die BHB-Konzentration im Blut sank mit FS-Supplementation. Die Grundfutterart hatte einen Einfluss auf die scheinbare Verdaulichkeit der Nährstoffe der Rationen. Die Schlachtausbeute war tendenziell bei der Zugabe von FS höher ($p < 0,1$). Im Pansensaft sank die Buttersäurekonzentration mit FS-Supplementation, die mikrobielle Gemeinschaft war jedoch nicht beeinflusst. Eine Interaktion der FS-Wirkung abhängig von der Grundfutterart konnte für die Anzahl der Entzündungsherde der Pansenwand gefunden werden. Das gleiche Ergebnis zeigten auch die Größen der Pansenzotten. Mit Maissilage als Grundfutter sank die Anzahl der Entzündungsherde mit FS-Zulage, wobei die einzelnen Pansenzotten größer und weniger wurden. Eine entgegengesetzte Wirkung der FS-Zulage konnte bei Tieren mit Grassilage als Grundfutter beobachtet werden. Ein Zusammenhang zwischen der Anzahl der Entzündungsherde der Pansenschleimhaut mit der Größe der Pansenzotten konnte in der vorliegenden Arbeit gezeigt werden. Außerdem konnte eine Korrelation zwischen der Gesamtkonzentration der kurzkettigen Fettsäuren und der Oberfläche der Pansenzotten pro cm^2 Pansenschleimhaut festgestellt werden.

Der Säure-Base-Haushalt der Bullen wurde von der FS-Zulage nicht beeinflusst, da weder die Blutgase, noch die pH-Werte im Pansensaft und Blut einen Effekt zeigten. Die kurzkettigen Fettsäuren im Pansensaft (außer Buttersäure) waren ebenfalls unbeeinflusst von der FS-Zulage.

In einem zweiten Experiment wurden sieben ruminal und duodenal fistulierte Milchkühe mit 7,4 kg (Trockensubstanz) Grassilage und 4,2 kg Kraftfutter gefüttert. Die Fumarsäurezulage betrug jeweils 0 g, 300 g bzw. 600 g pro Tier und Tag. In dem Kurzzeitversuch wurden die Kühe zufällig in drei verschiedenen Fütterungsgruppen verteilt. Von jedem Tier wurden Darmchymus, Milch, Blut und Urinproben gesammelt.

Im Pansensaft sanken der pH-Wert sowie die Essig- und Buttersäurekonzentrationen durch die FS-Zulage. Der Anteil der Propionsäure stieg mit FS, wobei die mikrobielle Gemeinschaft

sowie die scheinbare Verdaulichkeit im Pansen der einzelnen Nährstoffe nicht beeinflusst wurden. Der Verlauf des Propionsäureanteils im Pansensaft über die Zeit nach der Fütterung lässt vermuten, dass der Fermentationsweg von FS von dem der Stärke, welche als isokalorischer Ausgleich gefüttert wurde, variiert. Einen Einfluss von FS auf die Blutgase der Milchkühe konnte nicht festgestellt werden. Jedoch sanken mit FS die BHB-Konzentration im Blut und der pH-Wert im Urin der Kühe. Eine Korrelation zwischen der Netto-Säure-Base-Ausscheidung im Harn sowie dem pH des Harns mit der BHB-Konzentration im Blut der Kühe konnte in der vorliegenden Arbeit gezeigt werden.

Die FS senkte ferner die Milchfettkonzentration, wobei die anderen Parameter der Milchleistung unbeeinflusst blieben.

In beiden Experimenten konnten keine Unverträglichkeiten bzw. Akzeptanzprobleme bei den Tieren gegenüber der FS beobachtet werden. Zwar wurde der Säure-Base-Haushalt der Milchkühe durch FS beeinflusst, aber eine Azidose konnte in keinem der beiden Versuche festgestellt werden. Die fehlenden Effekte der FS-Zulage auf die Blutgase der Tiere zeigen, dass zu den jeweiligen Probenahmen keine akuten Azidosen vorlagen. Das Auftreten einer subakuten Pansenazidose kann vermutlich ebenfalls ausgeschlossen werden, da keines der Tiere eine typische verringerte Futteraufnahme zeigte.

Nur die Schleimhaut der Pansenwand der Bullen zeigte grundfutterabhängige Wirkungen der FS. Bei allen anderen Parametern war die FS-Wirkung unabhängig von der gefütterten Grundfutterart. Die dosisabhängige Wirkung von FS wurde im Kurzzeitversuch mit Milchkühen deutlich. Eine Adaptation der Pansenmikroben an die FS-Zulage während des Langzeitversuchs kann daher nicht ausgeschlossen werden. Jedoch waren die Mikrobengemeinschaften in beiden Versuchen unbeeinflusst von der FS-Supplementierung.

Aufgrund der Ergebnisse dieser Arbeit kann festgehalten werden, dass bis zu 300 g FS pro Tier und Tag über längere Zeit ohne auffällige Gesundheitsprobleme gefüttert werden können. Eine höhere Leistung der Tiere aufgrund von Energievorteilen durch die FS-Zulage konnte jedoch nicht festgestellt werden. Zur Langzeitwirkung von FS auf Milchkühe, zu einer FS-Zulage von 600 g pro Tier und Tag, sowie zur langfristigen methanreduzierenden Wirkung von FS sind weitere Studien notwendig.

Schlagwörter: Fumarsäure, Pansenfermentation, Säure-Base-Haushalt

Contents

Abbreviations

AIA	acid insoluble ash
AUC	area under the curve
BAQ	base-acid quotient
BE	base excess
BHB	β-hydroxybutyrate
CH_4	methane
CO_2	carbon dioxide
COI	center of inflammation
DM	dry matter
DMI	dry matter intake
EFSA	European Food Safety Authority
FA/FMA	fumaric acid
FAO	Food and Agriculture Organization
FS	Fumarsäure
G	grass silage
GE	gross energy
GfE	Gesellschaft für Ernährungsphysiologie
GHG	greenhouse gases
H_2	hydrogen
IPCC	Intergovernmental Panel on Climate Change
K	potassium
LSMEANS	least square means
M	maize silage
MA	malic acid

ME	metabolisable energy
Na	sodium
NABE	net acid-base excretion
NEFA	not esterified fatty acids
NEL	net energy of lactation
NH_3-N	ammonia nitrogen
N_2O	nitrous oxide
SARA	subacute ruminal acidosis
SCFA	short chain fatty acids
SEM	standard error of means

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1 Introduction

1.1 Methane emissions from ruminants

Over the last decades, the global warming effect received more attention. Responsible for this phenomenon are, amongst others, greenhouse gases (GHG). The GHG with the largest impact on climate change is carbon dioxide (CO_2). Besides CO_2, methane (CH_4) and nitrous oxide (N_2O) are the most important GHGs. These gases increase the conductivity of the atmosphere, and therefore, decrease the possibility of heat emissions (Flachowsky and Meyer 2008), hence this is called the global warming effect. CH_4 has about 23 times and NO_2 about 296 times more climate potential than CO_2 (IPCC 2006).

Agriculture and especially ruminant livestock production contribute substantially to CH_4 emissions. About 33% of the annual CH_4 production can be attributed to animal production (IPCC 2006). Especially the gases originating from the digestion processes of ruminants contribute to the emissions. The formation and eructation of CH_4 result in a loss of 6 to 8% of the gross energy (GE) in ruminants (Boadi et al. 2004; Flachowsky and Brade 2007). Therefore, a possible reduction of CH_4 formation in the digestive tract of ruminants would have a positive effect on both ecology and economy.

Methanogenesis is part of the anaerobic fermentation in the digestive tract. About 90% of the gastrointestinal CH_4 is produced in the rumen (Murray et al. 1976). During the fermentation, primary digestive microorganisms degrade the feed into amino acids and sugars (Boadi et al. 2004). These products of bacteria, protozoa and fungi are fermented into short chain fatty acids (SCFA), hydrogen (H_2) and CO_2. The H_2 production is thermodynamically unfavorable, but in the ruminal fermentation it is one of the major end products produced by protozoa and fungi. H_2 can not accumulate in the rumen (Boadi et al. 2004) and directly after production, it is used by the *Archaea* (Martin et al. 2010). Therefore, methanogenesis is the last step of the anaerobic reduction of biomass: $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$ (Moss et al. 2000) and requires H_2. Hence, the main substrates for the methanogenesis in the rumen are H_2 and CO_2. However, the *Archaea*, are able to use formate or acetate as a hydrogen source (Baker 1999; Castillo et al. 2004).

Fermentation pathways with acetate and butyrate as end product produce H_2 and, therefore, induce methanogenesis (Moss et al. 2000). However, the inhibition of these reactions would decrease the activity of the cellolytic bacteria (Asanuma et al. 1999; Castillo et al. 2004). In contrast, fermentation pathways with propionate as product for the host animal use H_2 in another way: $C_6H_{12}O_6 + 4\ H \rightarrow 2\ C_3H_6O_2$ (propionate) $+ 2\ H_2O$ (Boadi et al. 2004). It is known that an increase in proportions of propionate in the rumen leads to a decrease of acetate and / or butyrate (Martin et al. 2010). Therefore, a ration which supports the propionate production should reduce the CH_4 eructation (Beauchemin et al. 2008).

1.2 Approaches to reduce methane emissions from ruminants

During the last decades, different approaches to reduce CH_4 emissions have been introduced. There were two different attempts to reduce the CH_4 production per kg of animal product: Directly by reduction of the CH_4 formation or indirectly through increased productivity of the animal and, consequently, less CH_4 production per unit meat or milk (Boadi et al. 2004).

Optimization of the diet composition and improving of feeding management are the most intensive investigated mitigating strategies during the last years (Martin et al. 2010). By improving the feeding efficiency, it is possible to reduce the CH_4 output per kg animal product (Benchaar et al. 2001). Changes in the forage to concentrate ratio affected the CH_4 emissions (Johnson and Johnson 1995). An increase in concentrate proportion by 61% reduced the CH_4 emissions by about 4% of GE intake in heifers (Beauchemin and McGinn 2005). Not only could the forage to concentrate ratio, but also the kind of forage have an impact on CH_4 formation. The different carbohydrate profiles of grass and maize silage suggest different CH_4 emissions (Beauchemin et al. 2008). Fiber rich roughage is known to have more CH_4 production as consequence (Flachowsky and Brade 2007). High contents of grain or starch result in lower CH_4 emissions (Kolver and Aspin 2006). Theoretically, there are three reasons why maize leads to less CH_4 eructation than grass silage (Beauchemin et al. 2008):

- Higher starch content supports the propionate production, which is known as competitive pathway in H_2 use to CH_4 production (Boadi et al. 2004).

- The ruminal fermentation is restricted because of less ruminal residence time. Post-ruminal digestion is supported, which is in comparison to ruminal fermentation energetically more effective.
- The higher energy content of the feed supports lower CH_4 emissions per unit animal product.

However, there are not enough studies available which compare grass and maize silage (Beauchemin et al. 2008).

The replacing of fibre rich diets with starch or concentrate rich diets could reduce the CH_4 production by ruminants, but this would disable one of the advantages of ruminants over poultry and pigs. Ruminants can digest fibrous feeds with help of the microorganisms in their rumen (Taube et al. 2014). This enables the ruminants to process low quality roughage in animal protein (milk and meat) which can be utilized by humans. Therefore, the feeding of ruminants with fibre rich diets decreases competition over food sources with non-ruminants and humans (Flachowsky et al. 2013). Ruminant nutrition provides the opportunity for using grassland as feed source (Taube et al. 2014). About 70% of the world agricultural area is grassland (FAO 2008). Therefore, other mitigating strategies of CH_4 production in the rumen were considered to minimize the disadvantages of ruminants without reducing their advantages (Flachowsky et al. 2013).

Manipulation of the microbial fermentation in the rumen by additives or other biotechnological technics is promising (Martin et al. 2010). Strategies to affect the microbial community or its fermentation activity always pursue one of the following objectives (McAllister and Newbold 2008):

1. Inhibition of methanogens (makes it necessary to redirect H^+ ions into other products)
2. Decreasing the production of H_2 in the rumen
3. Providing alternative electron sinks in concurrence to the methanogens.

Due to the complexity and diversity of the rumen bacteria and methanogens, the potential of most CH_4 reducing strategies is limited (Martin et al. 2010).

An approach is the supplementation of substances which could handicap the CH_4 production. The application of antibiotics like ionophroes (e.g. Monensin) was tested as an approach to

reduce methane emissions (McAllister and Newbold 2008), but the use of them in animal diets is forbidden in the EU (EU Commission 2003). Ionophores improve the efficiencies of milk and meat production, but furthermore, the application of antibiotics had the side effect of reduced ruminal fermentation or reduced fiber digestion (Beauchemin et al. 2008). The addition of lipids to reduce CH_4 emissions has side effects like less milk fat in dairy cows and depressed dry matter intake (DMI) when lipids were supplemented more than 6-7% of DMI (Beauchemin et al. 2008). Therefore, it can only be used carefully.

Another approach to reduce the methanogenesis is the utilization of H_2 from bacteria others than the *Archaea* (Asanuma et al. 1999). Therefore, alternative electron acceptors with high H_2 affinity and a rapid utilization by bacteria are needed (Castillo et al. 2004).

The propionate formation in the succinate-propionate pathway consumes H_2 and is competitive to the methanogenesis (Moss et al. 2000). This pathway is the reductive or reverse citric acid cycle which is used to produce succinate or propionate (Castillo et al. 2004). Organic acids are key intermediates of this pathway because they are propionate precursors.

1.3 Fumaric acid

Fumaric acid (FA) is a 4-C dicarboxylic acid ($C_4H_4O_4$) and, like other organic acids categorized as a preservative. Its use is allowed in livestock production in the EU (EFSA 2013). Neither fumarate nor its metabolites are expected to accumulate in the animal tissue (EFSA 2013). It is a natural compound of plants and animals and a stereoisomer of maleic acid (Buntenkötter 1979). In 1832, FA was first isolated by Winkler from Fumitory (*Fumaria officinalis L*) (Rudy 1967). FA is classified as a strong acid (pk_a = 3.03; Engel et al. 2008). In the succinate-propionate pathway (the reverse citric acid cycle) (Castillo et al. 2004), FA is converted by the fumarate reductase under H_2 consumption to succinate (Lopez et al. 1999; Asanuma and Hino 2000) and subsequently to propionate. Therefore, one mole of fumaric acid can catch one mole of H_2 away from methanogenesis (Ungerfeld et al. 2007). Stoichiometrically, per mol converted FA to propionate, the CH_4 production in the rumen would be reduced by 6.4 L (1mol of gas, 25.6 L at standard 39°C and standard pressure (Yang et al. 2012)). Moreover, propionate is a glycogenic substance (Asanuma and Hino 2000) and

one of the SCFA which are used as energy source by ruminants. Therefore, the conversion of FA has an energy benefitting side effect for the host animal.

Already in 1968, FA was used prophylactic against beef ketosis (Buntenkötter 1979). FA has antibacterial effects and is used amongst others in food technology, nutrition of pigs and for acidification of milk for calves (Günther 1979; Kirchgessner and Roth 1979; Seibold and Ruch 1979).

1.4 Fumaric acid as feed additive to reduce methane emissions and its effect on the animal`s metabolism

In the past, malic acid (MA) and FA seemed to be the most potential CH_4 reducing organic acids (Callaway and Martin 1996). Newbold et al. (2005) found that FA is the most potential of them to reduce CH_4 *in vitro*. The addition of FA *in vitro* increased the propionate and decreased the CH_4 production (Asanuma et al. 1999). From their studies, they derived two hypothesis: Either that FA was reduced to succinate while utilizing H_2 or that FA reduced other equivalents which were otherwise used to produce H_2 (Asanuma et al. 1999). The reduction of FA supports a low partial pressure of H_2 in the rumen (Castillo et al. 2004).

During the last years, various *in vitro* and *in vivo* studies were conducted with FA as potential CH_4 reducer. The reduction potential measured *in vitro* was between 2 and 38% (Carro and Ranilla 2003; Kolver et al. 2004), whereas Wallace et al. (2006) could not underline the CH_4 reduction potential of FA *in vitro*. *In vivo,* the reduction potential alternated between not detectable and 50% (Beauchemin and McGinn 2006; Wallace et al. 2006). Bayaru et al. (2001) could demonstrate that a FA supplementation of 2% of DMI reduced 23% CH_4 in ruminal fermentation of steers. In goats, the CH_4 production could be reduced by 15 to 18.5% when FA was supplemented with 2.5 or 5% of DMI, respectively (Chethan et al. 2013). In contrast, the supplementation of 6% FA on DM basis showed no detectable CH_4 reduction in steers by McGinn et al. (2004). The differences of the observed CH_4 reduction varied among others because of different species, diets, experimental durations or methods of measurement. Castillo et al. (2004) concluded that “dietary factors such as forage: concentrate ratio, and forage or cereal grain type may alter the response” of FA. Hence, it is known that the effects of FA depend on the fed diet (Sirohi et al. 2012).

Most of the studies investigating the effects of FA focused on its CH_4 reduction potential. Only few studies examined the productivity of ruminants while feeding organic acid supplementations (e.g. Kolver and Aspin 2006). More research is needed about the effects of FA on beef cattle performance (Castillo et al. 2004). Supplementation of FA constitutes an additional energy source, because of its conversion to propionate in the rumen, and the potential lower energy loss via CH_4 formation. Kolver and Aspin (2006) conducted the first study with fumarate in diets for dairy cows. They supplemented fumarate with a dose of 5% of DM in a short-term experiment (15 days). No effects of fumarate on CH_4 emissions, ruminal fermentation, digestibility and milk production could be found (Kolver and Aspin 2006). To evaluate possible CH_4 mitigating strategies, it is necessary to examine them over a long time, e.g., the entire lactation or fattening period (Martin et al. 2010).

Most of the studies on FA were conducted over less than 30 days, but not over a long time period (e.g., Bayaru et al. 2001; McGinn et al. 2004). More long-term studies are needed (Beauchemin et al. 2008) to examine the possible long-term effects of FA on the animal or adaptations of the microbial population in the rumen. Little is known about the consequences for the organism of the animal and especially for the rumen microbiota when FA is fed over a long time period. Isobe and Shibata (1993) concluded due to their *in vivo* study, that the microbial population and the fumaric acid metabolism in the rumen might be changed when FA was supplemented.

The apparent digestibility of diets supplemented with FA was also investigated in several *in vivo* studies (Isobe and Shibata 1993; Beauchemin and McGinn 2006; Molano et al. 2008), but their results were inconsistent, though the studies were made with different species. Isobe and Shibata (1993) found promoting effects of FA on microbial cellulose digestion in goats. Beauchemin and McGinn (2006) could not find higher total tract digestibility for growing beef fed FA, but they hypothesized increased ruminal fermentation due to the higher total SCFA concentrations.

Most of the earlier studies investigating the effects of FA, demonstrated side effects of this acid on ruminal fermentation. The SCFA profiles were shifted in the direction of propionic acid in cattle when FA was supplemented to the diet up to 2% of DM of the diet (Bayaru et al. 2001). This assumption could be confirmed by Castillo et al. (2004) and others. Regarding the pH value in the rumen, the results of these studies varied widely (Isobe and Shibata 1993;

Asanuma et al. 1999; Zhou et al. 2012). Asanuma et al. (1999) found increased pH values with up to 30 mM fumarate, the salt of FA, supplementation in *in vitro* studies. Therefore, the authors recommend feeding the salt of FA in *in vivo* studies to prevent low pH values in the rumen when feeding the free acid. Decreased pH values can result in an acidosis (Kricziokat et al. 2009) which leads to a lower fiber digestion (Asanuma et al. 1999; Newbold et al. 2005). Furthermore, not in all studies the free acid was used. Some *in vivo* studies were conducted with the salt of FA, fumarate (Li et al. 2012). However, Newbold et al. (2005) found that the free acid FA is more effective in CH_4 reduction than its salt. Beauchemin and McGinn (2006) fed beef cattle approximately 1% of DM of the diet sodium bicarbonate in addition to the 2.9% FA supplementation, to neutralize the acidity of FA. Wallace et al. (2006) encapsulated FA in an oil to buffer its acidity. However, there exist also studies, which found increasing pH values when FA was added as acid (Li et al. 2009). Li et al. (2009) found *in vitro* increasing levels of pH independent of the chemical origin of FA: sodium fumarate or acid form.

It was found that FA has dose depending effects *in vitro* (Asanuma et al. 1999). The same observations were made by Li et al. (2009) in an *in vitro* study with up to 24 mM FA or MA. The CH_4 production decreased with increasing levels of the organic acid. Additionally, the level of application of FA had significant influence on pH 3h after supplementation and several other parameters. Carro and Ranilla (2003) could confirm this observations with decreased acetate:propionate ratios with increasing doses of fumarate *in vitro*. *In vivo*, Newbold et al. (2002) found dose-depending effects when the forage of sheep was supplemented up to 8% of DM of FA. The ruminal pH and total SCFA decreased with increasing levels of FA.

1.5 Acid-base balance as evidence of acidosis

The acid-base balance of animals is a complex system and its primary objective are constant pH values in blood and, therefore, in the organism (Silbernagl and Despopoulos 2003). Disturbances of the acid-base balance can result in alkalosis (increased blood pH) or acidosis (decreased blood pH) (Silbernagl and Despopoulos 2003). Decreased base-excess in body fluids relative to the acids are defined as an acidosis (Owens et al. 1998). Most of the

enzymatic reactions in organism depend on a closely range of pH. Therefore, disturbances in the acid-base balance have consequences for the whole organism (Fürll 1993).

Owens et al. (1998) summarized anorexia, decreased feed intake, diarrhea, and lethargy as diagnostic indications of acidosis of feedlot cattle. For ruminants, acidosis is separated in different forms: acute (or metabolic) and chronic (subacute) acidosis (Owens et al. 1998). An acute acidosis is obvious as an overt illness. In contrast, the chronic acidosis is not as obvious. The nomenclature of non-acute acidosis in cattle is complicated. In literature, the names chronic acidosis, subacute, (Owens et al. 1998) subclinical (Nocek 1997) or chronic-latent (Gäbel 1990) were used. The subacute ruminal acidosis (SARA) is defined as extended time periods with ruminal pH values below 5.5 – 5.6 (Mutsvangwa and Wright 2003). It occurs, when SCFA production exceeds the ability of the rumen to absorb or neutralize acids (Beauchemin 2007). Symptoms of SARA are, amongst others, reduced feed intake, reduced fiber digestion and milk fat depression (Plaizier et al. 2008).

The critical pH value in rumen fluid to define acidosis is not standardized until now. The threshold is depending on the sampling techniques and is not consistent (Nocek 1997; Plaizier et al. 2008). It ranges between pH 5.2 and 6.0 (Plaizier et al. 2008). Throughout a day, the ruminal pH fluctuate ± 1.5 units depending on feed intake (Beauchemin 2007). Gianesella et al. (2010) found a negative correlation between blood and ruminal pH. Nocek (1997) postulated that the ruminal pH is the only diagnostic test for subclinical acidosis.

The analyses of blood gases provide an instrument to estimate possible acidotic burdens (Brown et al. 2000). To diagnose an acidosis, blood gas analyses are practical, because their analyses is less invasive than rumen pH analysis (Gianesella et al. 2010). Especially for the acute form, blood gas analytic gives useful information (Fürll 1993). Blood gases from jugular or mixed venous blood give information about the total body acid-base status (Day 2002). Most important blood gases to diagnose acid-base disorders are pH, $_PCO_2$ and HCO_3^-. The reason is that "changes in HCO_3^- and $_PCO_2$ dictate the four primary acid-base disturbances as well as the direction of compensation in an attempt to maintain pH" (Day 2002). CO_2 and HCO_3^- are the main buffering agents in blood (DeRouchey et al. 1998). Additionally, the base excess (BE) is important to evaluate the metabolic, non-respiratoric aspects of acid-base balance (Deegen 1982). BE gives information how many mM of strong acid were needed to adjust one liter of blood to pH 7.40 (DeRouchey et al. 1998). Therefore,

the blood acid-base balance is also of interest, because it is closely associated with ruminal pH (Castillo et al. 2008).

Additionally, the pH in urine, the net acid-base excretion (NABE) and the base-acid quotient (BAQ) in the urine are indicators for changes in acid-base balance (Fürll 1993). The reason is that the kidneys are essential regulatory ways for ruminants acid-base balance (Kutas 1966). Metabolic acidosis is known to be associated with decreased pH values of urine (Kricziokat et al. 2009).

In 1965, Kutas (1965) introduced the easy to be determined NABE in urine for veterinary medicine. NABE is more sensitive on acute acidotic burden than the urinal pH, which is more inert because of the buffering capacity (Fürll 1993). The NABE is the total sum of the excreted H^+ ions and it is calculated as follows: NABE = Sum Acids – Sum Base (Fürll 1993). Fluctuated diuresis as a result of acid-base disturbances do not have an considerable influence on the NABE, but the additional forming of the BAQ (BAQ = base number / acid number) completely undo the influence of intensity of diuresis (Fürll 1993). For chronical acid-base disturbances, the buffering capacity and the SCFA concentrations in rumen fluid are more sensitive than the NABE (Fürll 1993). Nevertheless, with decreasing NABE, the β-hydroxybutyrate (BHB) concentration in blood increased (Fürll 1993).

Additional parameters of acidosis diagnosis are e.g. ketone bodies in blood and milk fat concentration (Kricziokat et al. 2009). Milk fat percentage and BHB in blood are known to be related to ruminal acid load (Enemark et al. 2004). Due to the consequences of acidotic burden for the rumen wall (Gäbel 1990), histopathological analysis of them could offer valuable clues about the acid-base balance in the rumen.

2 Scope of the thesis

As shown in the introduction, the attempt to reduce CH_4 emissions revealed different strategies in ruminant nutrition. A promising point is to support the alternative electron acceptors in concurrence of methanogens. Fumaric acid seems to be a feed additive with a high potential to reduce CH_4 formation in the rumen. However, most of the studies focused on the prevention of CH_4 production and were conducted *in vitro*. *In vivo* experiments with consideration of the whole organism of the animal are rare. Therefore, the objective of this thesis was to study the influence of fumaric acid on cattle and dairy cows with a focus on its potential side-effects on health and performance. The main questions were:

1. Is the acid-base balance of fattening bulls and lactating cows affected by fumaric acid? Does feeding the strong acid fumaric acid induce an acidosis or any other health impairment?

2. What are the consequences for the rumen mucosa and the ruminal fermentation when fumaric acid is supplemented to diets for fattening bulls and dairy cows?

3. Are the effects of fumaric acid dose-depending and do the effects of fumaric acid depend on the silage type which is fed as roughage (grass or maize silage)?

4. Do the animals have an energy benefit when supplemented with FA?

For the investigation of these questions, two experiments were conducted as dose-response trials.

The first experiment was conducted as a long-term study with growing bulls, fed two different roughage types: grass silage and maize silage. Thus, in this experiment, it was the aim to study the effects of fumaric acid depending on the roughage type (diet-dependence). Also questions one and four were focused in this experiment. Therefore, the trial was conducted over the whole fattening period of bulls to study the long-term effects of this acid on health and the performance parameter.

Additionally, a second (short-term) experiment with lactating cows was arranged. Cows were equipped with two cannulas: A cannula on the dorsal sac of the rumen and another cannula at the proximal duodenum. This second trial focused on the first and second question. The aim was to measure the ruminal fermentation pattern and the effect of FA on the rumen microbiota. Referring to question four, it was also the aim to get additional information about the effects of FA on milk composition.

The present thesis was developed in the context of a joint research project. In this project, next to the present trials, the microbial collective of Bacteria and Archaea was analyzed after feeding fumaric acid. The results of the present *in vivo* experiments with fumaric acid can be compared with the results of the *in vitro* fumaric acid experiments of the project partner at the Institute for Physiology, University of Veterinary Medicine Hannover Foundation, Germany. Therefore, the used concentrations of fumaric acid in the experiments in this thesis agreed with the *in vitro* experiments on the Institute for Physiology, University of Veterinary Medicine Hannover Foundation, Germany.

3 Paper I

87. Leads fumaric acid to a metabolic acidosis when fed to growing bulls?

(Führt Fumarsäurefütterung bei wachsenden Bullen zu metabolischen Azidose?).

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Introduction:

The methane reduction potential of the propionate precursor fumaric acid (FA) was frequently tested *in vitro* and suggested as a feed additive. However, unexpected side effects of this additive were only rarely addressed. Especially the strong acid character of FA may have the potential to influence the rumen pH and metabolism (Newbold 2005). Therefore it might increase the risk of metabolic acidosis. Hence, the aim of the present study was to examine the effects of increasing levels of FA on animal health over the whole fattening period of bulls. In addition, each dietary FA level was tested together with maize (M) or grass (G) silage which are well known to affect rumen pH and metabolism.

Methods:

A total of 59 growing Holstein bulls were randomly arranged into eight different feeding groups (mean starting weight 266 kg). In a dose-response experiment with up to 300 g FA per day four groups got M and the others G *ad libitum*. Venous blood samples were collected on experimental days 0, 70, 140 and 210 (only from those bulls with less than 580 kg live weight) to analyse pH, base excess (BE), and standard HCO_3 to diagnose a potential metabolic acidosis. A total of five animals per group fed M or G without or with 300 g FA/d were slaughtered at 580 kg live weight without fasting and samples of rumen papillae and rumen fluid were collected. The pH of the rumen fluid was measured immediately and the papillae were examined for pathological lesions. The number of centres of inflammation (COI) per 10 fields of sight was enumerated.

Table 1: Results of blood gas analysis for the eight feeding groups with maize (M) or grass (G) silage as roughage (LSMEANS ± SEM)

Group	n	Blood - pH	HCO_3 mmol/l	base excess mmol/l
M0	31	7.355 ± 0.009	28.5 ± 0.5	5.3 ± 0.5
M100	30	7.329 ± 0.009	27.9 ± 0.5	4.2 ± 0.6
M200	30	7.328 ± 0.009	27.4 ± 0.5	4.1 ± 0.5
M300	30	7.331 ± 0.009	27.6 ± 0.5	4.2 ± 0.5
G0	22	7.342 ± 0.010	29.1 ± 0.6	5.9 ± 0.5
G100	26	7.330 ± 0.009	28.2 ± 0.5	4.9 ± 0.5
G200	26	7.356 ± 0.009	29.6 ± 0.5	6.3 ± 0.5
G300	28	7.344 ± 0.009	28.1 ± 0.5	4.8 ± 0.5
ANOVA probabilities				
Silage		0.379	0.028	0.030
FA		0.844	0.962	0.568
Silage x FA		0.078	0.111	0.254

Results:

Standard HCO_3 and BE were significantly influenced by silage type (Table 1). An interaction between FA and silage on COI was observed (Table 2). All rumen pH values are on a low level and no treatment related changes could be observed.

Conclusions:

The silage dependent alterations in standard HCO_3 and BE might be related to the differences in chemical composition.

Table 2: pH of the rumen fluid and centre of inflammation on rumen papillae of slaughtered bulls (Means ± SEM; n = 5)

Group	pH	COI[#]
M0	5.83 ± 0.11	3.6 ± 0.9[a]
M300	5.92 ± 0.08	1.6 ± 0.8[ab]
G 0	5.87 ± 0.12	0.6 ± 0.4[b]
G 300	5.90 ± 0.08	3.0 ± 0.0[ab]
ANOVA (probabilities)		
Silage	0.907	0.221
FA	0.572	0.754
Silage x FA	0.815	0.003

[abc] values with different superscripts within a column are significantly different ($p<0.05$)

The low level of rumen pH was independent of FA addition. The observed interaction between silage type and FA for COI could approve that the effects of FA depend on the diet. Nevertheless the differences between COI of group M300 and G300 were not significant.

The determined factors indicate that long term FA-supplementation up to 300 g per day did not increase the risk of metabolic acidosis for growing bulls.

References:

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4 Paper II

Influence of various amounts of fumaric acid on performance and parameters of the acid–base balance of growing bulls fed with grass or maize silage

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Abstract:

The aim of the present study was to determine the effects of the potential methane reducer fumaric acid on the fattening performance and acid–base balance of growing bulls fed two different silage types as roughage (maize and grass silage). A total of 62 fattening bulls (German Holstein breed, initial body weight: 266 ± 42 kg), randomly assigned to eight feeding groups, received four levels of fumaric acid (0, 100, 200 or 300 g/d) at each silage type. The daily feed and water intake and the live weight were measured over the whole testing period of 280 days. In blood samples, blood cells and blood gases as a parameter of acid–base status were analysed. Feed and faeces were collected to determine the apparent nutrient digestibility. Five animals from each group fed maize or grass silage, supplemented with 0 g or 300 g fumaric acid, were slaughtered at 580 kg body weight. After slaughter, rumen fluid pH was measured and dressing percentage was calculated. Neither the total feed intake (8.81 ± 0.07 kg/d) nor the daily weight gain (1277 ± 24 g/d) was influenced significantly by treatments. Fumaric acid supplementation did not influence the erythrocyte count or the blood gas concentration. The silage type significantly influenced the apparent digestibility of the whole diet. The dressing percentage was slightly higher ($p < 0.1$) after fumaric acid supplementation. No signs of an incompatibility to fumaric acid on the animals were observed over the whole experimental period. However, it seems to be necessary to conduct more long-term studies with different silage types and addition of organic acids combined with direct measurements of methane.

Keywords: acid–base equilibrium; bulls; fattening performance; fumaric acid;

grass silage; maize silage

Introduction:

For ruminants, the production of the greenhouse gas methane implies a gross energy loss of 6 to 8% (Flachowsky and Brade 2007). Hence, feeding strategies that help to reduce the methane (CH_4) production by rumen microbes are not only of environmental relevance but also save metabolisable energy (ME) for production purposes.

The CH_4 reduction potential of organic acids such as fumaric acid (FMA) has been frequently measured in literature (Bayaru et al. 2001; McGinn et al. 2004; Wood et al. 2009). Because of its acidity (pKa = 3.03; Engel et al. 2008), FMA could have negative effects on the acid–base balance of the fed animals and acute acidosis could be a result of FMA supplementation (Wood et al. 2009). However, there are only a few *in vivo* long-term studies (Kolver and Aspin 2006) addressing general effects of FMA on health and performance.

In vitro studies showed that CH_4 reduction depends on the level of supplementation with FMA or fumarate (Asanuma et al. 1999; Carro and Ranilla 2003). On the other hand, roughage rich in fibre is responsible for higher methane production in ruminants (Flachowsky and Brade 2007). However, there are no studies available which compare grass and maize silage in combination with a feed additive which potentially reduces methane production (Beauchemin et al. 2008). An examination of the fattening performance and carcass conformation of fast growing bulls in response to diet types differing markedly in fibre content and supplemented with graded levels of FMA provides some indirect indications of methane production.

Overall, the aim of the present experiment was to investigate the interactions between dietary fibre type (maize and grass silage based diets) and various amounts of FMA on growth, health, acid–base balance and slaughter performance of bulls. Although methane production could not be measured in the present experiment, it is also important to know possible side effects of potential methane reducing feed additives.

Material and methods:

Experimental design

For this experiment, 62 growing fattening bulls (German Holstein breed) with an average initial body weight of 266 ± 42 kg (age: 228 ± 3 days) were randomly assigned to roughage diets with different fibre and starch contents (maize or grass silage). Each diet type was tested without and with FMA supplementation (100, 200 or 300 g/d). Animals were housed in groups of seven or eight on slatted floors with concrete and rubber surfaces (5.25 m^2 per animal) in a barn located at the Experimental Station of the Institute for Animal Nutrition,

Friedrich-Loeffler-Institute (FLI), Federal Research Institute of Animal Health in Braunschweig, Germany.

The grass silage used for the present study was the first cut of the year. For the maize silage, the material used was harvested with the optimal moisture for ensiling. Roughage and water were available for ad libitum consumption. The daily feed and water intake was recorded by an automated feeding system with ear transponders (Insentec, Marknesse, The Netherlands). The bulls had access to 2.8 kg of concentrate per day containing mineral supplement and FMA and/or glycerol (Table 1). Glycerol was fed as isoenergetic compensation for FA to exclude effects of FA on performance.

The feed was provided via feeding stations (Type AWS HF 2ST, manufacturer: Insentec, Marknesse, The Netherlands). The composition of the different concentrates and the experimental design are summarised in Tables 1 and 2. The rations were calculated according to the recommendations given by the Society of Nutrition Physiology (GfE 1995). The body weight was measured weekly with cattle weighing scales.

Table 1: Experimental design and allowance of daily concentrate intake

	Experimental groups							
	M0	M100	M200	M300	G0	G100	G200	G300
Animals per group	8	8	8	8	8	7	7	8
Silage type (*ad libitum*)	Maize	Maize	Maize	Maize	Grass	Grass	Grass	Grass
Fumaric acid supplementation (g/d)	0	100	200	300	0	100	200	300
Glycerol supplementation (g/d)	300	200	100	0	300	200	100	0
	Daily concentrate allowance [kg/animal]							
Concentrate A	0	0.8	1.6	2.4	0	0.8	1.6	2.4
Concentrate B	2.4	1.6	0.8	0	2.4	1.6	0.8	0
Concentrate C	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Sampling and slaughtering

Samples of the silages were collected twice a week and concentrates were collected weekly and pooled within a month for nutrient and fibre analysis. To analyse potassium (K) and

sodium (Na), three pooled samples of the silages and concentrates were collected over the whole experimental period.

Blood samples were taken periodically (every 70 days) from Vena jugularis externa to monitor the development of animal health (Kraft and Dürr 2005). Blood gas statuses were taken to estimate the acid–base balance of the animals (Goad et al. 1998; Brown et al. 2000). A total of 2 ml of blood were collected in blood gas monovettes (Sarstedt AG & Co, Nümbrecht, Germany) and immediately placed on ice. The samples were analysed at the Clinic for Cattle, School of Veterinary Medicine, Hanover, Germany.

Table 2: Composition of concentrates

Components [%]	Concentrate A	Concentrate B	Concentrate C
Wheat	4.17	4.17	25
Rapeseed meal	29.17	29.17	
Dried sugar beet pulp	54.16	54.16	39.5
Fumaric acid *	12.5		
Glycerol †		12.5	
Premix ‡			18.75
Urea			8
Calcium-carbonate			8.75

Feed Grade, ADM Silo Rothensee GmbH & Co.KG, Magdeburg, Germany;
{Contained per kg premix: Ca, 250 g; P, 40 g; Na, 85 g; Mg, 35 g; vitamin A, 560,000 IU; vitamin D3, 70,000 IU; vitamin E, 1.05 g;Mn, 3 g; Zn, 5 g; Cu, 0.7

Due to the observation that the grass silage-fed animals had more fluid faeces and drank more water, faeces samples from all animals were collected on days 70, 140 and 210 of experiment. The faeces dry matter (DM) was determined. At the end of the feeding period, the faeces of the subsequently slaughtered animals were freeze dried for analysis of acid-insoluble ash (AIA) and for the calculation of the apparent digestibility (AD) of the total ration. On the day before faeces sampling, feed samples from the silages and the concentrates were taken to analyse the nutrient content and the AIA.

The first five animals of groups M0, M300, G0 and G300 (see Table 1), which attained 580 kg body weight were slaughtered without fasting in the slaughter house of the institute. Because of limited capacity, it was impossible to slaughter all animals. The pH of the rumen

fluid was measured approximately 30 min after time of death. The carcass dressing yield was calculated.

Balance experiment with wethers

A balance study to investigate the nutrient digestibility and to calculate the ME of the maize and grass silage was conducted according to the standards given by the German Society of Nutritional Physiology (GfE 2001). For every silage type, four adult wethers were adapted to the feed over 14 days. The animals were fed with restricted amounts of silage (860 g DM/d) two times a day (07:00 h and 14:00 h). To compensate the protein lack in maize silage, the wethers received additional 20 g urea per day. In an 8-day-collection period, the animals were kept in balance cages which allowed the quantitative collection of the faeces. The total faeces were pooled to one sample per animals. Feed samples were collected daily and were also pooled to one sample per collection period. The samples were homogenised and dried at 608C for 72 h. Afterwards, they were ground to pass through a 1 mm screen.

Analysis

According to VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungsund Forschungsanstalten) method No. 3.1, the DM of feed and faeces were determined by oven-drying for 4 h (103°C) (Naumann and Bassler 1993). To analyse ash, the samples were burnt at 5508C for 4 h (VDLUFA; method no. 8.1). Nitrogen was measured with a mixed catalyst Kjeldahl method according to VDLUFA method No. 4.1.1, and the crude protein content was calculated by multiplying nitrogen by 6.25. Ether extract was determined by method No. 5.1.1 (Naumann and Bassler 1993), with the Soxhlet method with petroleum ether as a solvent. Acid detergent fibre is shown exclusive of residual ash (ADFom) and was analysed according to Goering and Van Soest (1970). The analysis of neutral detergent fiber (NDFom) of maize silage was made with a heat stable amylase and expressed exclusive of residual ash (Van Soest et al. 1991). The NDFom of grass silage and the other feed components were analysed without a heat stable amylase (Van Soest et al. 1991) and also expressed without residual ash. The K and Na content of the feedstuff were analysed in the laboratory of the Institute of Animal Nutrition, Braunschweig according to the methods of the VDLUFA (Naumann and Bassler 1993).

The AIA was analysed with the 4N HCl-method, an adapted form of the method described by McCarthy et al. (1974) and Wünsche et al. (1984), where AIA is used as a natural marker. A total of 2 to 5 g of freeze dried faeces or feed were ashed for 5 h at 5508C. The ashes were boiled for 15 min with 4N HCl and the residues were neutrally filtered with ashless filter paper. The dried filters with residues were ashed once more to obtain the amount of AIA in faeces or feed. The nutrient content in faeces and in the feed samples was analysed in the same way as the monthly pooled samples (see the description above). The measurements of AIA are suited to predict the digestibility of ruminants (Thonney et al. 1985; Sunvold and Cochran 1991).

The red blood cells were analysed with an automated haematology analyser for Veterinary (Celltac alpha MEK-6450, Nihon Kohden Corporatin, Tokyo, Japan) and the blood gases by the blood gas system rapidlab 348 system (Siemens Healthcare Diagnostics Deerfield, USA).

Calculations and statistics

Metabolisable energy of maize and grass silage was calculated as recommended by the German Society of Nutritional Physiology (GfE 2001) using the results from balance experiments with wethers. Based on Mach et al. (2009), ME for FMA was calculated as 13.6 MJ/kg DM. The ME of glycerol was supposed to be 13.5 MJ/kg according to Südekum (2008). For the components of the concentrates, the ME was calculated with table values from the DLG (1997). The apparent digestibility of the different diets was calculated based on the AIA and nutrient content of the diet and the faeces:

AD [%] = *([(NN/IN)-(NF/IF)] / NN/IN)* * 100

where NN, nutrient content in the feed; IN, AIA content in the feed; NF, nutrient content in faeces; IF, AIA content in faeces. All data are given in percent.

The ME content of the total ration can be calculated with the so-measured AD and the regression equation given by the GfE (2001). For this calculation, the mean feed intake was taken from each tested animal during the last three days before the sampling.

Dressing percentage was calculated as quotient of warm carcass weight and slaughter weight.

All statistics, except for the blood analyses, were done with Statistica 8.0 for Windows operating system (StatSoft Inc., 1984–2007) using a two-way factorial design of analysis of variance (ANOVA) with the different silages and the FMA supplementation as factors. Significant mean value differences were evaluated by the Tukey-test. Linear regressions were made to analyse correlations and results were given in correlation coefficients r and significances as *p*-values.

The blood analyses and the DM of faeces were evaluated by SAS (9.1.3 Service Pack 4; SAS Institute Inc., Cary, NC, USA) using the mixed procedure. Fixed effects were the FMA supplementation, the silage type and the interaction of FMA 6 silage. Random effect for the analysis was the individual animal. The sampling time point (Day 0, 70, 140 and 210) was added as a covariate to minimise the individual animal effect and get Day 0 as base. The Tukey-test was chosen for post hoc-test. All statistical significances were accepted by $p < 0.05$ and trends with $p < 0.1$.

Results:

Growth performance

The experiment took a normal course and the animals showed no abnormalities. Three bulls were excluded from the analysis due to illness: Two animals of group G0 were excluded due to chronic claw inflammation (day 187 of experiment) and unexplainable growth retardation (day 283 of experiment). In group G300, one bull suffered from chronic pneumonia and dropped out of the experiment after 139 days.

The mean DM and nutrient content of the analysed silages and concentrates and their energy contents over the whole experiment are shown in Table 3. The silages were of normal quality, and the energy contents of concentrate A and B were 12.2 MJ/kg DM. On average, the animals consumed the restrictively offered concentrate entirely. Consequently, they consumed the designated amounts of FMA (Table 4). The mean daily dry matter intake (DMI, 8.81 ± 0.07 kg) was neither influenced by the FMA supplementation nor by the silage type. The mean silage DM consumption varied between 6.0 and 6.6 kg/d.

The daily water intake (DWI) of animals fed the maize silage was on average 6.3 kg lower ($p < 0.001$) than that of the animals fed the grass silage based diets (Table 4). Correlations between the DWI and the intake of crude ash, crude protein, NDF, K and Na ranged from 0.44 to 0.68 and were all significant ($p < 0.05$). Negative correlations were found for the correlation between DWI and AD of ether extract and crude protein. No correlation was found for DWI and the AD of organic matter.

The average daily weight gain was around 1.30 ± 0.02 kg/d (Table 4) and was not influenced by silage type or FMA supplementation. The calculated feed per gain and energy per gain were also not influenced by dietary treatments.

Nutrient digestibility

The AD of ether extract, ADFom and NDFom was significantly influenced by silage type ($p < 0.001$; Table 5). The AD of organic matter was slightly higher for grass fed animals ($p = 0.057$). Higher values of AD could be observed for the grass silage fed animals for all fibre fractions. The AD of ether extract was increased for the maize fed animals. No differences in AD were found for crude protein. The FMA supplementation did not influence the digestibility of the nutrients.

Table 3: Nutrient contents and digestibility of concentrates (n = 9) and the silages (n = 10)

	Concentrates			Silages	
	A	B	C	Maize	Grass
Dry matter [g/kg]	906	885	916	346	365
Nutrient content [g/kg DM]					
Crude ash	64	70	295	41	88
Crude protein	160	164	301	77	120
Crude fat	22	24	12	32	33
Crude fibre	133	137	73	195	275
Acid detergent fibre (ADF_{OM})	175	179	90	223	298
Neutral detergent fibre (NDF_{OM})	373	353	199	419	521
Potassium (n = 3)	10	11	6	12	31
Sodium (n = 3)	1	4	18	0	1
ME (MJ/kg DM)	12.2[+]	12.2[+]	8.1[+]	10.9[#]	9.9[#]
Digestibility of the silages[#]					
Organic matter				74.2	71.2
Ether extract				66.8	40.7

Notes: [+]Calculated with table values from the DLG (1997); [#]Measurements in wethers according to GfE (2001).

Positive linear correlations with the daily NDFom intake were found for the AD of NDFom and ADFom with correlation coefficients between 0.49 and 0.52 ($p < 0.05$). The AD of crude protein ($r = -0.66$) and ether extract ($r = -0.45$) correlated negatively with the NDFom intake ($p < 0.05$). There was no correlation between NDFom intake and AD of organic matter.

The ME for the total ration calculated with the AD predicted by the AIA-method was affected neither by silage type nor by FMA supplementation. The energy contents of the total rations ranged between 10.3 and 10.7MJ ME/kg DM for all examined animals on the considered experimental days (Table 5).

The values of DM of faeces were estimated over all sampling time points. The DM of faeces for the maize fed animals was 12.65 ± 0.33%, and for the animals fed grass silage 13.14 ± 0.36% (LSMEANS ± SEM). The DM of faeces was neither influenced by silage type nor by FMA supplementation.

Haematological profile

Since some of the animals did not reach the pre-assigned slaughter weight and were slaughtered before the last blood sampling on experimental day 210, the number of analysed samples (*n*) varied between the different feeding groups (Table 6). Most of the haematological profiles were within the reference range. This leads to the conclusion that the estimated statistical effects had no clinical relevance on animal health. The erythrocytes count and the analysed mean corpuscular haemoglobin concentration (MCHC) values were all higher than the reference area. In contrast, all mean corpuscular volume (MCV) and most of the mean corpuscular haemoglobin (MCH) were lower than the reference area (Table 6). This may be caused by an unknown systematic problem. Fumaric acid supplementation decreased the haemoglobin concentration (Hb), the packed cell volume (PCV; haematocrit), the mean corpuscular volume (MCV) and the mean corpuscular haemoglobin (MCH) significantly. The control groups showed a higher Hb than groups with FMA supplementation. The leucocytes count was higher for the animals fed grass silage ($p < 0.05$). A trend of an interaction between FMA and silage type was detected for the erythrocytes count ($p < 0.1$). The erythrocytes count of maize fed animals increased with FMA supplementation and decreased with FMA for the grass fed animals.

Blood gas analysis

The FMA supplementation did not influence blood gas results (Table 7). The measured blood gas values varied within a small range. The base excess and the standard bicarbonate were significantly higher for animals fed with grass silage ($p < 0.05$). A trend of an interaction of FMA 6 silage was found for blood pH and for partial pressure of CO_2 ($p < 0.1$). While blood pH for maize fed animals decreased with FMA supplementation, the pH tended to increase for the groups fed grass silage. The opposite results could be demonstrated for the CO_2-pressure.

Slaughtering results

The animals were slaughtered with a final body weight of 579 ± 1.6 kg (mean ± SEM). The measured rumen pH was not influenced by dietary treatments (Table 8). The dressing percentage was slightly increased due to FMA supplementation ($p = 0.054$) and this effect occurred at a slightly higher level when grass silage was fed as roughage ($p = 0.085$). For dressing percentage no interaction of FMA 6 silage were observed.

Table 4: Mean feed intake, liveweight gains and feed efficiencies in dependence on fumaric acid (FA) supplementation and roughage type (maize silage (M), grass silage(G)) (Means ± SEM)

	Experimental group								ANOVA (probabilities)		
Group	M0	M100	M200	M300	G0	G100	G200	G300	Silage	FA	Silage × FA
Animals per group	8	8	8	8	6	7	7	7			
Feed intake [kg DM[+]/day]											
Silage	6.64 ± 0.28	6.15 ± 0.11	6.24 ± 0.13	6.34 ± 0.24	6.18 ± 0.18	6.34 ± 0.27	6.21 ± 0.15	6.04 ± 0.11	0.476	0.355	0.568
Concentrate	2.46 ± 0.05	2.51 ± 0.01	2.51 ± 0.01	2.52 ± 0.02	2.50 ± 0.03	2.55 ± 0.01	2.53 ± 0.01	2.54 ± 0.01	0.084	0.192	0.938
Total	9.10 ± 0.28	8.66 ± 0.11	8.75 ± 0.13	8.86 ± 0.23	8.68 ± 0.19	8.89 ± 0.28	8.74 ± 0.15	8.58 ± 0.12	0.631	0.519	0.588
Fumaric Acid											
Dosage [g/day]	0^{d} ± 0	100^{c} ± 1	199^{b} ± 1	298^{a} ± 3	0^{d} ± 0	100^{c} ± 0	200^{b} ± 1	302^{a} ± 1	0.294	< 0.001	0.609
Content [g kg/DMI[#]]	0^{d} ± 0	11^{c} ± 1	23^{b} ± 1	35^{a} ± 1	0^{d} ± 0	12^{c} ± 0	23^{b} ± 1	34^{a} ± 3	0.382	< 0.001	0.374
Nutrient intake											
Water [kg/d]	19.2^{b} ± 2.5	16.8^{b} ± 1.8	19.8^{b} ± 1.9	18.7^{b} ± 1.3	25.9^{a} ± 2.9	26.6^{a} ± 2.7	23.5^{a} ± 1.7	28.8^{a} ± 0.9	< 0.001	0.937	0.483
ME[†] [MJ/d]	95.7 ± 2.7	91.4 ± 1.1	92.3 ± 1.3	93.3 ± 2.3	93.6 ± 2.0	93.7 ± 2.8	92.2 ± 1.6	90.6 ± 1.2	0.660	0.547	0.591
Crude protein [g/d]	955^{b} ± 23	929^{b} ± 11	935^{b} ± 13	938^{b} ± 18	1192^{a} ± 18	1200^{a} ± 30	1175^{a} ± 19	1150^{a} ± 14	< 0.001	0.462	0.480
Daily weight gain [g/d]	1330 ± 37	1366 ± 60	1307 ± 53	1330 ± 64	1253 ± 113	1285 ± 63	1185 ± 50	1281 ± 54	0.129	0.563	0.866
Feed per gain [kg DM/kg]	6.9 ± 0.3	6.4 ± 0.3	6.8 ± 0.3	6.8 ± 0.4	7.0 ± 0.5	7.0 ± 0.3	7.4 ± 0.2	6.8 ± 0.3	0.141	0.609	0.686
Energy per gain [MJ ME/kg]	72.4 ± 3.2	67.7 ± 2.8	71.3 ± 2.7	71.2 ± 3.8	73.8 ± 5.5	73.7 ± 3.0	78.4 ± 2.6	71.5 ± 3.3	0.131	0.613	0.696

Notes: [+]DM, Dry matter; [#]DMI, Dry matter intake; [†]ME, Metabolisable energy. Mean values with different superscripts within a row are significantly different (p <0.05).

Table 5: Apparent digestibility and metabolisable energy (ME) of total rations for groups fed no or the highest amount of fumaric acid (FMA) (Means ± SEM; n=5)

	Experimental group				ANOVA probabilities		
	M0	M300	G0	G300	Silage	FA	Silage × FA
Digestibility [%]							
Organic matter	71.4 ± 2.0	73.2 ± 1.8	77.0 ± 1.9	74.8 ± 1.1	0.057	0.911	0.262
Crude fat	70.3^{a} ± 2.8	70.6^{a} ± 2.2	59.6^{b} ± 2.8	57.0^{b} ± 2.6	0.000	0.667	0.576
Crude fibre	56.1^{b} ± 2.6	59.1^{b} ± 3.1	75.9^{a} ± 3.3	73.6^{a} ± 0.9	0.000	0.886	0.328
Crude protein	64.0 ± 2.7	58.5 ± 3.8	61.4 ± 4.6	60.1 ± 2.8	0.898	0.360	0.573
ADF_{OM}	53.1^{b} ± 3.1	56.8^{b} ± 3.6	74.5^{a} ± 3.0	72.4^{a} ± 0.7	0.000	0.784	0.322
NDF_{OM}	55.1^{b} ± 3.0	60.0^{b} ± 2.8	76.1^{a} ± 3.0	74.8^{a} ± 1.0	0.000	0.499	0.251
ME total ration [MJ/kg DM]	10.5 ± 0.3	10.7 ± 0.3	10.6 ± 0.3	10.3 ± 0.2	0.600	0.925	0.295

Note: Mean values with different superscripts within a row are significantly different (p <0.05).

Table 6: Red blood cell count and leucocytes in dependence on fumaric acid (FA) supplementation and roughage type (maize silage (M), grass silage(G)) corrected over the four sampling times (LSMEANS ± SEM)*.

	Experimental group								ANOVA probabilities		
	M0	M100	M200	M300	G0	G100	G200	G300	Silage	FA	Silage × FA
Animals per group	31	30	30	30	22	26	26	28			
Leucocytes [10^9/l] (8.0-10.0)	10.1 ± 0.5	8.9 ± 0.5	8.6 ± 0.5	9.5 ± 0.5	10.1 ± 0.6	9.2 ± 0.5	9.6 ± 0.5	9.1 ± 0.5	0.043	0.936	0.690
Erythrocytes [10^{12}/l] (6.0-8.0)	9.1 ± 0.2	9.0 ± 0.2	9.1 ± 0.2	9.0 ± 0.2	8.9 ± 0.2	9.2 ± 0.2	8.5 ± 0.2	9.2 ± 0.2	0.255	0.924	0.086
Hemoglobin [g/dl] (8.0-14.0)	13.1 ± 0.3	12.3 ± 0.3	13.0 ± 0.3	12.5 ± 0.3	13.1 ± 0.3	12.0 ± 0.3	12.4 ± 0.3	12.2 ± 0.3	0.655	0.031	0.197
PCV [+] [%] (25.0-35.0)	35.6 ± 0.8	35.5 ± 0.8	35.7 ± 0.8	33.6 ± 0.8	33.4 ± 0.9	33.8 ± 0.8	32.3 ± 0.8	33.0 ± 0.8	0.598	0.033	0.138
MCV [#] [μm^3] (40.0-60.0)	39.3^{ab}± 0.8	39.4^{ab}± 0.8	39.7^{a}± 0.8	37.3^{ab}± 0.8	37.4^{ab}± 0.9	37.2^{b}± 0.8	38.2^{ab}± 0.8	36.1^{ab}± 0.8	0.210	0.006	0.982
MCH [¶] [pg] (14.0-22.0)	14.5 ± 0.3	14.4 ± 0.3	14.5 ± 0.3	13.9 ± 0.3	13.7 ± 0.3	13.7 ± 0.3	14.1 ± 0.3	13.4 ± 0.3	0.194	0.007	0.859
MCHC [†] [g/dl] (26.0-34.0)	37.0 ± 0.2	36.7 ± 0.2	36.7 ± 0.2	37.1 ± 0.2	36.8 ± 0.3	36.9 ± 0.2	37.1 ± 0.2	37.1 ± 0.2	0.858	0.317	0.486
PLT [‡] [10^9/l] (200-800)	384 ± 24	388 ± 28	414 ± 24	384 ± 26	468 ± 24	386 ± 26	418 ± 24	372 ± 25	0.229	0.332	0.140

Notes: [*]References areas in brackets (Dirksen et al. 1990); [+]PCV, Packed cell volume (haematocrit); [#]MCV, Mean corpuscular volume; [¶]MCH, Mean corpuscular haemoglobin; [†]MCHC, Mean corpuscular haemoglobin concentration; [‡]PLT, Platelets (thrombocytes); Mean values not sharing the same superscript are significantly different (p <0.05).

Table 7: Results from blood gas analysis and rectal temperature in dependence on fumaric acid (FA) supplementation and roughage type (maize silage (M), grass silage(G)) (LSMEANS ± SEM)

	Experimental group								ANOVA probabilities		
	M0	M100	M200	M300	G0	G100	G200	G300	Silage	FA	Silage × FA
Animals per group	31	30	30	30	22	26	26	28			
RT [+] [°C]	38.8 ± 0.1	38.9 ± 0.1	39.0 ± 0.1	38.9 ± 0.1	38.8 ± 0.1	38.9 ± 0.1	38.8 ± 0.1	38.7 ± 0.1	0.321	0.608	0.164
pH	7.355 ± 0.009	7.329 ± 0.009	7.328 ± 0.009	7.331 ± 0.009	7.342 ± 0.010	7.330 ± 0.009	7.356 ± 0.009	7.344 ± 0.009	0.379	0.844	0.078
CO_2 [#] [mm Hg]	60.0 ± 1.3	62.3 ± 1.4	62.4 ± 1.4	61.6 ± 1.4	63.8 ± 1.6	63.9 ± 1.5	61.9 ± 1.5	60.5 ± 1.4	0.657	0.743	0.071
pO_2 [*] [mm Hg]	42.6 ± 1.7	46.1 ± 1.8	43.9 ± 1.8	42.8 ± 1.8	40.9 ± 2.1	42.7 ± 1.9	42.9 ± 1.9	41.1 ± 1.9	0.358	0.095	0.744
HCO_3a [§] [mmol/l]	32.7 ± 0.6	31.0 ± 0.6	30.9 ± 0.6	30.9 ± 0.6	32.6 ± 0.7	31.8 ± 0.7	32.9 ± 0.7	31.3 ± 0.6	0.202	0.548	0.499
HCO_3s [~] [mmol/l]	28.5 ± 0.5	27.9 ± 0.5	27.4 ± 0.5	27.6 ± 0.5	29.1 ± 0.6	28.2 ± 0.5	29.6 ± 0.5	28.1 ± 0.5	0.028	0.962	0.111
BE [^] [mmol/l]	5.3 ± 0.5	4.2 ± 0.6	4.1 ± 0.5	4.2 ± 0.5	5.9 ± 0.5	4.9 ± 0.5	6.3 ± 0.5	4.8 ± 0.5	0.030	0.568	0.254
O_2-Sat. [$] [%]	66.9 ± 2.5	69.6 ± 2.9	66.9 ± 2.5	66.5 ± 2.7	63.7 ± 2.5	65.9 ± 2.7	67.8 ± 2.5	64.8 ± 2.6	0.288	0.117	0.352

[+] 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

Discussion:

The objective of the present study was to estimate the influence of a directly fed acid (FMA) on the rumen fermentation and consequently on the acid–base balance.

Therefore, a control substance, for which FMA was substituted, should not have acidic attributes at the moment of feeding. Moreover, this substance should not bring additional nutrients such as crude protein or starch into the ration. For that reasons, the 3-carbon compound glycerol was used as control substance. It will be fermented to propionic acid (Kijora et al. 1998; Krehbiel 2008), one of the main short chain fatty acids (SCFA) resulting from rumen carbohydrate degradation. The resulting acidic attributes were not the subject of this study.

With regard to the early exclusion of three bulls (two from Group G0 and one from Group G300) due to different types of illness, it seems to be reasonable to assume that dietary treatments were not responsible although all three bulls were fed the grass silage-based diets.

During the whole experimental period, no acceptance problems due to FMA supplementation were found. This was in accordance to the fact that FMA is categorised as a harmless feed additive (Buntenkötter 1979). The initial assumption that the animals would not accept the free acid and would consequently have lower feed intake (Wallace et al. 2006) was not proven (see Table 4). In accordance with Molano et al. (2008), who found that the DMI was only depressed in wether lambs when the FMA supplementation was higher than 4% of DM, no significant differences in DMI were found in the present study (see Table 4). The observed comparable DMI of groups fed grass or maize silage was not expected because in former studies it was found that the silage DMI increased with increasing proportions of maize silage in the ration and that the DMI of fattening bulls was higher when maize silage was fed (Aston and Tayler 1980; Izumi et al. 1982; Browne et al. 2000).

The daily weight gain in the present study is comparable to previous studies with Holstein bulls at the Institute of Animal Nutrition in Braunschweig (Dänicke et al. 2002; Schumann et al. 2007; Meyer et al. 2008). The absence of silage-type effects on weight gain do not conform to the observations of Aston and Tayler (1980), who found decreased gains for grass fed bulls. Dose-depending effects of FMA were found in former *in vitro* studies (Asanuma et

al. 1999; Lopez et al. 1999; Carro and Ranilla 2003), but in the present study this could not be confirmed.

The lack of interaction of FMA 6 silage on weight gain was unexpected. This could indicate that FMA acts differently *in vivo* and that more studies comparing *in vitro* and *in vivo* conditions are needed. The missing dose-response effect could also be based on the fact that FMA had the same influence on the growing of the bulls as glycerol. Castillo et al. (2007) found that malic acid, a precursor for propionic acid like FMA, had no positive effects on the growth performance of fattening bulls. This supports the results of the present study.

In the present study, the AD of NDF_{om} and ADF_{om} was not influenced by FMA supplementation (see Table 5), which confirms the results of Bayaru et al. (2001). However, a reduced fibre digestion caused by FMA supplementation was expected by Wood et al. (2009). For that reason they used an encapsulated form of FMA to reduce this risk of rumen acidosis and a consequent decrease in fiber degradation. But in our study, no such effects were observed although FMA was not supplemented in encapsulated form. In contrast to our results, where FMA supplementation had no influence on the digestibility of the tested nutrient, Bayaru et al. (2001) found an increased AD for crude protein with FMA supplementation.

The differences between the AD from maize and grass silages are consistent to those found by O'Mara et al. (1998). The ADF_{om} and NDF_{om} of grass silage were better digested (see Table 5), which could be an explanation why the silage type did not have a significant influence on the daily weight gain. However, this disagrees with the lower ME of the grass silage determined by the wether experiment (see Table 3). The reason of the different results can be seen in the fact that the wether experiment and the AIA analyses to calculate AD are two different methods with different goals. To calculate the ME of silages, wethers were fed restrictively and the silages were offered separately. In contrast, the bulls were fed *ad libitum* and the AD was calculated for the complete ration. A possible reason for the effect of the silage type on AD could be the different amounts of NDF_{om} in the silages. Strong correlations between daily NDF_{om} intake and AD were found.

Although faeces consistency was only spot-checked for quantitative evaluation, it appeared that bulls fed the grass silage-based diets excreted faeces with a lower DM content than their

maize silage fed counterparts. But the spot-checked analyses could not confirm these observations. The higher water intake of the grass silage fed animals could also be a result of the higher content of crude protein, ash or K in this silage. It is known that there is a high correlation between crude protein and K in feed and the water intake from cows (Kume et al. 2010). The amount of urine from the animals could not be measured in this experiment, thus it cannot be attested that the animals with higher water intake urinated more than the others. Further studies are needed to compare the different silages parallel over the long-term with view to water intake.

In the present experiment, neither the pH-value of blood nor of rumen fluid was influenced by feeding (Tables 7 and 8). The animals did not show the typical decrease in blood pH and bicarbonate (Goad et al. 1998; Brown et al. 2000), which can be observed for animals with acute acidosis. The influence of the silage type on blood standard bicarbonate and on base excess might be a reflection of the higher starch content in the maize silage because it increases the risk of acidosis (Owens et al. 1998). Nevertheless, this contradicts the observations of AD. Mazzenga et al. (2009), who worked also with cattle fed with maize silage, found blood pH values in the same range than in our experiment. The supplementation of FMA did not influence the blood bases, which contradicts results of Castillo et al. (2007), who found for malic acid a tendency of decreased blood bases.

The low level of rumen fluid pH after slaughter can have several reasons (see Table 8). On the one hand, it could be a result of the non-restricted feeding of the animals before slaughtering. Observations from previous studies suggest an increased pH after supplementation of FMA or other organic acids (Isobe and Shibata 1993; Callaway and Martin 1996; Molano et al. 2008). But it could be expected that the organic acids operated as a pH buffer (Castillo et al. 2004) when they were fermented. This contradicts the fact that FMA is given to the animals as an acid. Therefore, Isobe and Shibata (1993) supposed that FMA stimulates the saliva production; but this could not be monitored in the present study.

On the other hand, in past it was observed that glycerol supplementation decreased the rumen pH (Kijora et al. 1998; Mach et al. 2009; Wang et al. 2009). Therefore, it was expected that the differences between groups M0 and G0 were significant because the high content of starch in maize silage is known to decrease the rumen pH and increase the risk of acidosis. Hence, the expected interaction of FMA × silage on rumen pH was not confirmed.

Another reason for the comparatively low levels of rumen pH can be the time period between the death of the animals and the sampling of rumen fluid. The necessary active transport, which is needed for the absorption of SCFA (Gäbel and Sehested 1997), stops at the time of death. But the microbial fermentation does not stop and still produces SCFA. So it can be assumed that the decreased pH values are due to an increased amount of SCFA, which normally would be absorbed.

Kirchgeßner and Roth (1979) found that supplementation of FMA has no effect on meat and carcass quality of pigs. The results of the present study could confirm this for some carcass characteristics of bulls (empty body weight and abdominal fat; data not shown), even though the dressing percentage tended to be higher after supplementation of FMA (p = 0.054) and after feeding grass silage. The later finding disagrees with the study of Aston and Tayler (1980) who found no influence of silage type on dressing percentage. The effect of FMA on dressing percentage could mean that the animals got more energy for meat production. The difference seems not to be large (see Table 8), but for groups receiving grass silage plus FMA this means 10 kg more carcass weight. This observation could also be a consequence of the higher AD values for the grass fed animals. Unfortunately, there are no other studies available on dressing percentage of bulls in which grass and maize silage were compared. Therefore, a definite conclusion cannot be drawn.

In the present study, the hypothesis of an interaction between FMA and silage type could not be proved by most of the results. Nevertheless, Garcia-Martinez et al. (2005) found in an *in vitro* experiment that fumarate had the greatest effect in high-forage diets. Also Kolver and Aspin (2006) found *in vivo* that the mode of action of fumarate also depends on the diet. But this phenomenon could not be confirmed with the current experiment.

Conclusions:

Overall, it can be concluded that FMA can be fed to growing bulls with a live weight of 270 to 580 kg at 35 g per kg DMI without any apparent signs of incompatibility. None of the tested parameters showed that the supplementation of FMA increased the risk of acidosis for growing bulls. No dose-response effects were found. The growth and carcass yield were not significantly influenced by FMA in comparison to glycerol supplementation. Only the

dressing percentage showed a tendency to increase with FMA. For FMA supplementation up to 300 g/d, the hypothesis of an interaction between silage type and FMA could not be confirmed. Reasons for the missing effect could be underdosing of FMA, or that glycerol influenced the observed parameters in the same way as FMA. Besides testing higher doses of FMA in diets based on maize or grass silage, it would be also important to measure the methane emissions to prove if FMA is as an efficient methane reducer *in vivo* as *in vitro*.

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5 Paper III

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

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 [pka = 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.

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/d/animal.
† Treatment 300: 300 g FA/d/animal.
‡Treatment 600: 600 g FA/d/animal.
§ one animal had to be excluded because of losing its cannula.

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.

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 analyzing 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), Crconcentrations 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 ll 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 ll with a final concentration of 1x PCR buffer; each dNTP 0.2 mmol/ll; forward and reverse primer 0.05 lmol/ll; HotStarTaq DNA polymerase, 0.025 U/ll (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/ll) 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 ll. 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 ll. Single-strand DNA (ssDNA) was achieved from dsDNA by k-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 k-exonuclease buffer, 2.5 U k-exonuclease (New England Biolabs GmbH, Frankfurt, Germany) in a total reaction volume of 40 ll 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 behavior 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.

Table 2: Primers for 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)	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	Schwieger and Tebbe (1998)
	Com2-Ph *rv*	907-926	CCG TCA ATT CCT TTG AGT TT	
archaea	Com1m *fw*	519-536	CAG C(A/C)G CCG CGG	Boguhn *et al.* (2010)
	Com2m-Ph *rv*	907-926	CCG CCA ATT CCT TTA	

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 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).

Net 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:

$$\text{DMF (kg/day)} = \frac{\text{chromium application [mg/d]}}{\text{duodenal chromium concentration [mg/gDM]}} / 1000.$$

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:

FOM [kg/d] = OM intake [kg/d] – (Duodenal OM flow [kg/d] – Microbial OM According to Schafft (1983) microbial OM was determined by the following equation:

Microbial OM [kg/d] = 11.8 * Microbial N [kg/d].

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

uCP [g/d] = (NAN flow at the duodenum [g/d]) * 6.25 – endogenous CP [g/d].

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:

Ruminally degraded CP [g/d] = CP intake [g/d] – UDP [g/d]

UDP [g/d] = 6.25 * (NAN at the duodenum [g] – Microbial N [g]) – Endogenous CP [g].

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

FCM [kg/d] = ((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 productmoment 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).

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/d/animal.
† Treatment 300: 300 g FA/d/animal.
‡ Treatment 600: 600 g FA/d/animal.

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, isobutyric 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 9 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 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).

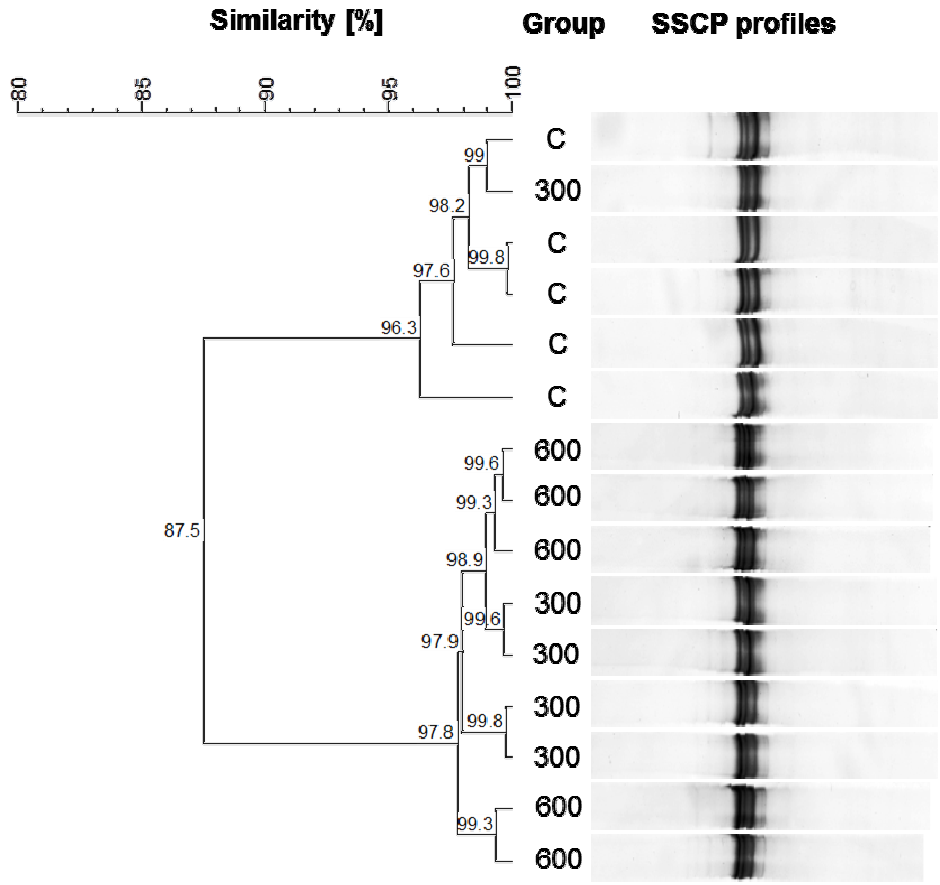

Figure 1: Dendrogram for the results of SSCP analysis of the community of archaea from treatments C, 300 and 600. Given are the similarities between the individual animals.

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		$6.08^{a} \pm 0.05$	$6.07^{a} \pm 0.06$	$5.93^{b} \pm 0.06$	0.008	< 0.001	< 0.001	0.207
NH_3-N	[mmol/l]	$7.6^{b} \pm 0.6$	$8.2^{a} \pm 0.6$	$7.4^{b} \pm 0.6$	< 0.001	< 0.001	< 0.001	< 0.001
Acetic Acid	[mol%]	$65.8^{a} \pm 0.5$	$64.0^{b} \pm 0.5$	$63.7^{b} \pm 0.5$	0.001	0.621	< 0.001	0.003
Propionic Acid	[mol%]	$16.5^{b} \pm 0.8$	$19.7^{a} \pm 0.8$	$20.2^{a} \pm 0.8$	< 0.001	< 0.001	< 0.001	0.001
Acetic:Propionic		$4.1^{a} \pm 0.2$	$3.3^{b} \pm 0.2$	$3.2^{b} \pm 0.2$	< 0.001	< 0.001	< 0.001	< 0.001
iso-Butyric Acid	[mol%]	$0.7^{b} \pm 0.0$	$0.8^{a} \pm 0.0$	$0.7^{b} \pm 0.0$	< 0.001	< 0.001	0.001	< 0.001
Butyric Acid	[mol%]	$14.4^{a} \pm 0.6$	$12.9^{b} \pm 0.6$	$12.9^{b} \pm 0.6$	< 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%]	$1.3^{b} \pm 0.1$	$1.7^{a} \pm 0.1$	$1.4^{b} \pm 0.1$	0.001	< 0.001	< 0.001	< 0.001
Total of SCFA	[mmol/l]	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/d/animal.

† Treatment 300: 300 g FA/d/animal.

‡ Treatment 600: 600 g FA/d/animal.

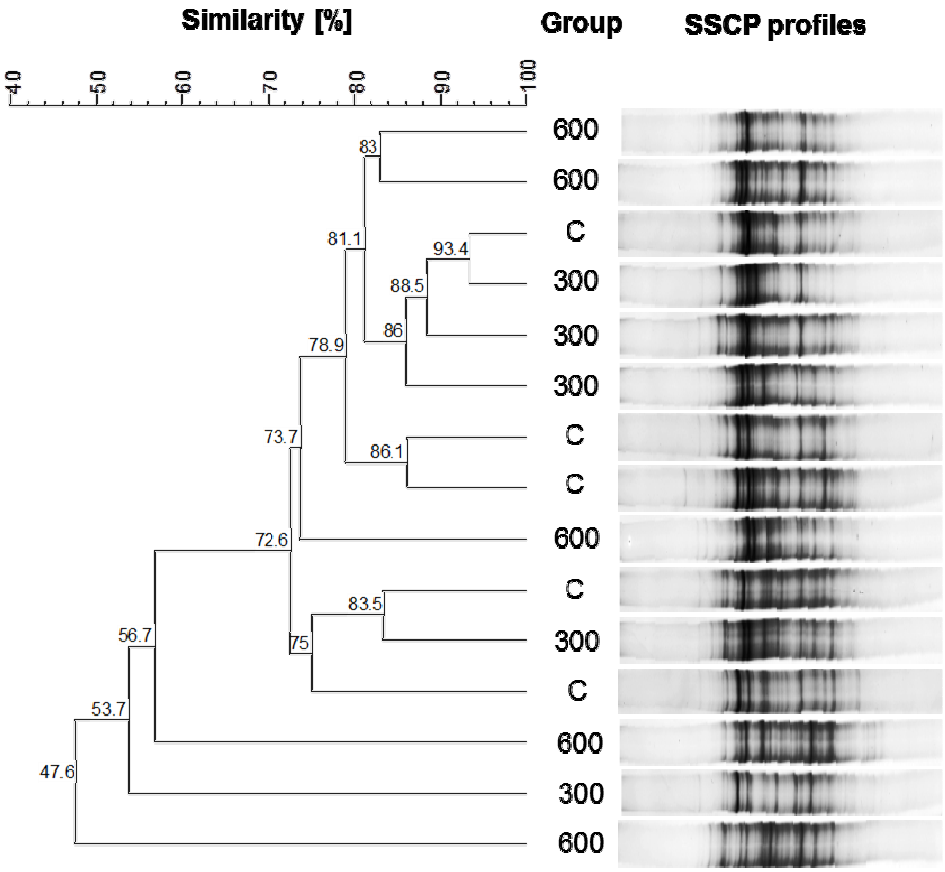

Figure 2: Dendrogram for the results of SSCP analysis of the bacterial communities from treatments C, 300 and 600. Given are the similarities between the individual animals.

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 acidbase 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 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.

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.

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 ¶ [mmol/l]		0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.454	0.658
BHB ¶ [mmol/l]		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/d/animal.

† Treatment 300: 300 g FA/d/animal.

‡ Treatment 600: 600 g FA/d/animal.

§ Reference range given by Clinic for Cattle, University of Veterinary, Foundation Hannover.

¶ No reference ranges available.

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	mmol/l	32.4 ± 0.8	30.7 ± 0.8	30.1 ± 0.7	0.105	< 0.001
HCO_3s	mmol/l	31.1 ± 0.8	29.7 ± 0.9	28.7 ± 0.7	0.125	0.003
BE	mmol/l	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/d/animal.
† Treatment 300: 300 g FA/d/animal.
‡ Treatment 600: 600 g FA/d/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 [m_M]	80 - 220	71.2 ± 9.8	48.0 ± 11.2	49.1 ± 9.7	0.123	< 0.001
Acid [m_M]	50 - 100	79.0 ± 6.6	76.5 ± 7.5	77.6 ± 6.5	0.960	0.219
Base [m_M]	150 - 250	158.4 ± 11.3	150.7 ± 11.9	157.8 ± 11.3	0.771	0.004
NH_4^+ [m_M]	< 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/d/animal.
† Treatment 300: 300 g FA/d/animal.
‡ Treatment 600: 600 g FA/d/animal.
§ Kraft and Dürr (2005).

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/d]					
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/d]	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/d/animal.

[†] Treatment 300: 300 g FA/d/animal.

[‡] Treatment 600: 600 g FA/d/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/d]	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/d]	255 ± 5.8	259 ± 5.5	247 ± 5.2	0.182	0.003
NAN [g/d]	242 ± 5.9	246 ± 5.6	234 ± 5.2	0.187	0.003
uCP [g/d]	1352 ± 29.9	1374 ± 28.2	1311 ± 26.3	0.190	0.003
UDP per CP [g/d]	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.

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 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).

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/d]	17.9 ± 1.0	19.6 ± 1.1	17.7 ± 1.0	0.208	0.013
Fat corrected milk[kg/d]	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/d]					
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 [m_M]	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.

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 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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6 Paper IV

Influence of fumaric acid on ruminal parameters and organ weights of growing bulls fed with grass or maize silage

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Abstract

The influence of the potential methane reducer fumaric acid (FA) on ruminal parameters, the rumen wall and organ weights was investigated in a long term study with growing bulls. Therefore, twenty bulls were fed with maize or grass silage as roughage and concentrate with or without 300 g FA per animal and day during the whole fattening period. After slaughtering, the organs were weighed and blood was analyzed for glucose, beta-hydroxybutyric acid (BHB) and non-esterified fatty acid (NEFA) levels. The ruminal fluid was analyzed for short chain fatty acids (SCFA), NH_3-N and the microbial community via single strand conformation polymorphism analysis. The rumen wall was examined histopathologically and the dimensions of the rumen villi were measured. FA decreased the blood BHB concentration and the butyric acid concentration in the ruminal fluid. The microbial community in the ruminal fluid was not influenced by FA. The rumen mucosa showed an interaction between FA and silage type for the center of inflammation. This interaction could also be found for the growth, length and surface of the rumen villi. The results of the rumen villi show that the influence of FA depends on the roughage used in the diet. In addition it could be shown that the villi size corresponds to the centre of inflammation of the rumen mucosa.

Keywords: Fumaric acid, growing bulls, ruminal parameters, slaughtering results, silage

Implications

Supplementation of the diet of fattening bulls with a strong acid such as fumaric acid (FA) could have consequences for the organism, especially for the rumen. FA is a potential methane reducer and has to be tested in a long term study for its side effects to preserve animal health. Therefore, its influence and interaction with the roughage fed to the bulls should be investigated. The organ weights, and mainly the rumen mucosa may give information about the long term effects of FA.

Introduction

The loss of energy for cattle as a result of erupted methane (CH_4) is estimated between 6 and 8% of gross energy (Flachowsky and Brade, 2007). Thus, propionic acid precursors have often been discussed as possible reducer of CH_4 from ruminal fermentation. In vitro studies show that fumaric acid (FA) is one of the most effective acids to reduce CH_4 emissions (Callaway and Martin, 1996, Martin, 1998). FA is a key intermediate in the succinate-propionate pathway, an alternative electron sink reaction for CH_4 production (Martin, 1998). Only few studies have investigated the influence of organic acid supplementation on animal productivity (Kolver and Aspin, 2006). Not much is known about the consequences for animal health (Castillo *et al.*, 2007). Especially the effects of supplementing a strong acid to the acid-base balance of the animals (FA: pka= 3.03; (Engel *et al.*, 2008)) are not yet well understood. Strong acidity has been discussed (Wood *et al.*, 2009) as one reason for the ruminal acidosis occurring in animals. The rumen is a complex ecosystem which developed over a long period of time (Hungate, 1966). Manipulations, like feeding of FA, might destabilize this ecosystem. About 85% of short chain fatty acids (SCFA) produced in the rumen are absorbed by the rumen papillae (Mach *et al.*, 2005). Supplementation of FA might lead to a change in the SCFA profile. It is known that propionic and butyric acid stimulate the growth of rumen villi, enlarging the absorption surface for ruminants (Mach *et al.*, 2005). Histological lesions in the rumen wall are, among others, one indication of acidosis (Owens *et al.*, 1998). The amount of non fiber carbohydrates (NFC) in the ration also influences the growth of the rumen villi (Mach *et al.*, 2006). Furthermore, it is known that CH_4 production is higher in fiber rich roughage (Flachowsky and Brade, 2007) and in consequence the reduction potential is higher. Therefore, the present study was conducted with a low fiber roughage (maize silage) as well as a high fiber carbohydrates (HFC; grass silage) roughage. Little literature is available reporting on the supplementation of FA to ruminants over a long period of time.

A growth experiment with fattening bulls was carried out to address the interactions between silage type and FA supplementation. While performance data was recently reported in Remling *et al.* (2011), the present investigation aimed at an examination of rumen microbial community and fermentation parameters, histopathological and histomorphometrical features of the rumen wall, and organ and tissue weights indicating the effects of dietary treatments.

Material and methods

Experimental design

Twenty out of a total of 62 fattening bulls of a growth experiment examining the interactions between silage type and FA addition (Remling *et al.*, 2011) were used for the present study. These 20 growing bulls (German Holstein breed) represented the average (live weight basis) of four different feeding groups: maize silage without (M0) or with 300 g FA per day (M300) and grass silage without (G0) or with 300 g FA per day (G300). The average initial body weight was 279 ± 38 kg (age: 232 ± 24 days).

Table 1 Experimental design with the eight different feeding groups and their allowance of daily concentrate intake

Group	M0	M300	G0	G300
Animals per group	5	5	5	5
Fumaric acid supplementation [g/d]	0	300	0	300
Glycerol supplementation [g/d]	300	0	300	0
Concentrate allowance [kg/animal/d]				
Concentrate A	0	2.4	0	2.4
Concentrate B	2.4	0	2.4	0
Concentrate C	0.4	0.4	0.4	0.4
Silage (ad libitum)	Maize	Maize	Grass	Grass

Experimental details were presented recently by (Remling *et al.*, 2011). Briefly, in a barn located at the Experimental Station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Federal Research Institute of Animal Health in Braunschweig, Germany, 62 animals were housed in groups of seven or eight animals. The barn was equipped with slatted floors with concrete and rubber surfaces (5.25 m^2 per animal).

The animals were equipped with ear transponders (Insentec, Marknesse, The Netherlands) to determine the daily feed and water intake. The bulls had access to 2.8 kg of concentrate containing mineral supplement and 300 g FA (Novus Deutschland GmbH, Gudensberg, Germany) per day, respectively 300 g glycerol (GLY: Concerine CD 80 Feed Grade, ADM Silo Rothensee GmbH & Co. KG, Magdeburg, Germany) per day as isocaloric compensation (see Table 1). Roughage and water were available for ad libitum consumption. The animals

were applied with energy and minerals according to the recommendations of the Society of Nutrition Physiology (GfE, 1995). The composition of the different concentrates is shown in Table 2. Water, roughage and concentrates were provided via feeding stations (Type AWS HF 2ST, Insentec, Marknesse, The Netherlands). The animals were slaughtered in the slaughter house of the institute at a mean body weight of about 580 kg.

Table 2: Composition of the three different concentrates

Components [%]	Concentrate A	Concentrate B	Concentrate C
Wheat	4.17	4.17	25
Rapeseed meal	29.17	29.17	
Dried sugar beet pulp	54.16	54.16	39.5
Fumaric acid	12.5		
Glycerol		12.5	
Premix [1]			18.75
Urea			8
Calcium-carbonate			8.75

[1] per kg Premix: Ca, 250 g; P, 40 g; Na, 85 g; Mg, 35 g; vitamin A, 560000 IU; vitamin D3, 70000 IU; vitamin E, 1050 mg; Mn, 3000 mg; Zn, 5000 mg; Cu, 700 mg; I, 50 mg; Co, 25 mg; Se, 25 mg.

Slaughtering and sampling

The animals had access to feed until slaughtering and five animals of each group were slaughtered in the institute's slaughter house. The carcasses, the viscera (full and empty; except liver and spleen), the different fat regions and the organs were weighed separately. Blood samples were collected to analyse beta-hydroxybutyric acid (BHB), non-esterified fatty acid (NEFA) and glucose. Rumen fluid was collected directly after opening the rumen to analyse short chain fatty acids (SCFA) and Ammonia-N (NH_3–N). A total of 100 ml of rumen fluid were immediately frozen at -18 °C for single strand conformation polymorphism analysis (SSCP) of the microbial community. For the morphological and histological examinations two pieces of about 5 x 8 cm of the rumen mucosa wall were excised from the caudal ventral ruminal sac according to Zitnan *et al.* (2003) and one part from the pilae ruminis. The mucosa was washed with 0.9 % NaCl solution and then clamped in a plastic frame and fixed in a 4 % formaldehyde solution.

Analyses

Glucose, BHB and NEFA in blood serum were analysed photometrically (Eurolyser CCA 180 VET; Greiner Diagnostik GmbH, Bahlingen, Germany). A gas chromatograph equipped with a flame ionization detector was used to analyse the short chain fatty acids (SCFA) of the ruminal fluid (Geissler *et al.*, 1976). NH_3–N in rumen fluid was analysed according to DIN38406-E5-2 (1998).

The samples of rumen fluid were prepared as described recently (Remling *et al.*, 2013, Riede *et al.*, 2013) to analyse the microbial community by means of single strand conformation polymorphism analysis (SSCP). Briefly, genomic DNA was extracted and the 16 S rDNA was amplified with polymerase chain reaction (PCR). Polyacrylamide gel electrophoresis was conducted after a single strand digestion. The results of the SSCP were visualized via silver staining (Riede *et al.*, 2013).

The rumen mucosa was histopathologically examined for inflammation. It were graded as follows: 0 = no visible lesions, 1 = few inflammatory infiltrates, 2 = some inflammatory infiltrates, 3 = = several inflammatory infiltrates. The number of centres of inflammation (COI) per 10 fields (10x magnification) was enumerated on both the villous area at the caudal ventral ruminal sac and the villi free area on the pilae ruminis. From the villous area of the ruminal sac, three parts of 1 cm^2 each were cut out. Each villus was dissected directly from the mucosa and transferred to white paper with a scale and photographed. The photographs were analyzed with the program ImageJ 1.45s (Wayne Rasband, National Institutes of Health, USA). The length, width and surface (two-dimensional) of the villi were measured and the number of villi per cm^2 was counted.

Calculations and statistics

The size of the rumen villi was calculated by measuring the pixel on the photographs which had been converted to values in mm with help of the scale. The surface of the whole rumen villi was measured with the tracing tool of ImageJ. The measured cm^2 of the surface was doubled due to the fact that the photographs present only one dimension of the villi as described by Hofmann and Schnorr (1982). To calculate the villous surface per cm^2 rumen mucosa wall the surfaces of the individual villi were multiplied with their number.

All statistics (except for SSCP analysis) were performed with Statistica 8.0 for Windows operating system (StatSoft Inc., 1984-2007). A two-way factorial design of the analysis of variance (ANOVA) was used with the different silages and the FA supplementation as factors (except rumen papillae histopathological examination). A Tukey-test was conducted as post-hoc test. The histology was evaluated with a contingence table which is based on the comparison between absolute frequencies for the particular classes with the expected frequencies and a Pearson's chi-squared test. For the statistical analysis of the SSCP, 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 were performed with dendrograms based on similarity matrices calculated using the Pearson product-moment correlation coefficient. The clustering algorithm was an unweighed pair group method using arithmetic averages (UPGMA). Statistical analysis was carried out using dissimilarity matrices following the method of Anderson (2001). All statistical significances were accepted by $P < 0.05$ and trends with $P < 0.1$.

Results

Weights of the carcasses

The weights of the different empty organs are given in Table 3. The 20 animals were slaughtered with a mean final body weight of 579 ± 2 kg (mean ± SEM), as described by Remling *et al.* (2011). FA did not effect the carcass composition and organ weights. Neither the silage type nor the FA supplementation influenced the empty body weights or the abdominal fat (Table 3). Effects were only found for the weights of the thymus and pancreas. The thymus weights were increased ($P = 0.01$) by maize silage. An interaction of FA and silage was found for the pancreas weight ($P = 0.004$). FA supplementation decreased pancreas weight when maize silage was fed and increased it with grass silage. The groups M0 and M300 were significantly different.

Blood

The concentrations of BHB, NEFA and Glucose in blood serum are shown in Table 4. The BHB values decreased significantly in response to FA supplementation ($P < 0.05$). The remaining parameters were not influenced.

Rumen fluid

SCFA and NH_3-N. The concentrations of ruminal SCFA and NH_3-N are shown in Table 5. The percentage of butyric acid decreased in the FA supplementation group ($P = 0.002$) and was higher for the animals fed maize silage ($P = 0.007$). The highest percentage of butyric acid was found in group M0. There were no interactions between silage and FA for SCFA in rumen fluid.

SSCP. Bacteria profiles were not affected by FA supplementation ($P = 0.239$; see Figure 1). However, there was an effect of silage type on microbial community ($P = 0.011$). No interaction of FA and silage type was found ($P = 0.802$). Regarding Archaea, neither the FA supplementation ($P = 0.645$) nor the silage type ($P = 0.410$) had a significant influence on the microbial community (Figure 2). An interaction of FA and silage type could not be determined for Archaea ($P = 0.533$).

Table 3 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on carcass composition and organ weights (Means; n=5)

Silage	Maize		Grass		s.e.m.	ANOVA (probabilities)		
Fumaric acid [g/d]	0	300	0	300		Silage	FA	Silage × FA
Live weight at slaughtering [kg]	578	581	578	579	2	0.873	0.646	0.692
EBW [kg]	507	510	511	518	2	0.205	0.260	0.635
Abdominal fat[1] [kg/100 kg EBW]	8.1	7.4	7.6	6.8	0.3	0.396	0.275	0.951
Rumen [kg/100 kg EBW]	1.70	1.60	1.80	1.80	0.04	0.087	0.815	0.730
GIT [kg/100 kg EBW]	4.4	4.6	4.7	4.7	0.1	0.223	0.474	0.460
Liver [g/100 kg EBW]	1590	1565	1682	1676	29	0.094	0.789	0.877
Lung [g/100 kg EBW]	693	782	731	777	19	0.651	0.091	0.576
Kidneys [g/100 kg EBW]	239	237	247	253	5	0.251	0.844	0.677
Heart [g/100 kg EBW]	460	426	453	433	9	0.991	0.182	0.724
Thymus [g/100 kg EBW]	116[a]	96[ab]	87[b]	91[b]	4	0.010	0.218	0.060
Pancreas [g/100 kg EBW]	115[a]	94[b]	98[ab]	111[ab]	3	0.928	0.436	0.004
Spleen [g/100 kg EBW]	253	218	218	220	7	0.195	0.177	0.140

EBW: Empty body weight (difference between live weight and the weights of the contents of the gastrointestinal tract and of the urinary bladder); GIT: Gastrointestinal tract

[1] Sum of the fat of the kidney cavity and the fat covering the gastrointestinal tract.

[a,b] Values within a row with different superscripts differ significantly at $P<0.05$

Table 4 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on BHB, NEFA and glucose in serum (Means; n=5)

Silage	Maize		Grass		s.e.m.	ANOVA probabilities		
Fumaric acid [g/d]	0	300	0	300		Silage	FA	Silage × FA
BHB [mmol/l]	0.78	0.61	0.65	0.52	0.04	0.127	0.038	0.831
NEFA [mmol/l]	0.20	0.20	0.20	0.22	0.01	0.529	0.652	0.589
Glucose [mg/dl]	95.9	93.6	91.3	84.6	3.0	0.284	0.473	0.722

BHB: beta-hydroxybutyric acid; NEFA: Non-esterified fatty acid

Table 5 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on NH3-N and SCFA concentrations and percentage of rumen fluid of the slaughtered animals (Means; n=5)

Silage	Maize		Grass		s.e.m.	ANOVA probabilities		
Fumaric acid [g/d]	0	300	0	300		Silage	FA	Silage x FA
NH_3-N [mmol/L]	10.25	13.08	8.92	2.63	1.66	0.073	0.581	0.157
Accetic acid [mol%]	61.2	62.9	62.5	64.8	0.7	0.245	0.136	0.840
Propionic acid [mol%]	18.1	20.2	19.8	20.0	0.4	0.344	0.165	0.256
iso-Butyric acid [mol%]	0.68	0.62	0.73	0.58	0.03	0.847	0.054	0.331
Butyric acid [mol%]	17.2^{a}	13.7^{b}	14.2^{b}	12.5^{b}	0.5	0.007	0.002	0.210
iso-Valeric acid [mol%]	1.21	1.08	1.24	0.73	0.10	0.450	0.134	0.359
Valeric acid [mol%]	1.71	1.52	1.61	1.43	0.07	0.535	0.212	0.961
Sum of SCFA [mmol/L]	130.3	132.1	134.4	136.6	2.4	0.407	0.693	0.971

SCFA: short chain fatty acids

^{a,b} Values within a row with different superscripts differ significantly at $P<0.05$.

Table 6 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on pathohistology from rumen papillae (frequency; n=5)

Group	diagnosis	M0	M300	G0[1]	G300	p[2]
villous area	no visible lesions	0	1	2	0	0.581
	few IF	0	0	0	0	
	several IF	3	4	3	5	
	many IF	2	0	0	0	
villi free area	no visible lesions	1	2	1	2	0.981
	few IF	1	2	1	1	
	several IF	3	1	2	2	
	many IF	0	0	0	0	

IF: inflammatory infiltrates

[1] G0 villi free area with n= 4.

[2] p = probability of Pearson Chi-square.

Rumen papillae

Histopathology. The results of the histopathological examination of the rumen mucosa are shown in Table 6. The integrity of the rumen mucosa was not influenced by the different feeding groups. The count of COI in the samples of villi free area was also not influenced. An interaction between FA and silage type was found for the samples of the villous area in the rumen mucosa (Table 7). The maize-fed animals showed more COI without FA, while the grass-fed animals showed the opposite result.

Villi number and size. The FA supplementation had no significant influence on the rumen mucosa villi number and size (Table 8). The silage type influenced all measured parameters except the number of villi per cm^2 ($P < 0.05$). The villi were smaller for the animals fed maize silage. An interaction of silage type and FA was found for all measured parameters except the villi surface per cm^2 mucosa ($P < 0.05$). The maize fed animals had bigger villi due to FA supplementation, in contrast to the grass silage fed animals where villi were smaller.

Table 7 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on the count of centres of inflammation [per 10 counted fields] of rumen papillae (Means; n=5)

Silage	Maize		Grass		s.e.m.	ANOVA (probabilities)		
Fumaric acid [g/d]	0	300	0	300		Silage	FA	Silage x FA
Villous area	3.6[a]	1.6[ab]	0.6[b]	3.0[ab]	0.4	0.221	0.754	0.003
Villi free area	0.8	0.2	1.5	1.4	0.3	0.151	0.585	0.696

[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

Table 8 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on rumen villi count, growth and surface (Means; n=5)

Silage	Maize		Grass		s.e.m.	ANOVA (probabilities)		
Fumaric acid [g/d]	0	300	0	300		Silage	FA	Silage × FA
Count per cm^2	29.3	25.4	20.9	35.9	1.9	0.774	0.141	0.013
Villi length [mm]	5.0^{b}	5.4^{b}	6.5^{a}	5.6^{ab}	0.1	0.000	0.894	0.000
Villi width [mm]	2.2^{c}	2.7^{b}	3.4^{a}	2.8^{b}	0.1	0.002	0.417	0.013
Surface per villi [mm^2]	18.0^{b}	24.7^{b}	35.0^{a}	26.7^{ab}	1.4	0.000	0.732	0.003
Villi surface per cm^2 rumen mucosa [mm^2]	562^{b}	595^{ab}	699^{ab}	978^{a}	55	0.016	0.141	0.247

[a,b] Values within a row with different superscripts differ significantly at $P<0.05$.

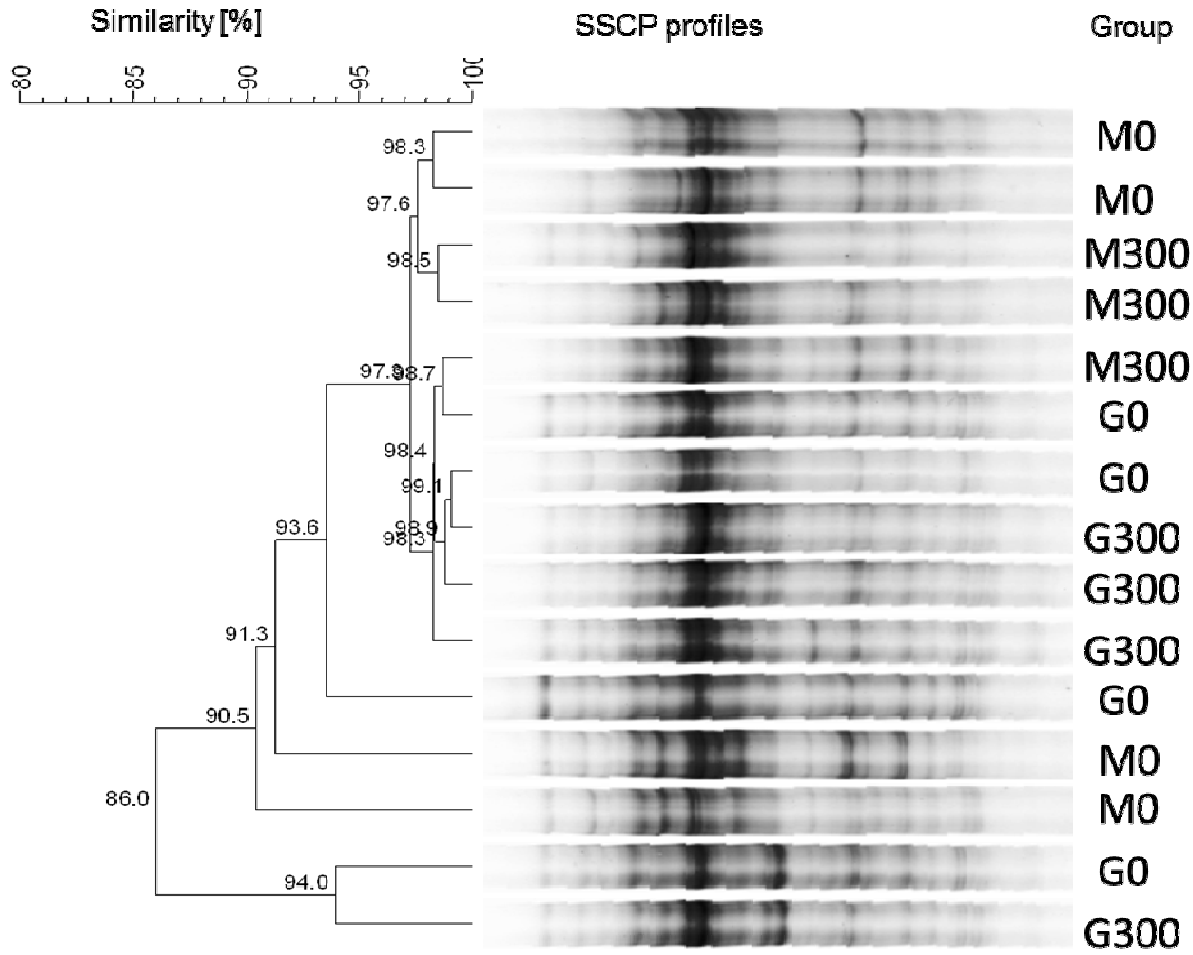

Figure 1: Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on the community of Bacteria (dendrogram for the results of SSCP analysis). Given are the similarities between the individual animals.

Discussion

The objective of the current study was to investigate the influence of a long-term supplementation of FA on the organ weights of bulls with special attention to the rumen, its mucosa and the composition of rumen fluid. Data on fattening performance and the acid-base balance of the animals were reported elsewhere (Remling *et al.*, 2011). Altogether, feeding 35 g FA per kg DMI for about 222 days did not result in intolerance and acidosis. There were no effects on daily weight gain, but the dressing, the quotient of warm carcass weight and slaughter weight, tended to be higher with FA supplementation (M0: 52.0 ± 0.3; M300: 52.4 ± 0.7; G0: 52.3 ± 0.6; G300: 54.0 ± 0.4 (Means ± SEM) (Remling *et al.*, 2011)). Carcass

weights show that feeding FA did not have an influence on body composition (see Table 3). This is in accordance with the observations of Kirchgessner and Roth (1979) who found no effects of FA on meat quality and carcasses, although their study was conducted with pigs and not with fattening bulls. No indications of long-term effects of FA on the organ weights could be found. The abdominal fat was not significantly influenced by FA supplementation and silage type. This could suggest that there was no energetic benefit for any of the different feeding groups. This corresponds with the results of Remling *et al.* (2011) who found that the daily weight gain with comparable daily ME intake was not influenced by FA. The results of the carcass weights, together with the results of Remling *et al.* (2011), suggest that FA could not reduce the CH_4 eructation insofar that the lower energy loss was measurable in the form of more abdominal fat or higher weight gain. The individual organ weights can provide information on animal metabolism and energy balance. For instance, the weight of the empty rumen tended to be higher for the grass fed animals (Table 3). In the present study, the bulls were fed according to feeding recommendations by the GfE (1995) and the difference in daily XP intake of the complete ration between grass and maize fed animals was significant, but not prominent (M0: 980 g/d; M300: 980 g/d; G0: 983 g/d; G300: 987 g/d/animal XP intake; ANOVA $P < 0.001$). Therefore, observations made by Stewart and Walsh (1967) regarding heavier rumen for calves fed a ration rich in protein can be applied to our study with growing bulls. In addition, the grass fed animals tended to have heavier rumina which might correspond with bigger rumen villi (Table 8). The significantly heavier thymus of maize fed animals and the trend of heavier lungs for animals fed with FA can not be explained.

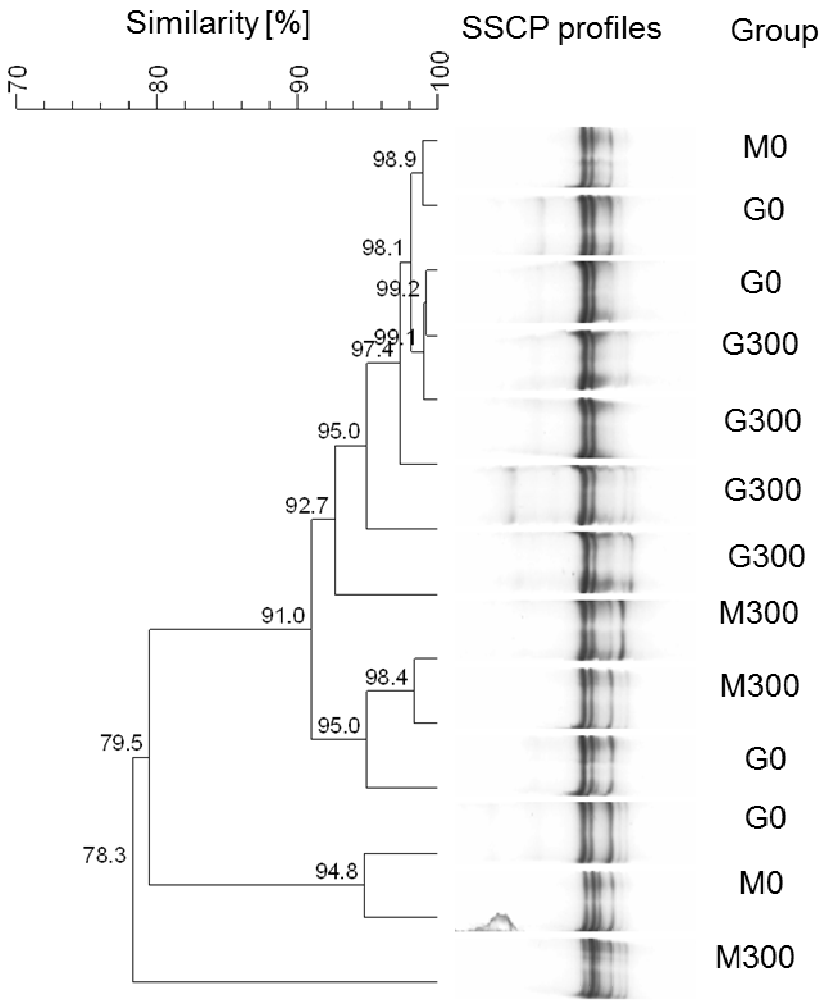

Figure 2 Effects of fumaric acid (FA) supplementation (0 or 300 g/animal/d) and roughage type (maize silage (M), grass silage (G)) on the community of Archaea (dendrogram for the results of SSCP analysis). Given are the similarities between the individual animals.

The results of blood glucose, NEFA and BHB are indicators for energy metabolism and give some evidence of the net energy balance as well as the fat depots. The results are in line with the results of an experiment in which FA was fed to fistulated dairy cows (Remling *et al.*, 2013). Supplementation with FA had no significant effect on plasma glucose and NEFA, which is an indicator for lipolysis (Cincovic *et al.*, 2012). This lack of effects on glucose and NEFA agree with Carrasco *et al.* (2012) who did not find an influence of malic acid (MA), a propionate precursor like FA, on the blood glucose of heifers. However, this contradicts the results by Bayaru *et al.* (2001) who found increased plasma glucose levels for steers fed with FA. The results of NEFA suggest that lipolysis was neither influenced by FA nor silage type. The values of BHB decreased with FA supplementation (Table 4). It can be assumed that this

is a result of less butyrate in the rumen fluid for the animals with FA supplementation (Table 5; Huhtanen *et al.*, 1993, Nielsen *et al.*, 2003, Kraft and Dürr, 2005). This is consistent with Hernández *et al.* (2011) and could show that BHB increases in blood in response to increased butyrate production. The decreased butyric acid concentrations in the present study are in line with Sirohi *et al.* (2012). BHB is also an indicator for the capacity of the liver to metabolize NEFA. Higher NEFA concentrations result in fatty liver with higher liver weights (Cincovic *et al.*, 2012). The higher BHB values for animals without FA supplementation do not conform with the corresponding liver weights, which were not influenced by FA. However, there was a trend ($P = 0.094$) of higher liver weights for the maize fed animals who also had slightly more BHB in plasma, although this effect was not significant ($P = 0.124$). The comparison of the BHB values in plasma and the liver weights of the carcasses tends to be consistent. SCFA which were metabolized in the liver were not influenced by the silage type. Therefore, these results could not explain the trend for the liver weights.

Less butyric acid concentrations for the grass fed animals are in accordance with Owens *et al.* (2009). They found the highest butyric acid concentrations in cattle fed with maize silage. The single values of the rumen fluid have to be considered with caution, because they present only spot samples. In order to get more representative results for the SCFA in rumen fluid, multiple samples should be taken over several hours. However, in the present study all samples were taken in the same way and therefore the differences and significations between the feeding groups can be considered. The time between animal death and sampling of rumen fluid was as short as possible. This was necessary since the active absorption of SCFA across the rumen wall (Gäbel and Sehested, 1997) stops at time of death. The production of SCFA from bacteria continues as long the bacteria survive and could shift the proportion of the SCFA. The sum of SCFA is in line with Remling *et al.* (2013) and Aschemann *et al.* (2012), who collected the samples at seven different time points after feeding. Nevertheless, the missing effects of FA on SCFA are in line with Carrasco *et al.* (2012) and Chethan *et al.* (2013). Carrasco *et al.* (2012) could show that MA has no influence on the SCFA profile and Chethan *et al.* (2013) detected the same result by feeding goats with FA. However, these observations contradict Zhou *et al.* (2012) with increased levels of SCFA with fumarate supplementation in sheep and Remling *et al.* (2013) who could also detect effects of FA on the SCFA profile in dairy cows. Yang *et al.* (2012) could found higher concentrations of propionic acid and acetic acid in rumen fluid of goats after feeding fumarate. Mach *et al.* (2006) found lower SCFA

concentrations for bulls fed more non fibrous carbohydrates like maize. This observation could not be confirmed in the present study.

The missing effects of FA on the microbial community are in line with Remling *et al.* (2013). The microbial community was not influenced by feeding an acid over a long-term experiment. The significant effect of the silage type on the bacteria community could be a result of the different roughage types. It is known that the microbial community depends on the diet (Carberry *et al.*, 2012) and as shown by Remling *et al.* (2011), the ether extract and the fibre contents were different between the roughages. The results of SSCP of the present study and the analyses of the fed roughage (Remling *et al.*, 2011) are comparable with the results in Remling *et al.* (2013).

The histological diagnoses of the rumen mucosa did not differ between dietary treatments. Neither the FA supplementation nor the silage type had an influence on the intensity of rumenitis. However, there was no difference between the different sample locations (villous area or villi free area in the rumen). These results are in line with the missing effects of FA and silage type on the SCFA of rumen fluid. Effects on the integrity of the rumen wall were not expected without changes in ruminal environment. An interaction of FA and silage type was found for the COI on the rumen mucosa. In the villous area, significantly more COI were found in animals of groups M0 and G300. Although it contradicts the histological diagnosis, this interaction underlines that the effects of FA dependency on the diet (Kolver and Aspin, 2006). The opposite results of COI for maize and grass fed animals could be a consequence of the rumen papillae which are influenced by the NFC content of a diet (Mach *et al.*, 2006). No interaction was found for the samples of the villi free area. The villous mucosa is potentially more sensitive and prone to damage through the fed acid. Nevertheless, the higher count of COI did not lead to worsening of rumenitis as shown by histology, where no effects of the different feeding groups could be demonstrated. These results are in line with pH values from the rumen fluid of slaughtered animals which is also not influenced by the different feeding groups (Remling *et al.*, 2011). Therefore, the results of the present study do not support the hypothesis by Wood *et al.* (2009) that feeding of FA triggers an acidosis.

The FA supplementation did not have a significant influence on the rumen villi count, growth and surface. This could be a consequence of the unaffected SCFA concentrations (except butyric acid). SCFA are known to increase the perfusion of the rumen (Dobson and

Phillipson, 1956) which results in increased rumen villi growth (Warner *et al.*, 1956). Tamate *et al.* (1962) could show that propionic and butyric acid stimulated the growth of rumen papillae. This contradicts the results of the present study, because the villi of the grass fed animals had higher surfaces, although the maize fed animals had higher butyric acid concentrations. However, the results of Mach *et al.* (2005) suggest greater surface areas for animals fed with more non fibrous carbohydrates. This is opposite to the results of the present study. The interaction between FA and silage type on villi count, growth and surface per villi underline the hypothesis by Castillo *et al.* (2004). They found that dietary factors like forage and grain type have an effect on the response of feed additives like FA. In the present study, the growth of rumen villi increased for the maize fed animals and decreased for the grass fed animals with FA supplementation. At the same time, FA supplementation decreased the COI for maize fed animals and increased the COI for the grass fed animals with FA. This might lead to the assumption that the rumen villi with more COI were smaller than the villi from rumen walls with less COI. A close relationship between the SCFA profile and the rumen papillae number and size could not be found because of the missing effects of the treatments on the SCFA (except butyric acid). However, as described above, the SCFA profile is based on a spot sample. The rumen villi morphology reflects the long term effects of FA. Therefore, the results of the analyses of the rumen wall in the present study underline the observations that the mode of action of FA depends on the diet (Castillo *et al.*, 2004, Kolver and Aspin, 2006).

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7 General discussions

In this chapter, the most important conclusions are summarized and comparisons as well as additional findings are discussed. Therefore, the questions raised in chapter 2 will be answered.

7.1 Is the acid-base balance of fattening bulls and lactating cows affected by fumaric acid? Does feeding the strong acid fumaric acid induce an acidosis or any other health impairment?

Asanuma et al. (1999) wrote: "If fumarate is added to ruminant feed, its sodium salt should be used because fumaric acid decreases ruminal pH". This statement is based on an *in vitro* study leading to studies that were conducted with the salt of fumaric acid (FA) or encapsulated FA to prevent pH decreaes in rumen fluid and a potential acidosis (e.g., Castillo et al. 2004; Wallace et al. 2006). Newbold et al. (2005) underlined this assumption with the statement that organic acids are more effective when they are supplemented as free acid, but this may inhibit fiber digestion because of decreased ruminal pH values. However, there are inconsistent results of FA supplementation on ruminal pH in the literature. The ruminal pH was not influenced by 80 g FA per day in growing steers (McGinn et al. 2004) or by 175 g FA per day in heifers (Beauchemin and McGinn 2006), respectively. Chethan et al. (2013) found increased ruminal pH values with increased levels of FA (2.5% – 5% of dry matter (DM)) in goats. Similar effects of FA on ruminal pH were found in goats in the study by Isobe and Shibata (1993).

To estimate the effects of feeding the free acid FA, in the present thesis the acid-base balance of bulls and dairy cows was examined. Parameters to diagnose the acid-base balance are e.g. rumen fluid, urine, milk, and blood, especially blood gases. Already in 1967, Kutas (1967) found a correlation between acid-base balance in rumen and the acid-base status of urine. In the present thesis, the sampling of urine from bulls in the first experiment was not possible.

Table 1: pH values of rumen fluid, urine, and blood, as well as net acid-base excess (NABE) of urine and milk fat concentration of dairy cows (Means ± SEM)

Treatment	C	300	600	ANOVA	
n	7	6	7	FA	Period
pH rumen	6.08^{a} ± 0.05	6.07^{a} ± 0.06	5.93^{b} ± 0.06	0.008	< 0.001
pH urine	8.00^{a}± 0.17	7.70^{ab}± 0.19	7.51^{b}± 0.17	0.015	< 0.001
NABE	71.2 ± 9.8	48.0 ± 11.2	49.1 ± 9.7	0.123	< 0.001
pH blood	7.447 ± 0.008	7.446 ± 0.009	7.422 ± 0.007	0.080	< 0.001
milk fat [%]	4.7^{a} ± 0.1	4.2^{b} ± 0.1	4.2^{b} ± 0.1	0.018	0.868

NABE, net acid-base excess; ^{abc} significances with p < 0.05.

Therefore, the urine from dairy cows in the second experiment was analyzed for net acid-base excretion (NABE) and other parameters of acid-base balance (see Chapter 5). The supplementation of FA had no significant influence on the urine parameters of dairy cows, except for the pH of urine (see Table 7 in Chapter 5). The pH of urine decreased with FA supplementation (Table 1), which could be a sign of acidotic burden (Fürll 1993). Usually, the urine pH of cattle is greater than 8 (Hu et al. 2007). The pH values from cows in groups 300 and 600 are lower (7.70 and 7.51, respectively; see Table 7 in Chapter 5). Especially the metabolic acidosis is known to be associated with a decreased urine pH (Kricziokat et al. 2009). The NABE was not significantly influenced by the level of FA supplementation but the values of group 300 and group 600 (48.0 or 49.1 mMol, respectively; see Table 1) were lower compared to the control group without FA (71.2 mMol). The negative correlation of NABE in urine and β-hydroxybutyrate (BHB) in blood found by Fürll (1993) could not be confirmed by the present results. In the present study with dairy cows, a positive correlation of NABE and BHB could be found (r = 0.640, p = 0.002; see Table 2). However, the numerically higher value of NABE in group 600 is related to the decreased milk fat concentrations (Annison 1973). The milk fat concentration decreased from 4.7% in the control group to 4.2% for groups supplemented with FA (see Table 1). The determined positive correlation of milk fat percentage and ruminal pH from (Allen 1997) could not be underlined (r = 0.258; see Table 2). Nevertheless, pH of rumen fluid as well as the milk fat concentration decreased with increasing levels of FA (Table 1). The relation of milk fat and ruminal pH was expected, because low ruminal pH values depress fiber digestion which produces precursors for milk fat synthesis (Beauchemin et al. 2003). Therefore, it could be an indication of an acidosis, because acidotic animals have lower milk fat concentrations (Nocek 1997), but depressed

milk fat concentration alone is not a proof of subacute ruminal acidosis (SARA) (Kleen et al. 2003).

Kricziokat et al. (2009) could not find a strong correlation between the acid-base status of urine and the acid-base status of rumen. Therefore, in their field study they suggest that acid-base status of urine is not reliable to predict the acid-base status of rumen from individual animals. Due to the different forms of feeding of animals in Kricziokat's (2009) field study and the dairy cows in the present study and, especially, its consequences on ruminal fermentation, a comparison of both results has only limited validity. Nevertheless, in the present study, no correlation of pH values of urine and pH values of rumen fluid could be found ($r = 0.23667$, $p = 0.329$; see Table 2).

Table 2: Correlation of net acid-base excess (NABE) and pH of urine, ruminal pH, blood BHB and milk fat concentration. Given are correlation coefficients r.

	NABE	pH of urine	Ruminal pH	BHB	milk fat[a]
NABE	1.000	0.857*	0.111	0.660*	0.304
pH of urine	0.857*	1.000	0.237	0.518*	0.267
Ruminal pH	0.111	0.237	1.000	0.059	0.258
BHB	0.660*	0.518*	0.059	1.000	0.303
milk fat[a]	0.304	0.267	0.258	0.303	1.000

NABE, net acid-base excess; BHB, β-hydroxybutyrate; * significances with $p < 0.05$.

In the present study, the afore mentioned suggestion of Kricziokat et al. (2009) could not be confirmed. Both, ruminal and urine pH decreased and the NABE was on a low level. This is expected in relation with the ruminal pH values below 6.0.

Beauchemin et al. (2003) concluded that the risk of acidosis increased with increasing time periods on the day with ruminal pH below 5.8. The analysis of rumen fluid of dairy cows could demonstrate a depressed pH with FA supplementation (see Table 4 in Chapter 5). Though there were no significant differences between the pH values at the different time

points, their numerical differences in its chronically sequences are shown in Figure 2. Already 30 min after feeding, the ruminal pH of cows fed 600 g FA per day was lower compared to the other two feeding groups. The mean pH before feeding was nearly the same for treatment 0 (pH = 6.64) and 600 (pH = 6.65). This hypothesized that the decreasing effect of FA on ruminal pH is measurable only during the first 30 minutes after feeding. To underline this hypothesis, further studies with continuous measurements of the ruminal pH over the whole experimental period would be optimal.

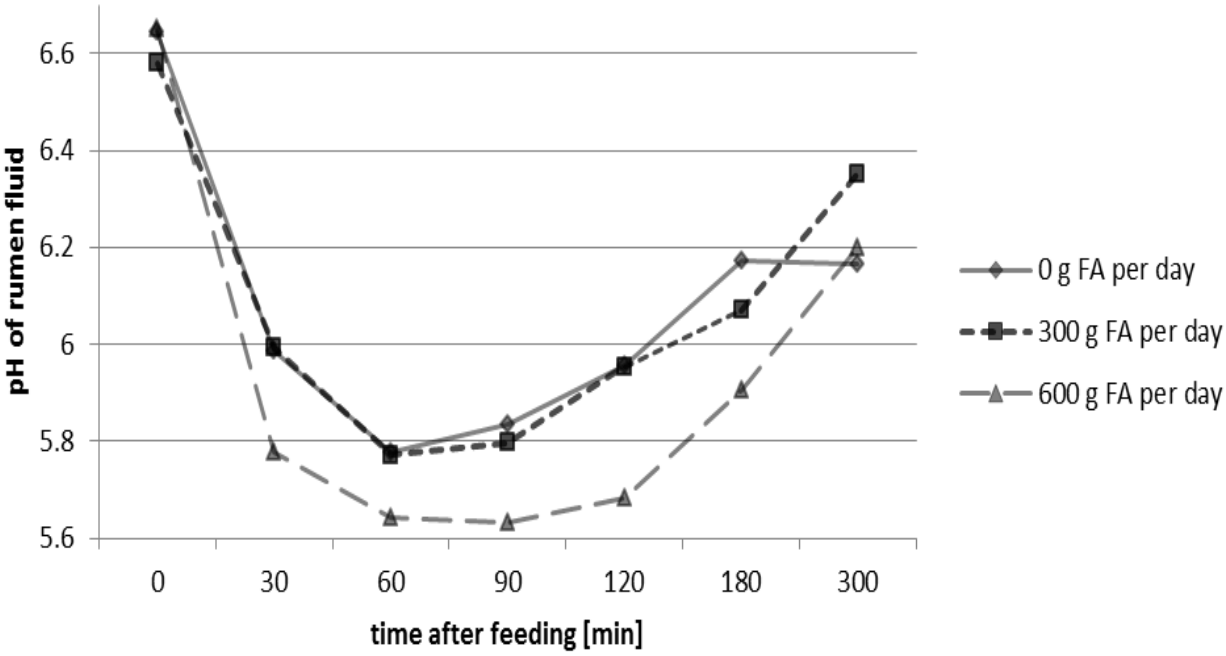

Figure 1: Effect of FA on ruminal pH in dependence of time after feeding for dairy cows. Given are LSMEANS of statistics from Chapter 5.

The ruminal pH of the animals in the group without FA (group 0) and with 300g FA per day and animal (group 300) were nearly the same, which can be underlined with their area under the curve (AUC) which is given in Table 3. Figure 1 shows that the ruminal pH did not decrease below 5.8 for groups 0 and 300. For group 600, no values of ruminal pH below 5.6 were observed. Although, the animals of group 0 and 300 got wheat starch (600g and 300g per day and animal respectively) as isocaloric compensation to FA, their ruminal pH did not decrease as much as in group 600. Wheat starch is rapidly digestible in the rumen and its disappearance *in situ* is nearly complete (Monteils et al. 2002; Castillo et al. 2009). Therefore, high starch contents are known to decrease the ruminal pH (Owens et al. 1998). Figure 1

demonstrates that FA is more potential to decrease the pH in rumen fluid as compared to starch. This could be a result of the acidic nature of FA (Engel et al. 2008). The observations of Molano et al. (2008), who found increased levels of ruminal pH when FA was supplemented on wether lambs, could not be underlined with the present thesis.
Other parameters for diagnosis of acidosis are ketone bodies in blood (Kricziokat et al. 2009). Combined with NABE, body BHB is related to the acid load of the rumen (Enemark et al. 2004). However, a prediction of the ruminal pH is not possible with this parameter (Enemark et al. 2004). In the present study with dairy cows, the BHB concentration correlates positive with NABE und pH of urine (Table 2). The ruminal pH decreased as well as the BHB concentration in blood. Nevertheless, no correlation of BHB ($r = 0.059$, $p > 0.05$) with pH in rumen fluid could be found.

Gianesella et al. (2010) found lower blood pH values when the ruminal pH dropped below 5.5. The blood pH of the cows in Chapter 5 tended to decrease with FA supplementation, although in none of the seven spot samples ruminal pH values were below 5.5. However, it was not possible to carry out continuous measurements of pH in rumen fluid of the animals. Therefore, it can not be excluded that ruminal pH-values under 5.5 occurred over the course of the day. In contrast, the ruminal pH of the growing bulls was not influenced by FA (see Chapter 4). This might be due to the fact that rumen fluid from the bulls was only taken as a spot sample at slaughtering. The validity of this value was already discussed in Chapter 4. Nocek (1997) determined that the ruminal cannulation is the preferred method of sampling ruminal fluid. With this method, the results of ruminal pH values were most representative. This additional information underlines the assumption that the results obtained with fistulated dairy cows (Chapter 5) are more meaningful than the results from the trial with fattening bulls (Chapter 4 and 6). That the blood pH was still in the reference range underline the assumption of Hu et al. (2007): “because maintenance of pH is a principal goal of homeostasis”.

In case of acute acidosis, the analysis of blood gases is essential (Fürll 1993). Therefore, in the present thesis, the blood gases were analyzed at every time of blood sampling to diagnose a possible acute acidosis. In the experiment with bulls (Chapter 4), the blood was collected every 70 days. Therefore, up to four blood samples per animal (depending on the achievement of slaughtering weight of 570 kg) were taken. This allows the control of possible indications of an acidosis at different time points of the experiment. The analysis of the individual blood

sampling days did not show a significant influence neither of FA nor of silage type on blood gases (data not shown). Therefore, it could be concluded that no acute acidosis appeared around the blood sampling days 70, 140 and 210 of the experiment with growing bulls. Although Fürll (1993) published that the blood gases have a limited validity for diagnosis of chronic acidosis, in the present study with growing bulls, the results of blood gases are in line with the other observations concerning acidosis.

Simultaneously, the daily feed intake of the growing bulls was observed as Hu et al. (2007) had determined a correlation between DM intake and acid-base status of the animals, especially, with blood pH and blood HCO_3. Reduced or inconsistent DM intake is known as one of the major indices of SARA (Nocek 1997). The expected depression of DM intake as consequence of feeding FA (Wallace et al. 2006) could not be underlined with the present thesis. The DM intake of the *ab libitum* fed growing bulls as well as the restricted fed dairy cows was not influenced by FA supplementation. Although the DM intake of the cows is not as meaningful as from the bulls because of their restricted feeding. The observations concerning the DM intake of restricted fed animals are not that meaningful as compared to studies with *ad libitum* feeding, but in the present thesis they were in line with the results of fattening bulls (see Chapter 4 and 5). The total daily feed intake of the bulls was not influenced by FA ($p=0.631$; see Table 4 in Chapter 4). This parameter contradicts the incidence of an acidosis. However, it is known that mean intakes have to be considered with caution to diagnose a SARA. Nocek (1997) showed that animals reduced their feed intake during the first few days when suffering from SARA but after this, they seemed to have a normal feed intake again. The daily observation of the animals and their feed intake did not show any abnormalities in the present studies. This is in line with Hu et al. (2007).

Inflammations in the rumen wall are typical for acidosis (Cernik et al. 2011). In the present study, the histopathological results of bull's rumen wall did not show an influence of FA on the count of center of inflammation (COI) (see Table 7 in Chapter 6). However, there was an interaction of FA and silage type. The most COI could be found for bulls fed grass silage supplemented with FA and for bulls fed maize silage without supplementation of FA. In consideration of the observations of Cernik et al. (2011), it could be assumed that FA did not result in an acidosis when starch rich silage is fed. A simple contemplation of the COI results hypothesized that FA reacts as a buffer and safes the rumen wall against inflammations, when the animals got maize silage as roughage. This observation is in line with the hypothesis of

Isobe and Shibata (1993) who found increased ruminal pH values after supplementation of diets with FA for goats and hypothesized that this is a consequence of higher saliva production. This result contradicts the observations in the present study. Increased ruminal pH could not be demonstrated in our experiment with growing bulls, but it has to be considered that there was only one spot sample of ruminal fluid. The increased number of COI after feeding a grass silage based diet with FA underlined the assumption of e.g. Wallace et al. (2006), who found acidosis in lambs after FA supplementation. However, the dimension of rumen papillae showed the same interaction of FA and silage type than the COI. The rumen wall is known to be able to "react" to diets. The rumen wall adapts on acidic burden with changes in length of rumen papillae (Allen 1997). Therefore, further experiments with FA and maize silage are needed, to clarify the interactions. Nevertheless, the number of COI has to be considered together with the other parameters of acid-base balance as mentioned above.

Pehrson et al. (1999) reasoned that an acidosis could be compensated for when the blood pH was not significantly reduced. Therefore, it can be assumed, that the acid-base balance was influenced by FA, but no metabolic acidosis and SARA seemed to be apparent. Further research is justified to clarify the mode of interaction between FA and different types of diet.

7.2 What are the consequences for the rumen mucosa and the ruminal fermentation when fumaric acid is supplemented to diets for fattening bulls and dairy cows?

In the present experiment with growing bulls, the rumen and its mucosa were examined after slaughtering. As shown in Chapter 6, the weight of the rumen was not influenced by FA in contrary to histopathology of its mucosa (Table 6, Chapter 6). The mucosa transports and metabolizes short chain fatty acids (SCFA) which were produced by ruminal fermentation (Nocek 1997). Increased SCFA concentration in rumen fluid stimulates the metabolism of rumen epithelia and the growth of papillae (Hofmann and Schnorr 1982) to increase the absorptive capacity. In the present study, FA affected the rumen papillae growth in dependence of the roughage type. FA fed animals had more, but smaller papillae when grass silage was fed. The opposite results could be demonstrated for maize silage fed animals (Table 8, Chapter 6). There was no interaction of FA and silage type for papillae surface per cm^2 of ruminal mucosa. The size and amount of rumen papillae depends on the diet (Dirksen et al. 1985). Feeding of energy-rich diets stimulates growth of papillae (Zitnan et al. 2003). In

the present study with growing bulls, the animals without FA got an isocaloric compensation (glycerol). Therefore, from a theoretical point of view, higher energy content could not be the explanation for increased sizes of papillae. However, potential energy savings caused by less CH_4 production cannot be excluded, although other parameters do not underline this assumption. Therefore, it can be hypothesized that FA had effects on ruminal papillae, which depend on the silage type, although the unaffected SCFA concentrations in rumen fluid of the bulls (Table 5, Chapter 6) do not underline this hypothesis. Nevertheless, Allen (1997) could demonstrate a positive correlation of ruminal papillae area with the rate of SCFA absorption. In the present study with growing bulls, the SCFA absorption was not measured. However, the total concentration of SCFA in rumen fluid correlates positively with the papillae surface [cm^2] per cm^2 of ruminal mucosa ($r = 0.748$, $p < 0.001$; see Figure 2). Hence, further studies with analysis of SCFA in rumen fluid and histopathological examinations of rumen mucosa after feeding FA are needed to explain these results.

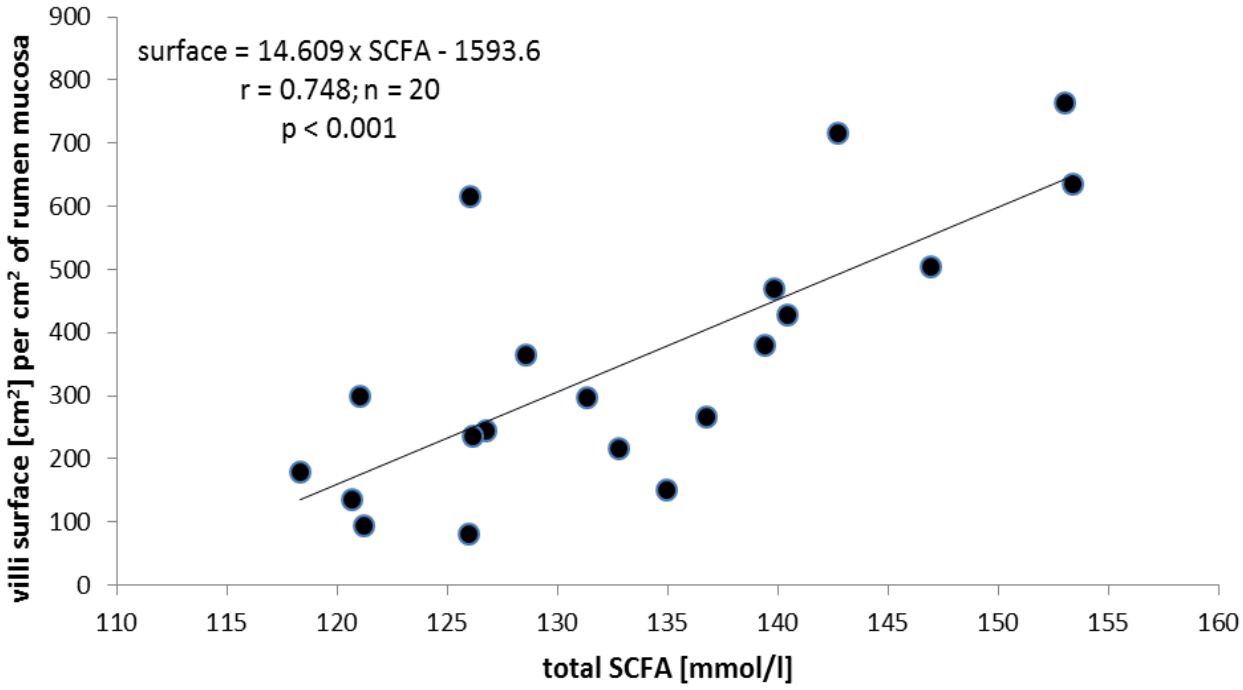

Figure 2: Relationship between the concentration of total short chain fatty acids (SCFA) in rumen fluid and the villi surface per cm^2 of rumen mucosa of all the slaughtered bulls (Linear correlation). Given are the mean values data from Tables 5 and 8 from Chapter 6.

Kolver and Aspin (2006) could not find an effect of fumarate on ruminal fermentation *in vivo*. In the present study with the spot sampled rumen fluid of growing bulls the SCFA

composition was also not affected by FA (Chapter 6). The apparent total tract digestibility of nutrients (organic matter, ether extract, crude protein, acid detergent fiber, and neutral detergent fiber) of growing bulls was estimated with acid insoluble ash (AIA) as marker (Table 5 in Chapter 4). It was not affected by FA as well as the apparent ruminal digestibility of nutrients (dry matter, organic matter, acid detergent fiber, neutral detergent fiber, crude fiber, and starch) of the dairy cows in Chapter 5 (Table 8). This observation shows that FA did not affect the digestibility in the long-term as well as in the short-term experiment, although the comparability of ruminal and total tract digestibility is limited.

The effects of FA on ruminal fermentation were basically examined in the experiment with dairy cows. Therefore, the results of rumen fluid analysis from the trial with bulls are not discussed here in detail.

The proportion of propionic acid increased more or less linear during the first 180 minutes after feeding for group 600 (Figure 3). This increase could not be demonstrated for the animals from group 0. Already before feeding, the proportion of propionic acid is higher for the animals fed with FA, although this is not significant. About 30 minutes after feeding, there is a trend of increased propionic acid concentration of group 600 in comparison to group 0 ($p = 0.070$). However, until more than 60 minutes after feeding, there were no differences between groups 300 and 600 ($p > 0.1$). More than 60 minutes after feeding, the proportion of propionic acid increased more for group 300. After more than 120 minutes, the proportion of propionic acid decreased for group 300, whereas the propionic acid proportion of group 600 still increased until it decreased more than 180 minutes after feeding. Due to the fact that the animals of group 300 got 300 g FA and 300 g starch per day, and the AUC from group 300 and 600 were not different (Table 3), it could be hypothesized that different fermentation pattern of FA and starch are the reason for this phenomenon. The proportion of propionic acid began to decrease again after more than 120 minutes after feeding for groups 0 and 300. This underlines the hypothesis.

Due to the higher proportions of propionic acid in groups 300 and 600 in comparison to group 0 (see Figure 3) it can be assumed, that the percentage of FA which is fermented to propionic acid is higher than that of starch. This underlines that FA is rapidly metabolized in the rumen (EFSA 2013) and mostly fermented to propionate (Asanuma et al. 1999), although Demeyer and Hendrickx (1967) found that fumarate can also be converted into acetate.

Enhanced amounts of propionic acid in rumen fluid can be associated with increased microbial protein synthesis in the rumen (Baker 1999). This assumption cannot be transferred to the present study with dairy cows, because the microbial crude protein production in the rumen was decreased with FA (see Table 9, Chapter 5) whereas the propionic acid amount increased (see Table 4 in Chapter 5).

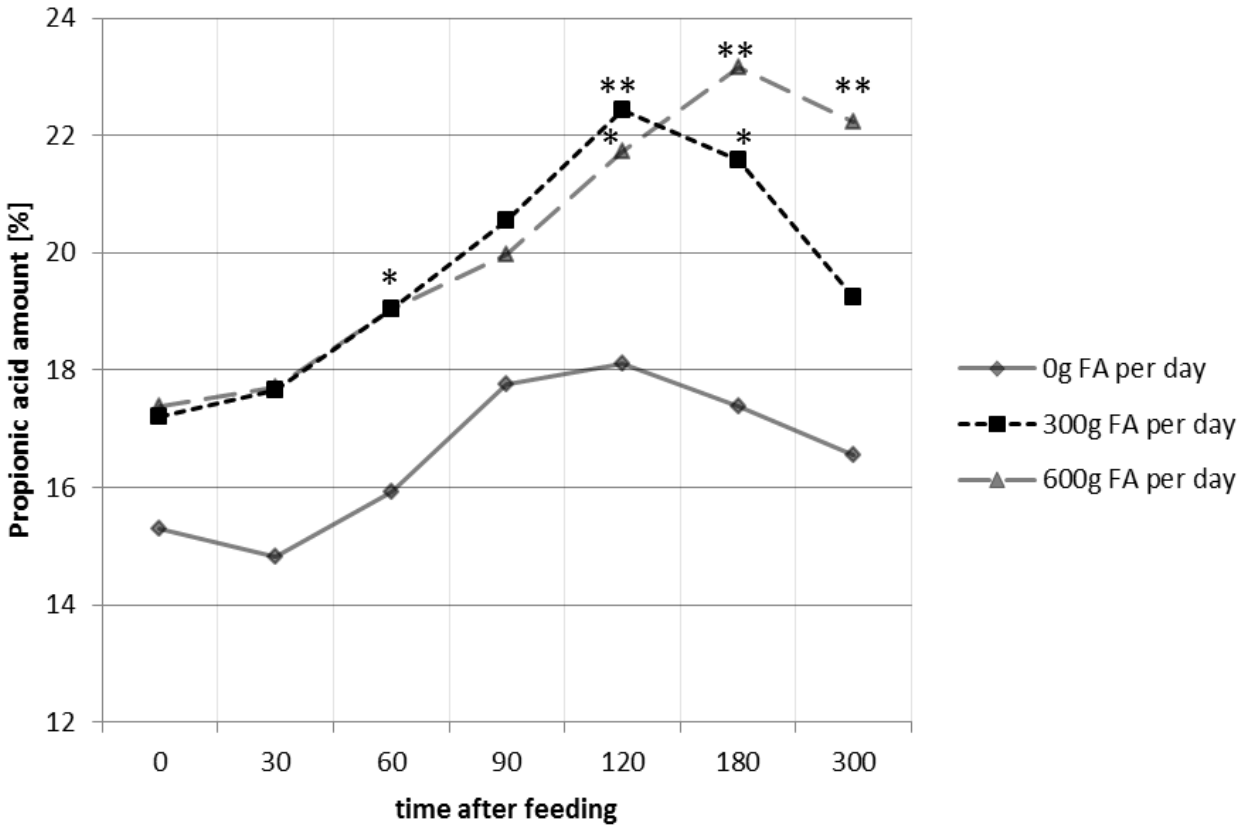

Figure 3: Effects of FA on ruminal propionic acid proportion in dependence of time after feeding of dairy cows. Given are LSMEANS from statistics of Chapter 5. Significant deviations from group 0 (without FA) at the same time point are indicated by asterix (*: p < 0.05; **: p < 0.001).

Lopez et al. (1999) found increased fiber digestion *in vitro* when fumarate was supplemented. This finding could not be confirmed in the present study. Neither the apparent digestibility of nutrients in bulls (Table 5 in Chapter 4), nor the apparent ruminal digestibility of nutrients in dairy cows (Table 8 in Chapter 5) were influenced by FA. This observation agrees with Newbold et al. (2005), who found no increased DM degradation when the free acid was used.

The microbial community did not show any effects (see Figures 1 and 2 in Chapter 5 and Figures 1 and 2 in Chapter 6) of FA supplementation in both present experiments. However, it has to be considered, that quantitative changes of the microbial community were not observed in both present experiments. These observations are in line with Riede et al. (2013), who also found no effects of FA on microbial community, although the propionic acid concentration increased. Therefore, further studies which estimate these changes in *in vivo* studies with FA supplementation are necessary to make a statement about the consequences of FA supplementation on the microbial community.

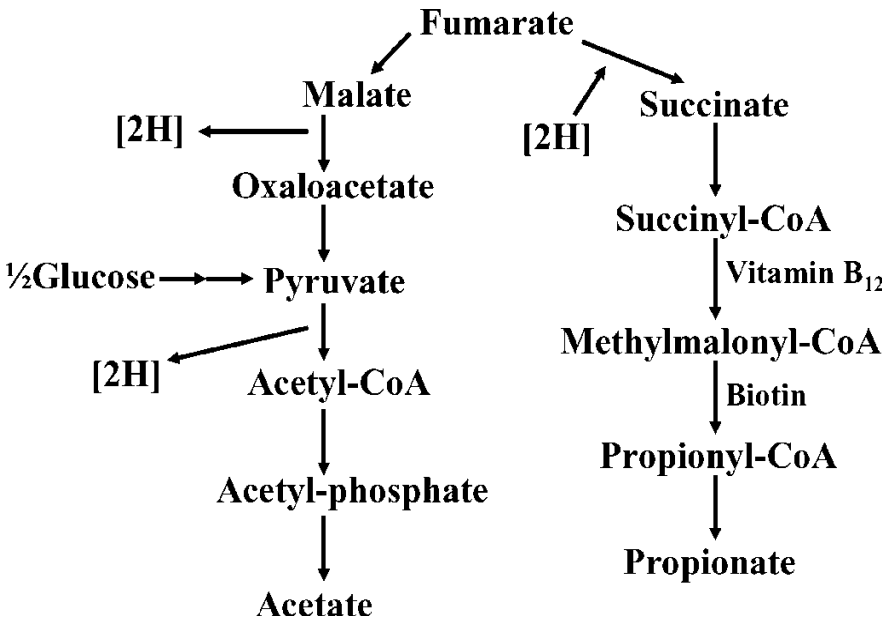

Figure 4: Ruminal fermentation pattern of FA (Ungerfeld et al. 2007)

7.3 Are the effects of fumaric acid dose-depending and do the effects of fumaric acid depend on the silage type which is fed as roughage (grass or maize silage)?

The first experiment with growing bulls (Chapter 3, 4 and 6) was conducted with two different silage types as roughage to verify the hypothesis of Garcia-Martinez et al. (2005) that the mode of action of fumarate depends on the diet. Garcia-Martinez et al. (2005) could demonstrate diet-depending responses to fumarate i*n vitro* with greatest effects in high-forage diets.

No interactions of FA and silage on performance parameters could be found in the present experiment with bulls (Chapter 4). Therefore, it could be assumed that FA acts different concerning the diet-dependence than its salt disodium fumarate, although in the present study, only grass and maize silage were compared. However, there are no *in vivo* studies available which compare the effects of the free acid FA supplemented to different diets and only a few studies are available investigating the effect of fumarate *in vivo*, as reported by Kolver and Aspin (2006).

In consideration of the fact that the methane (CH_4) production depends on the diet, possible mitigating strategies have to be tested with different diets before their conclusive evaluation. It could be proven in former *in vitro* and *in vivo* studies, that the greatest CH_4 reduction was possible in diets with high levels of CH_4 emissions (Kolver and Aspin 2006). Therefore, it was expected that there were interactions of FA and the silage in the experiment with growing bulls. As described in the introduction, the CH_4 emissions were expected to be greater when grass silage was fed as roughage. As a consequence, these silages should have greater reduction potential. Therefore, further studies are needed which compare different diets and their interaction with FA.

Additionally, it cannot be excluded, that the missing interactions of FA and silage type were a result of ruminal adaptation mechanism which occurred in the present long-term study. Ungerfeld et al. (2007) concluded in their meta-analysis *in vitro* that microbial adaptation to the fumarate fermentation could happen. The analysis of the microbial communities in the present thesis could also not demonstrate an interaction of FA and silage type (see Paragraph 3.2.2 SSCP Results in Chapter 3). However, the analysis of the microbial communities was made at the end of the experiment. To underline this hypothesis of microbial adaptive mechanisms *in vivo*, further studies with repeated analyses of the microbial community during the long-term study could be adjuvant.

As described above, FA had influence on the mucosa of the rumen wall. These interactions demonstrate a long-term effect of FA on rumen mucosa which depends on the silage type. Castillo et al. (2004) concluded that dietary factors like forage and grain type have an effect on the response of feed additives like FA. The present results of ruminal mucosa underline this statement.

The hypothesized dose-depending effects of FA (Asanuma et al. 1999; Carro and Ranilla 2003) could not be confirmed in the present experiment with growing bulls. FA had no influence on feed intake, live weight gain and feed efficiency (Table 4 in Chapter 4). For the haemoglobin content in the bulls' blood, decreasing values after the supplementation of FA could be found, but they were not significantly different between the individual feeding groups (Table 6 in Chapter 4). For the propionic acid precursor malic acid, Liu et al. (2009) found a dose-depending effect in steers. The dose-depending effects of FA and fumarate, which were found by Li et al. (2009) *in vitro* could not be underlined with the present study with growing bulls *in vivo*. However, Farkasova et al. (2008) could demonstrate dose-depending effects of glycerol, which was used as isocaloric compensation in the present study. Linear decreased ruminal pH values and increasing levels of total SCFA with glycerol supplementation could be found in steers fed up to 300 g glycerol per day (Wang et al. 2009). Therefore, it cannot be excluded, that the lacking effects of FA in the present study were a result of a compensation effect from glycerol and FA. This assumption can be supported by the fact, that most of the determined parameter in the present fattening study did not show an effect of FA.

Table 3: Effects of FA supplementation on ruminal fluid pH and concentrations of short chain fatty acids of dairy cows. Given are the area under the curve [pH∗minutes, %∗minutes, respectively] (Means).

Group	Fumaric acid [g/d]			ANOVA probabilities		
	0	300	600	FA	Period	FA × Period
pH	1823	1820	1769	0.039	0.012	0.199
Ammonia	3376	3058	3602	0.242	0.017	0.176
Acetic Acid	19286[a]	18643[b]	18578[b]	0.008	0.037	0.065
Propionic Acid	5037[c]	6136[b]	6374[a]	0.000	0.001	0.022
Acetic:Propionic	1169[a]	932[b]	893[b]	0.001	0.020	0.055
iso-Butyric Acid	212[ab]	237[a]	209[b]	0.039	0.010	0.038
Butyric Acid	4560	4300	4146	0.279	0.003	0.171
iso-Valeric Acid	344	331	338	0.944	0.049	0.605
Valeric Acid	442	588	482	0.161	0.001	0.136
Sum	39749	38496	38551	0.818	0.000	0.098

[abc] values with different superscripts within a column are significantly different ($p<0.05$)

In the experiment with dairy cows (Chapter 5), significant effects of FA could be found. Such clear dose-depending effects could be shown for the area under the curve (AUC) of the proportion of propionic acid (Table 3). The AUC was calculated to observe possible different courses of fermentation due to different fermentation pattern of the diet supplemented with FA in comparison to the control diet supplemented with starch. With increasing levels of FA, the proportion of propionic acid increased as well. Numerical dose-depending effects of FA could also be shown for the AUC of acetic acid and the ratio of acetic : propionic acid. The decreasing effects with FA supplementation were not significant between groups 300 and 600. The mean pH values of rumen fluid and urine decreased with increasing levels of FA (Table 4 and Table 7 in Chapter 5). In blood plasma, BHB and erythrocytes were also dose-depending influenced by FA.

The dose-depending effects of FA were more obvious in the short-term experiment under restricted feeding conditions than in the long-term study with *ad libitum* feeding. Potential adaptations of the microbial community after supplementation of FA, which were observed in *in vitro* studies (Isobe and Shibata 1993), could be the reason for the different findings in the present two studies concerning the dose-depending effects of FA.

Most of the previous studies with FA who postulated dose-depending effects were *in vitro* studies (Asanuma et al. 1999; Carro and Ranilla 2003; Li et al. 2009) or *in vivo* studies with focus on CH_4 reduction (Kolver and Aspin 2006). It was assumed that effects of fumarate are variable in *in vitro* and in *in vivo* studies (Ungerfeld et al. 2007). Therefore, it cannot be excluded, that a lack of dose-depending effects of FA, especially in the present long-term study, was caused by adaptive mechanisms as described by Ungerfeld et al. (2007). However, Ungerfeld et al. (2007) published a study about the effects of fumarate and not the free acid. Such adaptive mechanisms of the ruminal microorganism have already been discussed with respect to other potential methane reducers (e.g. Monensin; Johnson and Johnson 1995).

The results of the present thesis and the *in vitro* results of Becker (2012) and Riede et al. (2013) are comparable. The *in vitro* studies of Becker (2012) were conducted with samples of the silage from the present *in vivo* experiment. The analyses of the microbial collective with SSCP did not show an effect of FA. The *in vitro* studies of the thesis of Becker (2012) also found less difference of the effects of FA depending on maize or grass silage. However, rumen simulation technique (RUSITEC) experiments of Becker (2012) were conducted only

over 14 days and the experiments of the present thesis with growing bulls lasted on average 222 days. Therefore, the hypothesis that there are differences in the mode of action of FA *in vivo* and *in vitro* (Ungerfeld et al. 2007), cannot be underlined. The results of *in vitro* (Becker 2012) and the present *in vivo* studies with FA are in line with the results of fumarate, which were *in vitro* (Garcia-Martinez et al. 2005) and *in vivo* (Kolver and Aspin 2006) diet-depending. Nevertheless, no differences in effectiveness of FA depending on silage type could be found in the present experiment with growing bulls.

7.4 Do the animals have an energy benefit when supplemented with FA?

The growth of the bulls was not influenced by FA supplementation (Chapter 4). This observation is in line with Castillo et al. (2007), who found the same result for malic acid, a propionate precursor like FA. Neither the daily weight gain, nor the feed per gain were influenced by FA (Table 4 in Chapter 4). Therefore, the hypothesis of Kolver et al. (2004), that FA increased energy capture by improving supply of glucogenic compounds and reducing losses to CH_4 could not be underlined with the present study. However, Kolver et al. (2004) made an *in vitro* study. The carcasses and the organ weights were also not influenced by FA supplementation (Table 3 in Chapter 4). The carcass dressing showed a trend to increase with FA supplementation (Chapter 4). This indicated that there was more energy available for the animals fed FA. This contradicts the results of daily intake of metabolisable energy (ME). However, the ME was calculated with literature values and a wether experiment (Chapter 4). Therefore, it can not be excluded, that in the present study, the real available energy differed from the calculated values. Additionally, it might be possible that the fermentation of FA provides the animal with additional energy which was not taken into account when the diet was calculated. Chethan et al. (2013) described beneficial effects of FA on net energy retention, because of decreased CH_4 production and higher plasma glucose concentrations when FA was supplemented up to 5% of DMI to goats. In the present thesis, the plasma glucose concentrations neither from bulls nor from dairy cows were influenced by FA. In addition, it has to be considered, that in the present study with growing bulls (Chapter 4 and 6) FA supplementation was fed in comparison to a control substance (glycerol) as isocaloric compensation.

The energy status of an animal can be assessed on the basis of blood metabolites and milk composition (Heuer et al. 2000; Grabherr et al. 2009). Especially plasma glucose, not esterified fatty acids (NEFA), BHB and urinal ketone bodies correlated with the energy balance of the animal (Heuer et al. 2000). In the present study, the plasma concentrations of BHB, NEFA and glucose were measured in both experiments (Table 5 in Chapter 5 and Table 4 in Chapter 6). The results for the long-term and the short-term experiment were the same: Plasma glucose and NEFA concentration were unaffected by FA whereas the BHB concentration decreased with increasing levels of FA. The decreased BHB levels in the present studies indicate that FA could benefit the available energy for the cows or bulls, respectively. However, together with BHB, plasma NEFA and glucose are also indicator for metabolic status (Nielsen et al. 2003) and both parameters were not influenced by FA in the present studies. The comparable results of plasma BHB, NEFA and glucose could underline the mode of action of FA on these parameters.

Additional to blood metabolites, the fat:protein ratio in milk is a strong predictor of energy balance (Heuer et al. 2000). The milk fat:protein ratio in the present study with dairy cows was not significantly influenced by FA ($p = 0.170$), though it decreased numerically (fat:protein ratio of the different feeding groups: 0g FA = 1.55 ± 0.05; 300g FA = 1.43 ± 0.05; 600g FA = 1.37 ± 0.04 (LSMEANS ± SEM)). The milk fat content is negatively and the protein content positively correlated with the energy balance (Grieve et al. 1986). As shown in Table 10 in Chapter 5, the milk fat content decreased after feeding a diet supplemented with FA, but the milk protein content was not influenced by FA. The energy balance of the dairy cows, calculated with the energy content of their ration (see "Calculations and statistics" in Chapter 5) was not influenced by FA (Balance of energy of group 0: -2.77 ± 2.78 MJ net energy of lactation (NEL) / d, group 300: -5.79 ± 2.88 MJ NEL / d; group 600: -2.04 ± 2.79 MJ NEL / d; $p = 0.241$). Therefore the milk protein content is in line with the energy balance of the dairy cows, although the milk fat content contradicts it. However, Grieve et al. (1986) concluded, that the milk fat:protein ratio is a better predictor for energy supply of the cows than these parameter alone. The number of cows in the present study was limited ($n = 7$), therefore, the results concerning milk composition have to be considered carefully, although it is known, that the individual variation in milk fat and protein is small (Veerkamp et al. 1995). The numerical decreased fat:protein ratio in dairy cows and the trend for increased carcass

dressing in the bulls could be an indication for a higher energy supply of the animals fed diets supplemented with FA.

Due to a possibly higher energy supply of the animals in the present study, the possible CH_4 reduction by FA was stoichiometrical calculated. In sum, four mol of FA were needed to avoid the production of one mol CH_4 (Ungerfeld et al. 2007). The daily CH_4 production per animal ranges between 250 L and 500 L per day (Johnson and Johnson 1995). This corresponds to a mean amount of about 16 mol CH_4 per day. In the present studies, up to 2.6 mol (300 g) and 5.2 mol (600 g) FA were supplemented to the animal's diet, respectively. This resulted in a stoichiometrical CH_4 reduction by 4% and 8% respectively for the present studies. However, Wood et al. (2009) and Wallace et al. (2006) could detect much more CH_4 reduction *in vivo*, than calculated stoichiometrically. These studies demonstrate that stoichiometric calculations cannot be transferred to *in vivo* studies without a validation. In the present thesis it was expected, that higher CH_4 reductions occur, which are measurable in a higher performance. For instance, higher daily weight gain of the growing bulls or higher milk yields were expected. For an accurate calculation of the animals energy balance, CH_4 measurements would be interesting, but this was not the aim of the present thesis. At the end, only the tendency of increased dressing in bulls could underline this assumption. Hence, gradual differences were found, but resounding effects of FA on the energy balance of the animals are missing.

8 Conclusions

The aim of the present thesis was to evaluate the effects of fumaric acid (FA) on the health and performance of dairy cows and growing bulls. The examined parameters of the present thesis do not indicate any overt health or acceptance problems of the animals when FA was supplemented with the presented dosage.

However, the acid-base balance of the animals was influenced, without clear evidence of occurrence of metabolic or ruminal acidosis. In the present short-term experiment, it could be shown that the ruminal and urinal pH decreased with FA, whereas the blood pH was still in the reference range.

The mode of action of FA does not depend on the diet, except for its effects on rumen mucosa. If fiber rich silage is fed, FA decreases the dimension and the surface of rumen villi and increases the centre of inflammation of villious areas of rumen mucosa.

Dose-depending effects of FA could not be established in the long-term study. Therefore, microbial adaptation mechanisms could not be excluded and further studies to estimate this are needed.

However, the short-term effects of FA on blood metabolites and SCFA composition in rumen fluid are dose-dependent. FA decreases the ruminal pH and increases the concentration of propionic acid. However, the microbial community of Bacteria and Archaea was not affected.

The apparent total tract digestibility of the nutrients (organic matter, crude fat, crude fiber, crude protein, neutral detergent fiber and acid detergent fiber) was not influenced by FA. This observation was supported by the unaffected apparent ruminal digestibility of nutrients in the short-term experiment, whereas the production of microbial protein decreased.

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2011	N. Remling , S. Hachenberg, U. Meyer, M. Höltershinken, A. Beineke, G. Flachowsky, S. Dänicke 87. Leads fumaric acid to a metabolic acidosis when fed to growing bulls? (Führt Fumarsäurefütterung bei wachsenden Bullen zu metabolischen Azidose?). Proceedings of the Society of Nutrition Physiology, Volume 20 (2011). S. 109.
2011	Nicole Remling, Sabrina Hachenberg, Ulrich Meyer, Martin Höltershinken, Gerhard Flachowsky and Sven Dänicke Influence of various amounts of fumaric acid on performance and parameters of the acid–base balance of growing bulls fed with grass or maize silage Archives of Animal Nutrition. Volume 65. S. 386 – 401.
2013	N. Remling, S. Riede, P. Lebzien, U. Meyer, M. Höltershinken, S. Kersten, G. Breves, G. Flachowsky and S. Dänicke Effects of fumaric acid on rumen fermentation, milk composition and metabolic parameters in lactating cows Journal of Animal Physiology and Animal Nutrition. DOI: 10.1111/jpn.12152
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