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ORIGINAL PAPER

Effects of C18 long-chain fatty acids on population density and methane production in three rumen ciliate cultures

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ABSTRACT. The study was carried out to investigate the long-term (28 days) effects of different types and concentrations of C18 fatty acids (FA) on methanogenesis, fermentation, and microbial population in cultures of *Entodinium caudatum* (EC), *Eudiplodinium maggii* (EM), and *Epidinium ecaudatum* (EE). The ciliates were maintained *in vitro* on a basal 'caudatum' type culture medium, supplemented with a substrate mixture consisting of powdered meadow hay, wheat gluten (Sigma), crystalline cellulose (Sigmacell 20), and barley flour, together with an undefined prokaryotic population. Control cultures were maintained on the basal medium, whereas experimental ones were supplemented with stearic acid (SA), oleic acid (OA), linoleic acid (LN), and linolenic acid (LNA) at concentrations of 1, 5, 25, and 50 g/kg substrate mixture. After long-term cultivation, the following parameters were measured: pH, ammonia, short chain fatty acids (SCFA), methane concentration, and protozoal counts. Ciliates were enumerated under a light microscope, and methane level was determined by gas chromatography. Responses to fatty acids were species- and dose-dependent: EM was the most tolerant, whereas higher supplementation levels (≥25 g/kg) generally reduced protozoal density in EC and EE, with signs of gradual adaptation during long-term cultivation. Methane concentration decreased most markedly in EC at 50 g/kg, with reductions ranging from approx. 18 to 61% depending on the fatty acid. In EE, decreases occurred mainly under stearic acid supplementation and selected unsaturated fatty acid treatments, whereas total SCFA, pH and ammonia remained largely unchanged. Overall, C18 fatty acids can reduce *in vitro* methanogenesis, but the magnitude of the effect depends on protozoan species, fatty-acid type and dose.

Introduction

The ability of ruminants to utilise a variety of feeds results from the presence of a highly diverse microbial ecosystem in the rumen. Numerous interactions have been developed between specific groups of rumen microorganisms, e.g., interspecific hydrogen transfer (Cieślak et al., 2013). This interaction is particularly pronounced between representatives of *Archaea*, i.e., methanogens, and protozoa. Ciliates release H_2 and CO_2 as end products of their metabolism, which are subsequently

utilised by methanogens in energy-yielding processes, where an important step is the reduction of CO_2 to methane using H_2 (Hegarty, 1999). This relationship leads to close physical associations between protozoa and methanogens, occurring within both the intra- and extracellular space of ciliates (Vogels et al., 1980). Moreover, published findings indicate that such interactions are a very common phenomenon (Vogels et al., 1980; Williams and Coleman, 1997). On the other hand, methanogens associated with rumen protozoa are estimated to account for 9–37% of total ruminal methane production (McAllister and Newbold, 2008). This suggests that the level of methanogenesis in the rumen may depend on the abundance and proportion of ciliates associated with methanogens.

It is widely known that methane is a major greenhouse gas, and considerable efforts have therefore been undertaken to reduce its emissions to the atmosphere. It is also well established that certain feed additives, particularly lipids, may adversely affect ruminal protozoal populations, while simultaneously decreasing methane emission. According to several authors, lipid supplementation in ruminant diets seems to be a promising nutritional strategy to reduce methane production (Eugène et al., 2008). Dietary lipids can also limit energy losses in the host animal (Moss et al., 2000). However, available data are inconsistent, indicating both stimulatory and toxic effects of dietary lipids on ruminal protozoa, especially at high proportions of C18 fatty acids (Hristov et al., 2004; Cieslak et al., 2006; Kišidayová et al., 2006; Zhang et al., 2008). For example Cieślak et al. (2009a) demonstrated that the rumen ciliates *Entodinium caudatum* and *Diplоplastron affine*, together with their associated bacterial populations, showed distinct metabolic responses depending on the form and concentration of linoleic acid (LA). In addition, Zhang et al. (2008) have suggested that further research is required to clarify the effects of specific C18 fatty acids on methanogenesis, including their long-term impact. We hypothesised that increasing supplementation with C18 fatty acids, especially at 25 and 50 g/kg of DM, would reduce the density of rumen ciliate protozoa and decrease methane production *in vitro*, through both (i) inhibitory effects on protozoal growth, and (ii) disruption of protozoa-associated methanogenesis, with the extent of responses varying between ciliate species and showing signs of adaptation during long-term (28 days) cultivation. The objective of the present study was to evaluate the long-term effects of different types and concentrations of C18 fatty acids on population density, methane production, and other metabolic activities in cultures of *Entodinium caudatum*, *Eudiplodinium maggii*, and *Epidinium ecaudatum*.

Material and methods

Cultivation experiment

The ciliates *Entodinium caudatum* (EC), *Eudiplodinium maggii* (EM), and *Epidinium ecaudatum* (EE) (Foissner, 1996) were isolated at the Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, from the natural rumen fauna of slaughtered sheep the natural rumen fauna of slaughtered sheep and subsequently maintained in *in vitro* culture. The basal culture medium was composed of a '*caudatum*'-type mineral solution (Coleman et al., 1972) and a substrate mixture. The '*caudatum*' mineral solution contained g/l: K_2HPO_4 6.3, KH_2PO_4 5.0, NaCl 0.65, $CaCl_2 \cdot 6\ H_2O$ 0.09, $MgSO_4 \cdot 7\ H_2O$ 0.09, CH_3COONa 0.75 and distilled water. The substrate mixture consisted (%, w/w) of powdered meadow hay (60%), wheat gluten (16%; Sigma-Aldrich, Darmstadt, Germany), crystalline cellulose (Sigmacell 20; 12%; Sigma-Aldrich), and barley flour (12%). The chemical composition of meadow hay and barley flour is presented in Table 1. The cultures of protozoa were initiated and maintained under anaerobic conditions according to the method of Michałowski et al. (1986). Briefly, cultures were grown in 40-ml serum bottles sealed with rubber stoppers and aluminium caps (Merck, Darmstadt, Germany). The substrate

Table 1. Chemical (g/kg dry matter) and fatty acid composition (g/kg FA) of the experimental substrates

Item	Meadow hay	Barley flour
Dry matter	923.9	886.7
Organic matter	865.7	869.2
Crude ash	58.2	17.5
Crude protein	108.6	163.5
Crude fat	13.8	21.4
NDF	548.1	202.4
ADF	310.1	59.5
C16:0	251	239
C18:0	46	20
C18:1 *cis*-9	63	120
C18:2 *cis*-9, *cis*-12	199	555
C18:3 *cis*-9, *cis*-12, *cis*-15	374	47
Others	67	19

FA – fatty acids, NDF – neutral-detergent fibre, ADF – acid-detergent fibre, C16:0 – palmitic acid, C18:0 – stearic acid, C18:1 *cis*-9 – oleic acid (cis-9-octadecenoic acid), C18:2 *cis*-9, *cis*-12 – linoleic acid (*cis*-9, *cis*-12 – octadecadienoic acid), C18:3 *cis*-9, cis-12, *cis*-15 – α-linolenic acid (all-*cis*-9,12,15 – octadecatrienoic acid), others (C12:0 – lauric acid, C14:0 – myristic acid, C16:1 – palmitoleic acid, C18:1 *cis*-11 – *cis*-vaccenic acid)

mixture was supplied daily at 15 mg per culture flask (0.375 mg/ml/day). Control cultures were maintained on the basal medium alone, whereas experimental cultures were supplemented with stearic acid (SA), oleic acid (OA), linoleic acid (LN), and linolenic acid (LNA) at concentrations of 1, 5, 25, and 50 g/kg substrate mixture (corresponding to 0.1, 0.5, 2.5, and 5%, respectively). The concentrations of supplemented fatty acids (FA) were selected based on the data reported by Kišidayová et al. (2006). All the FA were purchased from Sigma-Aldrich: 'SRI Instruments, Torrance, CA, USA' stearic acid (85680, 985 g/kg), oleic acid (75090, 990 g/kg), linoleic acid (62230, 990 g/kg), and linolenic acid (L2376, 990 g/kg). Appropriate amounts of FA were dissolved in pre-warmed basal medium containing substrates using three 30-s cycles of probe sonication prior to feeding, as described by Hristov et al. (2004). Cultures were flushed daily with CO_2 after feeding. Every fourth day, cultures were transferred to fresh medium at a 1:1 ratio. For each ciliate species and treatment, three control flasks and three experimental flasks were maintained in parallel. After long-term cultivation, on day 28, the following parameters were determined: pH, ammonia and short chain fatty acid (SCFA) concentrations in the medium, protozoal counts, and methane concentration in the gas phase.

Analytical procedures

Samples of meadow hay and barley flour (n = 3) were analysed according to AOAC International (2005) procedures for method no. 934.01) and ash (AS; method no. 942.05). Crude protein (CP) was determined using a Kjel-Foss Automatic 16210 analyser (method no. 976.05), crude fat (CF) using a Soxtec System HT analyser (method no. 2003.05), and acid-detergent fibre (ADF; method no. 973.18). Neutral-detergent fibre (NDF) was determined following the method of Van Soest et al. (1991). Organic matter was calculated as the difference between dry matter and ash content. FA composition of the substrates and post-culture medium from long-term protozoal cultures (day 28) was determined according to the protocol published by Cieślak et al. (2009b). The pH was measured using a digital pH meter (Elmetron, Typ CP-104, Zabrze, Poland). Ammonia concentration was determined spectrophotometrically using the Nessler reagent, as described by Szumacher-Strabel et al. (2004). SCFA levels were quantified by liquid chromatography (model 2690; Waters Corporation, Milford, MA, USA) according to the method of Czauderna et al. (2008). Protozoal density was assessed by counting all ciliate cells in 0.1 ml of each sample under a light microscope (Carl Zeiss, Jena, Germany), following the method of Michałowski et al. (1986). All samples were analysed in triplicate, and the results were expressed as cells per ml (cells/ml). Methane was collected directly from the headspace of the serum bottles according to Angelidaki et al. (2009). A sterile gas-tight syringe was inserted through the rubber stopper to minimise disturbance of the internal environment. The collected samples were analysed by gas chromatography using an SRI 310 instrument (SIR Instruments, Torrance, USA) equipped with a thermal conductivity detector (TCD) and a Carboxen-1000 column (60/80 mesh, 15 FT × 1,8; SUPELCO, Merck). Nitrogen was used as the carrier gas at a constant flow rate of 30.0 ml/min. Oven temperature was programmed as follows: initially 180 °C for 1.5 min, followed by an increase at 20 °C/min to 220 °C. The injected gas samples had a volume of 1000 µl. Peaks were identified by comparison of retention times with certified gas standards (5.63% CO_2, 5.56% CH_4, 5.10% H_2, and balance N_2; Multa S.C., Poland) using Peak Simple software (Version 3.29).

Calculations and statistical analyses

General linear mixed models implemented in the Proc Mixed procedure of SAS (version 7.0; SAS Institute, Cary, USA) were used for data analysis, following the recommendations of Quinn and Keough (2002). Ciliate taxon (monoculture), type of C18 fatty acid, and supplementation level were considered as independent variables. All potential 2- and 3-way interactions were initially incorporated into the models. Non-significant interactions ($P > 0.05$) were removed, and the reduced models were subsequently re-fitted. Separate models were constructed for each response variable: protozoal counts, methane concentration and SCFA concentrations in the culture medium. For the latter variable, analyses were conducted both for total concentration and for individual acid fractions. Differences between treatment means were assessed using Tukey's post hoc test. Individual culture vessels (triplicate bottles) were included as a random effect. The differences were considered significant at $P < 0.05$.

Results

Effects of fatty acids on protozoa

The impact of FA was dependent on protozoan species, the type of FA, and their concentration. Supplementation of the EC culture with 25 and 50 g/kg FA resulted in a significant reduction in the protozoal abundance ($P < 0.05$), following

Table 2. Effects of different fatty acids (FA) on protozoal counts, methane concentration and fermentation parameters in *Entodinium caudatum* monocultures

Items	Level of FA supplementation (g/kg of food mixture)					SEM
	0	1	5	25	50	
C18:0 (SA)						
protozoa, ml^{-1}	6100^{a}	6333^{a}	5533^{a}	3866^{b}	2133^{c}	185.942
methane, mmol/l	0.54^{a}	0.51^{a}	0.53^{a}	0.49^{a}	0.21^{b}	0.034
total SCFA, mmol/l	20.64	19.84	20.05	19.99	20.02	0.133
A, mmol/l	17.16	16.58	16.65	16.69	16.80	0.102
P, mmol/l	2.17	2.06	2.06	2.11	2.08	0.027
butyrate, mmol/l	1.31^{a}	1.20^{ab}	1.34^{a}	1.20^{ab}	1.15b	0.023
A/P	7.90	8.06	8.10	7.92	8.08	0.076
ammonia, mmol/l	3.42	3.33	3.71	3.41	3.53	0.077
pH	6.20^{a}	6.17^{ab}	6.17^{ab}	6.18^{a}	6.16^{b}	0.007
C18:1 (OA)						
protozoa, ml^{-1}	6266^{a}	6233^{a}	5800^{ab}	5333^{b}	4866^{c}	153.994
methane, mmol/l	0.55^{ab}	0.58^{a}	0.53^{b}	0.52^{b}	0.45^{c}	0.013
total SCFA, mmol/l	21.96	21.53	22.30	22.14	22.10	0.115
A, mmol/ml	17.73^{a}	17.47^{a}	17.62^{a}	16.71^{b}	16.23^{b}	0.162
P, mmol/l	2.57^{b}	2.50^{b}	3.11^{b}	4.13^{a}	4.79^{a}	0.248
butyrate, mmol/l	1.66^{a}	1.56^{a}	1.57^{a}	1.31^{b}	1.07^{b}	0.061
A/P	6.89^{a}	7.02^{ab}	5.76^{b}	4.05^{c}	3.39^{c}	0.405
ammonia, mmol/l	3.38	2.98	2.96	2.47	2.45	0.173
pH	6.19	6.18	6.18	6.17	6.17	0.005
C18:2 (LN)						
protozoa, ml^{-1}	6233	6733	6433	5666	5350	146.237
methane, mmol/l	0.56^{a}	0.56^{a}	0.52^{ab}	0.48^{bc}	0.44^{c}	0.013
total SCFA, mmol/l	22.11	21.81	22.03	21.45	21.33	0.104
A, mmol/l	17.73^{a}	17.46^{a}	17.64^{a}	16.70^{b}	15.74^{c}	0.205
P, mmol/l	2.83^{c}	2.75^{c}	2.80^{c}	3.65^{b}	4.73^{a}	0.207
butyrate, mmol/l	1.54^{a}	1.61^{a}	1.58^{a}	1.10^{b}	0.86^{c}	0.082
A/P	6.28^{a}	6.36^{a}	6.30^{a}	4.59^{b}	3.33^{c}	0.330
ammonia, mmol/l	3.60	3.56	3.61	3.61	3.45	0.043
pH	6.26	6.25	6.23	6.23	6.22	0.010
C18:3 (LNA)						
protozoa, ml^{-1}	6133^{a}	5900^{a}	5066^{ab}	4366^{b}	4216^{b}	225.223
methane, mmol/l	0.52^{a}	0.51^{a}	0.45^{b}	0.39^{c}	0.37^{c}	0.017
total SCFA, mmol/l	22.31^{a}	21.85^{b}	21.55^{b}	19.79^{c}	19.36^{c}	0.348
A, mmol/ml	18.36^{a}	17.87^{ab}	17.63^{ab}	14.85^{ab}	13.78^{b}	0.543
P, mmol/l	2.62^{c}	2.67^{c}	2.56^{c}	3.73^{b}	4.62^{a}	0.249
butyrate, mmol/l	1.32^{ab}	1.30^{ab}	1.35^{a}	1.21^{b}	0.97^{c}	0.044
A/P	7.02^{a}	6.72^{a}	6.90^{a}	3.98^{b}	2.99^{b}	0.509
ammonia, mmol/l	3.72	3.61	3.74	3.57	3.42	0.052
pH	6.24	6.22	6.21	6.20	6.19	0.010

SCFA – short chain fatty acids, C18:0 (SA) – stearic acid, C18:1 (OA) – oleic acid, C18:2 (LN) – linoleic acid, C18:3 (LNA) – linolenic acid, A – acetate, P – propionate, A/P – acetate to propionate ratio, SEM – standard error of the mean; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

the addition of SA, OA, and LNA (Table 2). The strong effects ($P < 0.05$) were observed at 50 g/kg, with reductions of 65, 31, and 22% for SA, LNA, and OA, respectively. A similar, but less pronounced ($P < 0.05$) influence occurred at 25 g/kg, with decreases of 37, 29, and 15% for SA, LNA, and OA, respectively. In EM cultures, supplementation with the highest dose (50 g/kg) of all FA resulted in significant decreases ($P < 0.05$) in protozoal counts, ranging from 4 to 28% (Table 3). On average, adding 5, 25, and 50 g/kg SA reduced the EE population by 33, 37, and 41%, respectively ($P < 0.05$; Table 4). In addition, higher concentrations of OA and LNA (25 and 50 g/kg) significantly decreased ($P < 0.05$) EE counts by 8–26%.

Table 3. Effects of different fatty acids (FA) on protozoal population, methane concentration and fermentation parameters in *Eudiplodinium maggii* monocultures

Items	Level of FA supplementation (g/kg of food mixture)					SEM
	0	1	5	25	50	
C18:0 (SA)						
protozoa, ml^{-1}	1740^{a}	1563^{ab}	1560^{ab}	1526b	1246c	44.91
methane, mmol/l	0.73	0.70	0.70	0.68	0.66	0.011
total SCFA, mmol/l	15.10	14.30	14.92	15.10	15.49	0.123
A, mmol/l	12.61	12.10	12.58	12.39	12.69	0.079
P, mmol/l	2.18^{b}	2.02^{b}	2.10^{b}	2.44^{a}	2.56^{a}	0.058
butyrate, mmol/l	0.30^{a}	0.18^{b}	0.24^{ab}	0.27^{ab}	0.25^{ab}	0.015
A/P	5.79^{a}	6.00^{a}	5.99^{a}	5.07^{b}	4.96^{b}	0.129
ammonia, mmol/l	4.23^{b}	4.27^{ab}	4.56^{ab}	4.81^{ab}	4.95^{a}	0.097
pH	6.16	6.15	6.15	6.16	6.15	0.005
C18:1 (OA)						
protozoa, ml^{-1}	1783^{a}	1723^{a}	1796^{a}	1716^{a}	1600^{b}	29.726
methane, mmol/l	0.74^{a}	0.73^{ab}	0.70^{ab}	0.68^{ab}	0.66^{b}	0.011
total SCFA, mmol/l	15.72^{ab}	15.81^{ab}	16.25^{a}	14.94^{c}	15.40^{bc}	0.130
A, mmol/ml	13.21^{a}	13.29^{a}	13.74^{a}	12.33^{b}	12.15^{b}	0.171
P, mmol/l	2.09^{b}	2.11^{b}	2.06^{b}	2.25^{b}	2.92^{a}	0.090
butyrate, mmol/l	0.42^{ab}	0.41^{ab}	0.45^{a}	0.37^{bc}	0.32^{c}	0.013
A/P	6.34^{ab}	6.32^{ab}	6.69^{a}	5.52^{b}	4.16^{c}	0.255
ammonia, mmol/l	4.26^{c}	4.46^{bc}	4.80^{ab}	4.81^{ab}	4.93^{a}	0.076
pH	6.29	6.26	6.29	6.30	6.28	0.008
C18:2 (LN)						
protozoa, ml^{-1}	1716^{ab}	1846^{ab}	1883^{a}	1730^{ab}	1640^{b}	30.499
methane, mmol/l	0.75	0.78	0.76	0.76	0.73	0.011
total SCFA, mmol/l	14.92^{a}	14.90^{a}	14.45^{b}	14.68^{ab}	14.76^{ab}	0.058
A, mmol/l	12.73^{a}	12.54^{ab}	12.21^{b}	11.26^{c}	11.04^{c}	0.186
P, mmol/l	1.84^{c}	2.03^{c}	1.95^{c}	3.12^{b}	3.51^{a}	0.185
butyrate, mmol/l	0.34^{a}	0.33^{a}	0.29^{ab}	0.30^{ab}	0.21^{c}	0.013
A/P	6.92^{a}	6.19^{ab}	6.28^{b}	3.61^{c}	3.15^{c}	0.415
ammonia, mmol/l	3.85^{b}	4.19^{ab}	4.22^{ab}	4.54^{a}	4.65^{a}	0.088
pH	6.26^{a}	6.24^{ab}	6.24^{ab}	6.22^{ab}	6.20^{b}	0.007
C18:3 (LNA)						
protozoa, ml^{-1}	1753^{a}	1706^{a}	1666^{a}	1606^{a}	1253^{b}	50.516
methane, mmol/l	0.74^{a}	0.71^{ab}	0.66^{ab}	0.64^{b}	0.55^{c}	0.019
total SCFA, mmol/l	15.01^{ab}	14.45^{b}	14.55^{ab}	15.11^{ab}	15.16^{a}	0.101
A, mmol/ml	12.18^{a}	12.06^{ab}	12.27^{a}	11.13^{b}	10.75^{b}	0.173
P, mmol/l	2.50^{b}	2.09^{b}	2.02^{b}	3.83^{a}	4.31^{a}	0.264
butyrate, mmol/l	0.33^{a}	0.30^{ab}	0.25^{b}	0.15^{c}	0.10^{c}	0.025
A/P	4.92^{a}	5.77^{a}	6.11^{a}	2.90^{b}	2.50^{b}	0.414
ammonia, mmol/l	4.26^{b}	4.33^{b}	4.43^{b}	4.55^{ab}	4.79^{a}	0.058
pH	6.18^{a}	6.16^{a}	6.16^{a}	6.13^{ab}	6.12^{b}	0.010

SCFA – short chain fatty acids, C18:0 (SA) – stearic acid, C18:1 (OA) – oleic acid, C18:2 (LN) – linoleic acid, C18:3 (LNA) – linolenic acid, A – acetate, P – propionate, A/P – acetate to propionate ratio, SEM – standard error of the mean; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

Methane concentration

The EE control culture produced a significantly higher amount of methane (0.77 mmol/l) compared to the EM (0.74 mmol/l) and EC controls (0.54 mmol/l). As for protozoal population density, the effect of FA depended on protozoan species, FA type, and concentration. The greatest reduction in methane concentration was recorded in the EC monoculture (Table 2). Supplementation with 50 g/kg of the tested FA decreased ($P < 0.05$) methane production by 18–61%. Supplementation with 25 g/kg OA, LA, and LNA reduced ($P < 0.05$) methane production in EC cultures by 5, 14, and 25%, respectively (Table 2). Although FA addition decreased methane concentration in EM monocultures, significant effects were observed only for 50 g/kg OA (11%) and 25 and 50 g/kg LNA (14% and 26%, respectively; Table 3).

Short chain fatty acids concentration in protozoal cultures

The concentrations of almost all analysed SCFA were unaffected by the applied treatments. The only exception was butyric acid, whose concentration decreased in treatment following 2.5% LNA addition to the EC culture compared to the 0.5% level (Table 2), and after supple-mentation with 5% LNA in the EE culture compared to both the control and the 0.1% dose (Table 4).

Table 4. Effects of different fatty acids (FA) on protozoal population, methane concentration and fermentation parameters in *Epidinium* ecaudatum monocultures

Items	Level of FA supplementation, g/kg of food mixture					SEM
	0	1	5	25	50	
C18:0 (SA)						
protozoa, ml^{-1}	380^{a}	376^{a}	253^{b}	240^{b}	223^{b}	18.794
methane, mmol/l	0.78^{a}	0.78^{a}	0.67^{b}	0.51^{c}	0.49^{c}	0.034
total SCFA, mmol/l	15.10^{ab}	14.30^{b}	14.92^{ab}	15.10^{ab}	15.49^{a}	0.123
A, mmol/l	12.61^{a}	12.10^{b}	12.58^{ab}	12.39^{ab}	12.69^{ab}	0.079
P, mmol/l	2.18^{b}	2.02^{b}	2.10^{b}	2.44^{a}	2.56^{a}	0.058
butyrate, mmol/l	0.30^{a}	0.18^{b}	0.24^{ab}	0.27^{ab}	0.25^{ab}	0.015
A/P	5.79^{a}	6.00^{a}	5.99^{a}	5.07^{b}	4.96^{b}	0.129
ammonia, mmol/l	2.01^{a}	1.92^{ab}	1.91^{ab}	1.82^{ab}	1.77^{b}	0.042
pH	6.27^{a}	6.26^{a}	6.25^{a}	6.22^{b}	6.24^{ab}	0.007
C18:1 (OA)						
protozoa, ml^{-1}	390^{a}	413^{a}	383^{ab}	356^{b}	343^{c}	7.069
methane, mmol/l	0.76^{a}	0.76^{a}	0.76^{a}	0.66^{b}	0.61^{c}	0.017
total SCFA, mmol/l	15.72^{ab}	15.81^{ab}	16.25^{a}	14.94^{c}	15.40^{b}	0.130
A, mmol/l	13.21^{a}	13.29^{a}	13.74^{a}	12.33^{b}	12.15^{b}	0.171
P, mmol/l	2.09^{b}	2.11^{b}	2.06^{b}	2.25^{b}	2.92^{a}	0.090
butyrate, mmol/l	0.42^{ab}	0.41^{ab}	0.45^{a}	0.37^{b}	0.32^{c}	0.013
A/P	6.34^{ab}	6.32^{ab}	6.69^{a}	5.52^{b}	4.16^{c}	0.255
ammonia, mmol/l	1.92	1.84	1.87	2.02	1.83	0.029
pH	6.29^{a}	6.26^{ab}	6.26^{ab}	6.25^{b}	6.26^{ab}	0.005
C18:2 (LN)						
protozoa, ml^{-1}	391	356	380	373	366	4.891
methane, mmol/l	0.77	0.75	0.79	0.76	0.75	0.013
total SCFA, mmol/l	14.92^{a}	14.90^{a}	14.45^{b}	14.68^{ab}	14.76^{ab}	0.058
A, mmol/ml	12.73^{a}	12.54^{ab}	12.21^{b}	11.26^{c}	11.04^{c}	0.186
P, mmol/l	1.84^{c}	2.03^{c}	1.95^{c}	3.12^{b}	3.51^{a}	0.185
butyrate, mmol/l	0.34^{a}	0.33^{a}	0.29^{ab}	0.30^{ab}	0.21^{b}	0.013
A/P	6.92^{a}	6.19^{b}	6.28^{ab}	3.61^{c}	3.15^{c}	0.415
ammonia, mmol/l	1.95	2.03	2.03	2.18	2.28	0.056
pH	6.35^{a}	6.28^{b}	6.28^{b}	6.27^{b}	6.23^{b}	0.007
C18:3 (LNA)						
protozoa, ml^{-1}	383^{a}	406^{a}	363^{ab}	320^{b}	283^{c}	12.606
methane, mmol/l	0.78^{a}	0.77^{a}	0.68^{ab}	0.65^{ab}	0.61^{b}	0.019
total SCFA, mmol/l	15.01^{ab}	14.32^{b}	14.55^{ab}	15.23^{a}	15.16^{ab}	0.151
A, mmol/ml	12.18^{a}	12.06^{a}	12.27^{a}	11.13^{b}	10.75^{c}	0.173
P, mmol/l	2.50^{b}	2.09^{b}	2.02^{b}	3.83^{a}	4.31^{a}	0.264
butyrate, mmol/l	0.33^{a}	0.30^{a}	0.25^{b}	0.15^{c}	0.10^{c}	0.025
A/P	4.92^{a}	5.77^{a}	6.11^{a}	2.90^{b}	2.50^{b}	0.414
ammonia, mmol/l	1.87^{b}	2.01^{ab}	2.11^{ab}	2.13^{ab}	2.26^{a}	0.038
pH	6.30^{a}	6.27^{ab}	6.25^{b}	6.23^{b}	6.26^{ab}	0.009

SCFA – short chain fatty acids, C18:0 (SA) – stearic acid, C18:1 (OA) – oleic acid, C18:2 (LN) – linoleic acid, C18:3 (LNA) – linolenic acid, A – acetate, P – propionate, A/P – acetate to propionate ratio, SEM – standard error of the mean; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

Discussion

To exclude the possibility that uneven distribution of the examined FA occurred, which could obscure the results, FA content was determined in the post-culture medium on day 28 of the experiment. The results (data not shown) clearly indicated uniform distribution of the analysed FA in the culture medium.

Effects on fermentation parameters

In the monoculture incubations, ruminal pH remained largely stable over most supplementation levels of OA, LN and LNA acids, which was consistent with short-term protozoal monoculture studies where lipid addition did not significantly affect basic rumen parameters (Cieślak et al., 2006). Nevertheless, a pH reduction was observed in selected treatments, particularly in the LN supplementation in EE (pH 6.35 vs 6.23 at 0 vs 50 g/kg) and, to a lesser extent, in EM (pH 6.26 vs 6.20), while other fatty-acid variants showed only minor changes. Importantly, the most pronounced pH decreases were not associated with higher total SCFA concentrations and occurred despite unchanged or increased ammonia levels, making the classical explanation that higher SCFA production, combined with reduced NH_3 buffering, leads to pH decline unlikely to be the primary mechanism in these treatments. We further noted that EE cultures also had a lower baseline ammonia pool than the other monocultures, which may render this system more sensitive to small amounts of net acid equivalent. Since rumen ciliate taxa differ greatly in metabolic niche and functional efficiency, *Epidinium* is frequently associated with a profile richer in fibre- and carbohydrate-related enzymes, compared to *Entodinium*-type ciliates (Ivan et al., 2001), species-specific interactions with polyunsaturated fatty acids (PUFA) are plausible. We therefore hypothesised that the combination of a high-fibre/low-starch substrate, protozoal monocultures, and a strongly phosphate-buffered Coleman 'caudatum-type' medium attenuated the pH differences caused by fermentation, allowing a small but statistically detectable LN effect to emerge. A mechanistically possible factor is a direct 'acid-equivalent' effect of added free FA, whose apparent ionisation depends on micellar/membrane partitioning and supramolecular state in aqueous systems (Small et al., 1984). On the other hand, changes in the qualitative and quantitative composition of rumen microbial population can significantly affect SCFA production and their molar proportions (Ivan et al., 2001). However, Cieślak et al. (2006) observed no effect of FA on basic rumen parameters (SCFA, pH, and ammonia concentration) in monocultures of *Eremoplastron dilobum* during a short-term (24 h) incubation with the addition of 0.66 mg of rapeseed (OA source) and linseed (LNA source) oils corresponding to 5% DM. Similarly, the same group in another study (Cieślak et al., 2009a) did not find any changes in SCFA or ammonia concentrations during long-term (30-day) incubation of EE and *Diploplastron affine* monocultures supplemented with low levels of linoleic acid (0.05 µg per culture). Comparable results were reported for supplementation with evening primrose, borage and Saint-Mary's thistle seed oil (17.4 mg per culture; 5% DM), as well as for pure linoleic acid at 60 mg per culture, with no effects on SCFA content or molar proportions. However, Szumacher-Strabel et al. (2004) reported that a higher dose of unsaturated fatty acids (UFA) in the supplement was associated with stronger effects on protozoal populations and butyric acid concentration. This relationship was not explicitly observed in the present study, and interpretation is partly limited by the inability to distinguish between bacterial and protozoal-derived SCFA. Michałowski (1987) showed that rumen protozoa can markedly contribute to SCFA production, accounting for 30–46% of total SCFA. Considering the fermentation parameters, the contribution of other microorganisms should also be taken into account, particularly fungi, which can participate in SCFA synthesis (Saxena et al., 2010). Fungi were also present in the analysed cultures and were observed microscopically, although they were not quantified.

Effects on protozoan population

Earlier studies by Cieślak et al. (2009a) proved that the rumen protozoa (EE and *Diploplastron affine)* differ in their metabolic responses to the dose and form of linoleic acid supplementation. The same authors also showed that rumen protozoa may be a UFA reservoir for their host, depending on the feed ration. Nevertheless, the FA profile of a protozoan species is species-specific (Cieslak et al., 2009c), and they may account for up to 35% of total rumen microbial biomass (Williams and Coleman, 1992). The present results indicate that the effects of FA depend on their type, the duration of exposure, and the protozoan species. EC was negatively affected by higher doses of the FA tested. The greatest reduction in its population size was observed with 5% SA supplementation; however, in this monoculture, both 2.5 or 5% SA addition consistently decreased protozoan counts throughout the cultivation period. We also observed that the EC population gradually

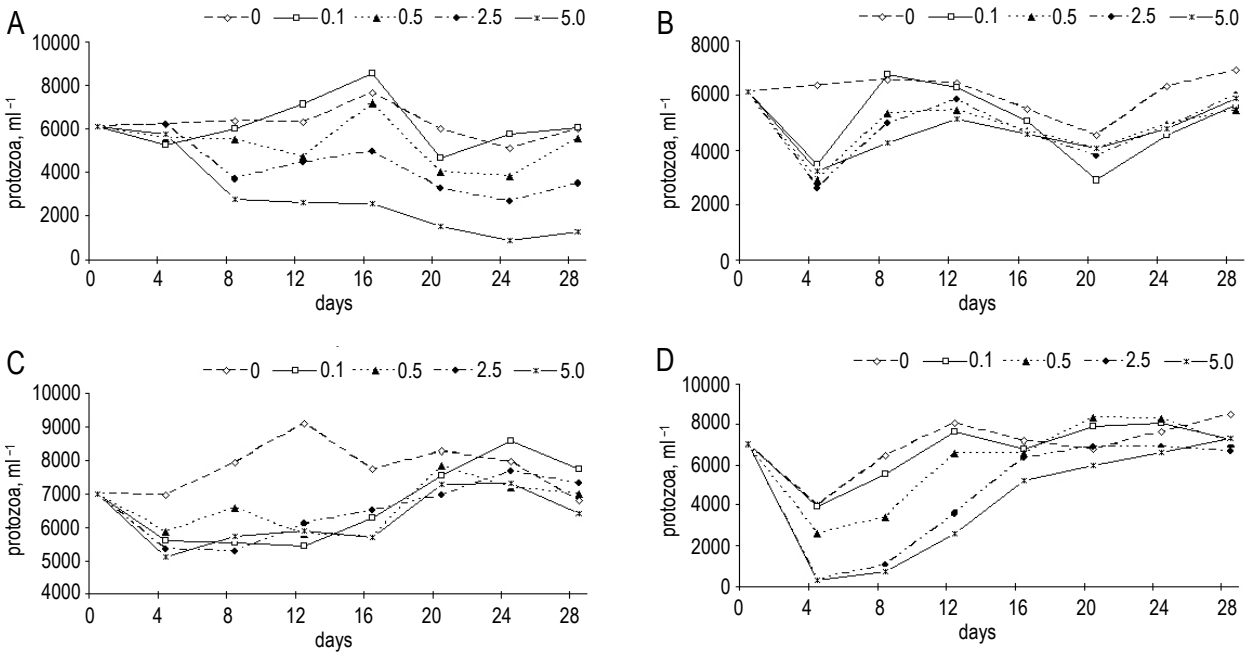

Figure 1. Changes in the number of *Entodinium caudatum* in the cultures fed different concentration (0, 0.1, 0.5, 2.5 or 5%) of stearic acid (A), oleic acid (B), linoleic acid (C), linolenic acid (D)

adapted to LN supplementation by day 20 of cultivation, regardless of its level (Figure 1). Statistically significant inhibition was observed in EC monocultures supplemented with 2.5% and 5% LNA during the 28-day incubation; however, adaptation to the tested FA occurred by day 16 at 2.5% LNA and by day 20 at 5% LNA. Similar adaptation trends were observed for EE with OA, LN, and LNA supplementation; however, in this culture, the time required for adaptation was different for individual FA (Figure 3). In contrast, the examined FA exerted no statistically significant effect on the population of EM (Figure 2). To date, most studies have reported that both saturated and unsaturated fatty acids, whether applied individually or in mixtures and in liquid or solid form, negatively affect total protozoal populations or dominant species under *in vitro* and *in vivo* conditions (Ivan et al., 2001; Hristov et al., 2004; Zhang et al., 2008).

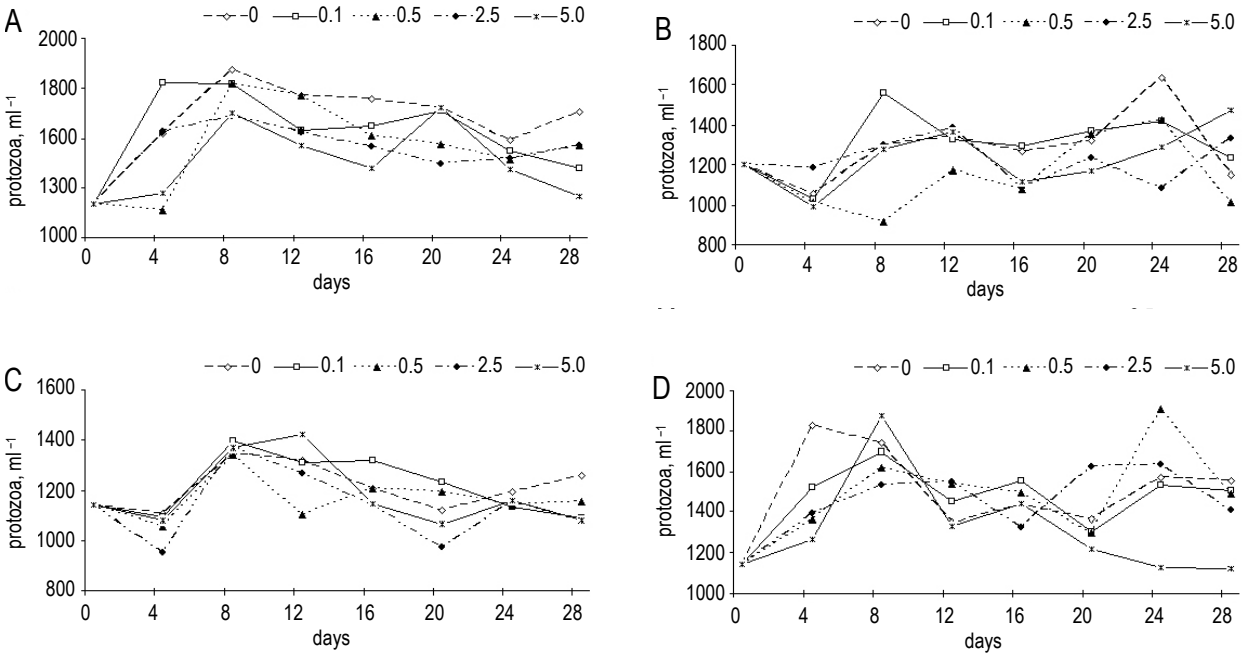

Figure 2. Changes in the number of *Eudiplodinium maggii* in the cultures fed different concentration (0, 0.1, 0.5, 2.5 or 5%) of stearic acid (A), oleic acid (B), linoleic acid (C), linolenic acid (D)

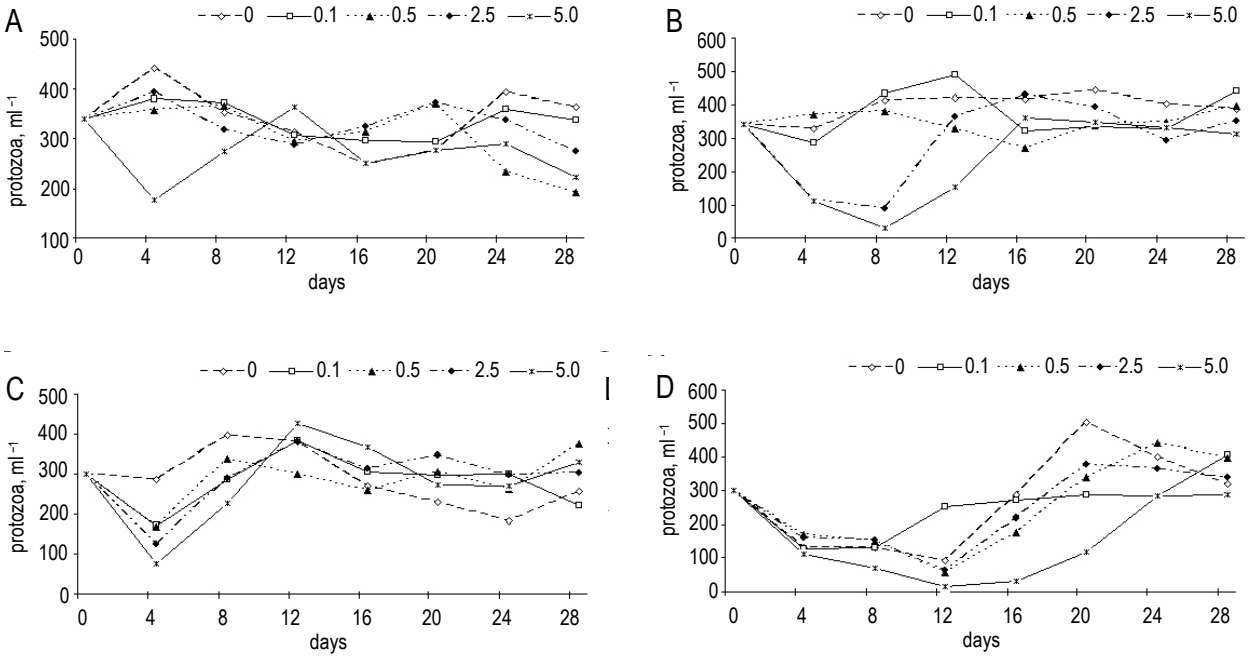

Figure 3. Changes in the number of *Epidinium ecaudatum* in the cultures fed different concentration (0, 0.1, 0.5, 2.5 or 5%) of stearic acid (A), oleic acid (B), linoleic acid (C), linolenic acid (D)

The current results suggest that the examined species are able to adapt to unfavourable environments, which is consistent with previous findings (Kišidayová et al., 2006). Some discrepancies between studies may arise from differences in the rumen fluid employed, including animal species, individual variation, and diet of the host. Moreover, some authors suggested that rumen microorganisms have a limited capacity to assimilate, transform, and utilise dietary lipids (Ivan et al., 2001). The FA supplementation level required to affect these processes has not been clearly defined. Ivan et al. (2001) proposed a critical concentration of 10 mg/l for LA, while Kišidayová et al. (2005) suggested that as little as 3 μg/l already inhibited the growth and development of *Diplodinium* sp. and *Entodinium* sp. Additionally, Ivan et al. (2001) have demonstrated that *Isotricha* sp., *Dasytricha* sp., and cellulolytic protozoa (*Polyplastron* sp., *Diplodinium* sp., *Enoploplastron* sp.) are more sensitive to LA than *Entodinium* sp. This indicates that there is substantial inconsistency in the reported FA effects on individual protozoan species, and the present study represents a further contribution to this ongoing discussion. The current findings confirm that protozoan species do not respond uniformly to the same FA. Here, LN was not the most toxic FA for any of the three examined rumen ciliate populations, although most FA concentrations initially reduced the abundance of EC and EE in monocultures. These findings partly align with the fatty acid toxicity ranking published by Henderson (1973), who identified linoleic acid as the most toxic C18-FA, a conclusion also supported by Hristov et al. (2004). Henderson (1973) further reported stearic acid as the least detrimental FA to protozoa. In contrast, in the present study, the most toxic FA for EC were, in descending order, SA, LNA, OA, and LN, whereas for EE, these were LNA, OA, and SA, with LN showing no adverse effect. EM was the most resistant species, with only SA and LNA reducing the counts of this protozoan species.

The discrepancies between our findings and those of earlier studies may be due to the use of monocultures, which eliminate interactions with other protozoan species. Similar responses have been reported for *Eremoplastron dilobum* (Cieslak et al., 2006), supporting this interpretation. Differences should also be expected between monocultures and mixed protozoan communities. In addition, *in vitro* systems exclude host-related factors, which can substantially influence experimental outcomes. Hence, confirmation of these results requires studies in monofaunated animals, which would allow unambiguous validation under *in vivo* conditions. Further work is also needed to elucidate the mechanisms underlying protozoan adaptation to culture conditions altered by FA supplementation.

Effects on methanogenesis

The process of methanogenesis removes hydrogen generated during microbial fermentation of feed components in the rumen, and the resulting methane volume is proportional to the concentration of hydrogen dissolved in the rumen (Czerkawski et al., 1972).

Methanogens living in symbiosis with ruminal microorganisms are integral to this process. They occur both on the surface and within the cytosol of ciliate protozoa and account for 9–37% of enteric CH_4 production (McAllister and Newbold, 2008). The present results indicate that FA supplementation reduces methane production in protozoal monocultures. In the EE culture, the greatest decrease occurred with 5% LNA and OA supplementation, whereas in the EC culture, 5% LN led to a statistically significant reduction in methane production. No statistically significant effect was found in the EM monoculture, although methane production was the highest of all controls. Meanwhile, the process of methanogenesis in the EC and EE monocultures was significantly reduced. Vogels et al. (1980) reported that EM was more frequently associated with methanogens, with 55% of EM cells harbouring them compared with EC and EE. Additionally, several studies have indicated that changes in methanogen abundance are not necessarily reflected in protozoal numbers or methane production (Machmüller et al., 2003), which complicates interpretation. One possible explanation is that methanogens present in rumen fluid do not fully represent the total rumen archaeal community (Zhu et al., 2007). Further studies should be carried out on the effects of long-chain FA on methanogenesis, with particular emphasis on their influence on individual methanogen families throughout the experimental period.

Conclusions

C18 fatty acids affected protozoal population density and methane formation in a manner dependent on protozoan species, fatty acid type, and dose. *Eudiplodinium maggii* (EM) appeared to be the most resistant species, whereas increasing fatty acid doses reduced the abundance of *Entodinium caudatum* (EC) and *Epidinium ecaudatum* (EE), and were accompanied by lower methane concentrations. The strongest inhibition was observed in EC monocultures supplemented at 50 g/kg substrate mixture. Since fermentation end-products (total short chain fatty acids, pH, and ammonia) changed only marginally, the anti-methanogenic effect likely reflects reduced protozoal biomass and disruption of protozoa-associated methanogenesis rather than a generalised change in bulk fermentation. Furthermore, evidence of time-dependent adaptation of EC and EE populations, particularly to linoleic acid and linolenic acid, suggests that the persistence of this effect should be verified in mixed protozoal communities and under *in vivo* conditions.

Conflict of interest

The Authors declare that there is no conflict of interest.

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