Letters in Applied Microbiology 2001, 32, 47–51

Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets

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0197/00: received 10 July 2000, revised 16 October 2000 and accepted 17 October 2000

F. DOHME, A. MACHMÜLLER, A. WASSERFALLEN AND M. KREUZER. 2001. The objective of the present study was to investigate the effects of seven different pure fatty acids on rumen fermentation using the rumen simulation technique (RUSITEC). The fatty acids were supplied to a complete ruminant diet at a proportion of 50 g kg^{-1} dietary dry matter and compared with an unsupplemented control. Methane release and methanogenic counts were suppressed by the fatty acids $C_{12:0}$, $C_{14:0}$ and $C_{18:2}$ whereas $C_{8:0}$, $C_{10:0}$, $C_{16:0}$ and $C_{18:0}$ showed no corresponding effects. Apart from $C_{12:0}$ and $C_{18:2}$, $C_{8:0}$ and $C_{10:0}$ also adversely affected ciliate protozoa suggesting independence from the methane-suppressing effect of medium-chain fatty acids (MCFA). Although MCFA but not $C_{18:2}$ reduced ruminal fibre degradation, the influence on other fermentation traits remained low. In conclusion, the supply of certain fatty acids to ruminant diets seems to have the potential to reduce methane release.

INTRODUCTION

Dietary fats have been identified as an efficient means of decreasing ruminal methanogenesis (Jouany 1994). In this context, several fats rich in medium-chain saturated fatty acids (MCFA; $C_{8:0} - C_{14:0}$) were found to inhibit methane production in rumen fluid (Dohme *et al.* 2000). To date, most studies have used coconut oil as the most common source of MCFA in animal feeding. On average, coconut oil includes 874 g MCFA kg^{-1}, mainly $C_{12:0}$ and $C_{14:0}$ (471 and 179 g kg^{-1}; Dohme *et al.* 2000). Compared with highly unsaturated oils, coconut oil was found to be equally or more effective against ruminal methanogenesis (Dong *et al.* 1997; Machmüller *et al.* 2000). However, it is unclear which of the MCFA in coconut oil are actually inhibiting methanogenesis. Therefore, the present study investigated the effects of individual MCFA on various rumen fermentation characteristics in comparison with long-chain saturated ($C_{16:0}$ and $C_{18:0}$) and polyunsaturated ($C_{18:2}$) fatty acids.

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MATERIALS AND METHODS

Experimental design and techniques

The present study was carried out with the ru simulation technique (RUSITEC). This model has described and validated by Czerkawski and Breckenr (1977) to be suitable for maintaining a normal run microbial community under strictly controlled condit (39 °C, anaerobic, artificial saliva [McDougall buffer] continuous movement of feed in fermenter) over exter periods of time. The system was equipped with fermenters, each with a capacity of 900 ml. The w experiment consisted of four independent incubation se In each incubation series caprylic acid ($C_{8:0}$), capric ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palr acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and linoleic acid (C_1 were compared with a complete but fatty acid unsup mented diet. In total, this resulted in four replicates treatment. Each of the four incubation series lasted 1 with the values on days 5–10 of each series servin calculate treatment values for analysis of variance. Init the fermenters were filled with 100 ml artificial saliva 800 ml strained rumen fluid obtained from a run fistulated non-lactating cow receiving a diet compose hay *ad libitum*, maize silage (3 kg d^{-1}) and cereal-b commercial concentrate (1 kg d^{-1}; 7·6 MJ kg^{-1} dry m (DM) net energy, 220 g kg^{-1} DM crude protein, 45 g

DM crude fibre). Furthermore, at the beginning of each incubation series two nylon bags, of 100-μm pore size, containing either solid rumen digesta or the respective diet were introduced. Thereafter, one bag was replaced daily (firstly the digesta-containing bag) allowing 48 h incubation for each feed bag. After each exchange of the bags, the fermenters were immediately flushed with nitrogen to re-establish anaerobic conditions. Artificial saliva was continuously infused into each fermenter at an average rate of 530 ml d^{-1}. Daily rumen fluid samples were taken directly from each fermenter prior to exchange of the bags.

The daily DM supply per fermenter consisted of maize silage (5·4 g), hay (1·2 g), barley (1·2 g), soybean meal (2·7 g) and potato protein (0·6 g). In seven of the eight treatment groups this basal diet was supplemented by individual fatty acids at a level of 0·54 g d^{-1} (50 g kg^{-1} DM). The purity of the fatty acids was ≥95% (Fluka Chemie AG, Buchs, Switzerland). The analysed average composition of the supplemented diets was (kg^{-1} DM): 958 ± 1 g organic matter (OM); 65 ± 3 g ether extract; 308 ± 7 g neutral detergent fibre (NDF); 216 ± 2 g crude protein and 19·9 ± 0·3 MJ gross energy. The respective values of the unsupplemented diet were (kg^{-1} DM): 956 g OM; 18 g ether extract; 326 g NDF; 222 g crude protein and 19·0 MJ gross energy.

Laboratory analysis and statistical evaluation of data

In each of the four incubation series rumen fluid was analysed daily for pH and ammonium (determined as ammonia), using a pH meter (model 632; Metrohm, Herisau, Switzerland) equipped with the appropriate electrodes, and volatile fatty acids (VFA), by gas chromatography (GC Star 3400 CX; Varian, Sugarland, TX, USA). Gas released daily from each fermenter was completely collected in gas-proof bags and quantified. Gas samples were analysed for methane and hydrogen by gas chromatography (model 5890 Series II; Hewlett Packard, Avondale, PA, USA). Hydrogen balance was calculated according to Demeyer (1991) considering VFA and methane produced by fermentation of carbohydrates and amino acids. Standard procedures were used to determine DM, total ash, ether extract and NDF in the diets (Dohme *et al.* 1999). Because $C_{8:0}$ and $C_{10:0}$ volatilized during extraction, ether extract was not determined in the respective diets. Crude protein content was assessed from the nitrogen content (6·25 ×N) determined with a C/N analyser (Leco-Analysator Typ FP-2000; Leco Instrumente GmbH, Kirchheim, Germany). Gross energy was measured with an adiabatic calorimeter (C700 T System; IKA-Analysentechnik GmbH, Heitersheim, Germany). Apparent degradation of OM and NDF was calculated from the nutrient contents present in the nylon bags before and after 48 h incubation.

Rumen ciliate protozoa and total bacteria were counted using Bürker counting chambers (Blau Brand, Wertheim, Germany) of 0·1- and 0·02-mm depth, respectively. Ciliates were enumerated each day and bacteria on days 1, 4, 7 and 10 of the incubation series. Cell counts of methanogens were determined as colony-forming units on the first and last day of each incubation series in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) filled with $N_2 : H_2$ (94 : 6, v/v). Methanogen cultivation and determination were performed as described by Dohme *et al.* (1999).

The treatment group and incubation series were considered as effects in analysis of variance using the general linear model procedure of SAS (Version 6·12; SAS Institute, Cary NC, USA). Multiple comparison among means was done with the Tukey method.

RESULTS AND DISCUSSION

Fatty acids and microbial populations

The present study analysed the effects of supplementary fatty acids on fermentation by a mixed rumen microbial population, using a rumen simulation model (RUSITEC). Methanogenic, protozoal and bacterial populations in rumen fluid responded differently to the supplementation of various individual fatty acids (Fig. 1). Compared with $C_{10:0}$ fatty acid, the number of methanogens was significantly lower than with $C_{12:0}$ and $C_{18:2}$ and numerically lower with $C_{14:0}$. All other treatment groups had intermediate values. The inhibiting effect of $C_{12:0}$ and $C_{14:0}$ fatty acids agrees with observations of Henderson (1973) who supplied different concentrations of these fatty acids to pure cultures of *Methanobrevibacter ruminantium*. Methanogens, as Archaea, have a different cell wall structure to that of bacteria (Miller 1995) and this may account for their susceptibility to certain fatty acids. In the present study, the observed promotion of the methanogenic population in diets supplemented with $C_{8:0}$ and $C_{10:0}$ may be due to the decline in numbers of protozoa, because these microorganisms also degrade Archaea to a certain extent (Newbold *et al.* 1996). Moreover, Miller *et al.* (1986) documented a stimulation of *M. ruminantium* growth by a mixture of isoacids, including isovalerate, which increased with $C_{8:0}$ treatment (Table 1).

Compared with the control, supplementation with $C_{8:0}$, $C_{10:0}$ and $C_{12:0}$ significantly decreased the concentration of protozoa, which confirms the results of Matsumoto *et al.* (1991). Newbold and Chamberlain (1988) observed a efficiency against protozoa in the order $C_{18:2} > C_{18:0} > C_{12:0}$. In the present study $C_{18:0}$ was completely ineffecti

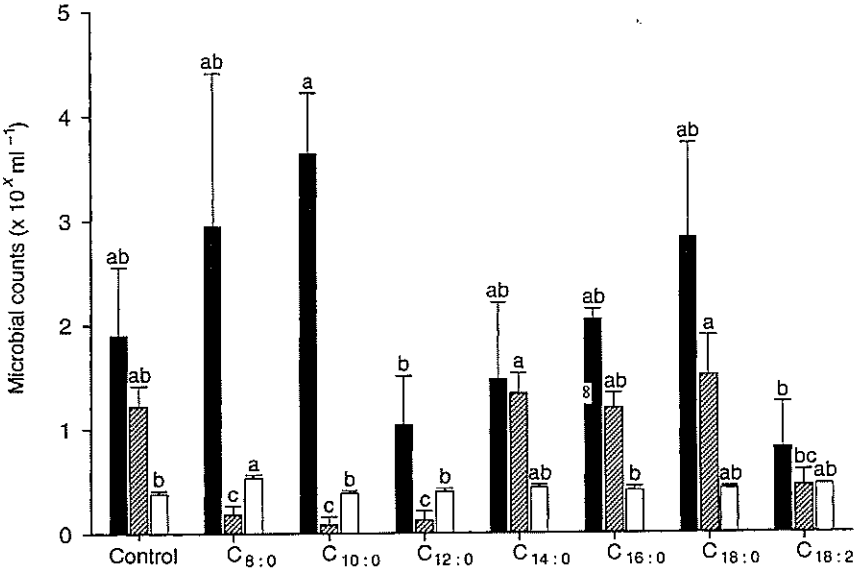

Fig. 1 Effect of individual fatty acids on rumen microbial counts. ■, Methanogens ($\times 10^5$ ml^{-1}); ▨, ciliate protozoa ($\times 10^4$ ml^{-1}); □, total bacteria ($\times 10^{10}$ ml^{-1}). Values represent the means of four incubation series, error bars indicate standard errors of the means. [a,b,c]Treatment means within the same group of micro-organisms not sharing a common superscript are significantly different at $P < 0{\cdot}05$ according to the Tukey test

Table 1 Effects of individual fatty acids on rumen fermentation*

	Treatment								
	Control	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:2}$	S.E.M.
Rumen fluid characteristics									
pH	6·97^{ab}	6·95^{abc}	7·02^{a}	6·85^{c}	6·96^{ab}	6·91^{bc}	6·95^{abc}	6·88^{bc}	0·021
Ammonium (mmol l^{-1})	10·9	10·3	9·6	10·0	10·9	11·5	11·0	10·5	0·43
Volatile fatty acids (mmol l^{-1})	110^{a}	112^{a}	91^{b}	114^{a}	109^{a}	118^{a}	110^{a}	118^{a}	3·3
acetate (mol %)	65·4	65·2	64·3	63·9	65·5	64·4	65·3	64·9	0·70
propionate (mol %)	18·7^{a}	14·3^{b}	8·8^{c}	17·8^{ab}	18·6^{a}	18·7^{a}	17·7^{ab}	20·2^{a}	0·76
butyrate (mol %)	12·3^{cd}	16·3^{b}	22·6^{a}	14·3^{bc}	12·3^{cd}	13·2^{cd}	13·3^{bcd}	10·9^{d}	0·67
isobutyrate (mol %)	0·3	0·4	0·4	0·3	0·4	0·3	0·3	0·4	0·05
valerate (mol %)	1·8^{ab}	1·6^{bc}	1·5^{c}	1·8^{ab}	1·9^{ab}	2·0^{a}	1·8^{ab}	1·8^{ab}	0·05
isovalerate (mol %)	1·44^{c}	2·13^{ab}	2·37^{a}	1·85^{abc}	1·35^{c}	1·34^{c}	1·50^{c}	1·82^{bc}	0·112
Nutrient degradation (after 48 h incubation)									
OM apparently degraded (%)	51·6^{bc}	51·3^{bc}	51·0^{bc}	53·8^{ab}	47·8^{c}	54·2^{ab}	51·4^{bc}	56·5^{a}	1·02
NDF apparently degraded (%)	24·0^{abc}	21·6^{bcd}	18·0^{cd}	15·5^{d}	21·4^{bcd}	28·8^{a}	26·5^{ab}	23·2^{abc}	1·49
Gaseous emission									
Methane (mmol d^{-1})	7·30^{abc}	8·80^{a}	8·20^{ab}	6·46^{bc}	6·42^{bc}	7·78^{ab}	7·86^{ab}	5·88^{c}	0·391
Hydrogen (mmol d^{-1})	0·16	0·07	0·12	0·10	0·18	0·23	0·07	0·05	0·073
Hydrogen balance									
produced (mmol d^{-1})	119·1	132·8	117·1	129·6	119·7	130·2	121·3	133·0	3·34
utilized (mmol d^{-1})	66·5^{ab}	74·1^{a}	64·0^{b}	65·7^{ab}	63·4^{b}	72·1^{ab}	69·1^{ab}	65·3^{ab}	2·01
recovered (%)	56·9	57·1	56·6	51·3	54·1	56·2	57·4	49·4	1·68

* Values represent the means of four incubation series.

S.E.M., Standard error of mean; OM, organic matter; NDF, neutral detergent fibre.

[a,b,c,d]Treatment means within the same line not sharing a common superscript are significantly different at $P < 0{\cdot}05$ according to the Tukey test.

against protozoa and $C_{18:2}$ was less toxic than $C_{8:0}$ – $C_{12:0}$. A simultaneous reduction in methanogenic and protozoal numbers, as found with $C_{12:0}$ and $C_{18:2}$, was expected considering that a certain proportion of methanogens are associated with protozoa (Jouany 1994). Nevertheless, the diverging effects of $C_{8:0}$, $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$

on methanogens and protozoa suggest that the mode of action of the individual MCFA against these microbes is not the same.

For total bacteria the significantly highest counts were found in $C_{8:0}$-treated rumen fluid and relatively small differences were noticed among the other treatment groups. In addition to their protozoa-suppressing effect $C_{10:0}$, $C_{12:0}$ and $C_{18:2}$ could also inhibit bacteria (Kabara 1978). This might explain why, in the present study, no general compensatory increase in the bacterial population was found, as might have been expected when protozoa are suppressed (Jouany 1994). Dong *et al.* (1997), applying 100 g coconut oil kg^{-1} feed, even observed a decrease in total bacterial counts.

Fatty acids and rumen fermentation characteristics

The effects of adding individual fatty acids on rumen fermentation are shown in Table 1. The pH was lowest with $C_{12:0}$ followed by $C_{18:2}$; for all other fatty acids the pH was 6·9 or above. Protozoa, known to assist in the stabilization of rumen pH (Jouany 1994), were not only low in number with $C_{12:0}$ and $C_{18:2}$ but also with $C_{8:0}$ and $C_{10:0}$, which indicates that other effects such as total VFA concentration might also have played a role in pH control. Rumen fluid ammonium concentration was not significantly affected by any of the fatty acids thus excluding major effects on ruminal feed protein degradation.

Total rumen fluid VFA concentration was similar in all groups except $C_{10:0}$. In addition, in this group there was a significant shift in the proportions of individual VFA, with significantly increased butyrate and isovalerate and decreased propionate and valerate. Significant variation in VFA was also observed with $C_{8:0}$ but not with other fatty acids. Generally, suppressing the protozoa should shift fermentation towards more propionate (Jouany 1994), but this was only observed to any extent in the case of $C_{18:2}$. The differences between treatment groups concerning the VFA profile might have resulted from complex inter-relationships in the microbial populations. For example, $C_{10:0}$ and $C_{12:0}$, while inhibiting several species of microbes, stimulate others (e.g. *Butyrivibrio*) when added in concentrations of 0·01–0·1 g l^{-1} (Van Nevel and Demeyer 1988).

Apparent degradation of OM was lowest with $C_{14:0}$ and highest with $C_{18:2}$. Since $C_{8:0}$, $C_{10:0}$ and $C_{18:2}$ are liquid at rumen temperatures, they might have flowed out of the nylon bags, which could have resulted in over-estimation of the OM degradation observed in these treatment groups as these fatty acids are probably not degraded. The apparent degradation of NDF was reduced with all MCFA, particularly $C_{10:0}$ and $C_{12:0}$, although at a generally low level. This agrees with the observations of Ushida *et al.* (1992) who supplied Ca-salts of $C_{8:0}$, $C_{10:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ to sheep and noted a decreased fibre digestion only with MCFA. However, polyunsaturated fatty acids are also known for their adverse effects on fibre digestion (Tamminga and Doreau 1991).

Fatty acids and ruminal methane release

Compared with the unsupplemented control, the addition of $C_{16:0}$ and $C_{18:0}$ had no significant effect on methane release from rumen fluid (Table 1); neither did these fatty acids change other fermentation traits. Demeyer and Henderickx (1967) also observed little or no suppression of methane production when supplying $C_{18:0}$ to a washed rumen cell suspension. In contrast, Blaxter and Czerkawski (1966) found each of the fatty acids used in the present study to cause some degree of methane inhibition. It is likely that the level of fatty acid supplementation and the environmental conditions play an important role in modulating the influence of the fatty acids and could account for these divergent results.

Compared with the $C_{16:0}$ and $C_{18:0}$ treatment groups, daily methane release from rumen fluid was on average 25% lower with $C_{18:2}$ and about 18% lower with $C_{12:0}$ and $C_{14:0}$. As the major proportion of fibre fermentation takes place in the rumen, approximately 90% of total tract methane release originates from the rumen (Murray *et al.* 1978). Adding fats such as coconut oil may cause a shift of fibre fermentation to the hindgut (Sutton *et al.* 1983). However, whole body methane release of lambs was also found to be suppressed by coconut oil supplementation (Machmüller *et al.* 2000).

Methane release and the number of methanogenic organisms were altered almost in parallel. The calculated formation rate varied from 0·03 to 0·18 nmol methane per methanogenic cell but did not show significant treatment differences. In contrast, the relationship between protozoal count and methane release differed greatly between fatty acids. This indicates that MCFA that suppressed methane production did not necessarily reduce the protozoal count ($C_{14:0}$ fatty acid), whereas in turn $C_{8:0}$ and $C_{10:0}$, which had a significantly negative effect against protozoa, were ineffective in relation to methanogenesis. This confirmed previous observations, gained from comparing faunated and defaunated rumen fluid (Dohme *et al.* 1999), that methane suppression by fats rich in MCFA is generally independent of simultaneous effects on protozoa. The level of methane suppression found in the present study with MCFA was lower than that observed in previous studies where coconut oil was supplemented at a level guaranteeing an approximately similar supply of MCFA (Machmüller *et al.* 1998; Dohme *et al.* 1999). Accordingly, Kabara (1984) noted that $C_{12:0}$ esterified within a monoglycerid is more active against

bacteria than when supplied as a free fatty acid, possibly because of a biologically important role of the hydroxyl groups.

The substrates for methanogenesis are mainly derived from fibre degradation when monosaccharides are converted, via pyruvate, into acetate, carbon dioxide and hydrogen. The suppression of methane release observed with $C_{12:0}$ fatty acid was probably associated with the simultaneously occurring reduction in fibre fermentation. Nevertheless, this neither affected the daily production nor release of gaseous hydrogen. When regarding the shifts in the calculated amounts of hydrogen utilized and recovered, this probably indicates that, in the case of the methane-suppressing fatty acids ($C_{12:0}$, $C_{14:0}$ and $C_{18:2}$), alternative hydrogen sinks, such as reductive acetogenesis (Van Nevel and Demeyer 1995), were promoted. The actual fate of the hydrogen has to be followed in further studies.

ACKNOWLEDGEMENTS

The authors are grateful to Professors Braun and Wanner and their staff at the Faculty of Veterinary Medicine, University of Zurich for providing rumen fluid and performing VFA analyses.

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