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METHODS

Methods of Emulsifying Linoleic Acid in Biohydrogenation Studies In Vitro May Bias the Resulting Fatty Acid Profiles

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Abstract The effects of three emulsifying methods on ruminal fatty acid biohydrogenation (BH) in vitro were compared. Using a static in-vitro gas test system, four replicates of each treatment were incubated in buffered ruminal fluid. Hemicellulose (300 mg dry matter) was supplemented either with or without linoleic acid (9*c*12*c*-18:2, 5% in diet dry matter) and incubated for 4 and 24 h. Three methods of emulsifying 9*c*12*c*-18:2 were tested: (1) ethanol, (2) Tween® 80, and (3) sonication. The products were then compared to non-emulsified 9*c*12*c*-18:2. Out of the three emulsifying methods tested, ethanol and sonication resulted in stable 9*c*12*c*-18:2 emulsions, indicating good 9*c*12*c*-18:2 distribution, while the Tween® 80 emulsion was less stable. BH was strongly inhibited by treating 9*c*12*c*-18:2 with ethanol and sonication at different steps of the BH-pathway, resulting in changed concentrations of certain BH intermediates. The fatty acid profile generated from the major BH-pathways of 9*c*12*c*-18:2 with Tween® 80 was comparable to that without emulsification after 24 h of incubation. We conclude that it is not recommended to emulsify lipids before incubating them in vitro when investigating fatty acid BH. If emulsification of 9*c*12*c*-18:2 is necessary, Tween® 80 seems to be the method that interferes least with BH.

Keywords Emulsion · Ruminal biohydrogenation · Ethanol · Tween® 80 · Sonication · Linoleic acid · C18-fatty acids

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Abbreviations

BH	Biohydrogenation
FAME	Fatty acid methyl esters
LNA	Linoleic acid
PUFA	Polyunsaturated fatty acids

Introduction

Investigations on the ruminal biohydrogenation (BH) of polyunsaturated fatty acids (PUFA) are commonly conducted using in-vitro techniques, as well as other methods, due to the lack of rumen-fistulated animals. Often, PUFA are emulsified before being administered to the various types of fermenters. The most common emulsifying methods applied in such studies so far are ethanol [1], Tween® 80 (commercial non-ionic surfactant) [2], and sonication [3]. The emulsification of fatty acids is used to promote fatty acid distribution in the incubation liquid; in addition, this technique allows for a more precise dosage of the lipids when using micropipettes. However, fatty acid emulsification methods might have additional side effects such as affecting the microbial metabolism. Therefore, they could alter the extent and kinetics of ruminal lipolysis and BH [4]. Such side effects are unwanted, as in-vitro studies aim to simulate in-vivo conditions as well as possible. Furthermore, in trials using ruminants, the emulsification of dietary fatty acids is only very rarely required.

There are indications of side effects on some ruminal microbes due to certain fatty acid emulsification methods but, to the authors' knowledge, no investigation has been carried out so far to investigate their actions on microbial BH-pathways or their implications for the interpretation of

the fatty acid results obtained. It has been shown that concentrations of linoleic acid (9*c*12*c*-18:2) up to 0.8 mg ml^{-1} were appropriate, i.e., they revealed no inhibitory effect on ruminal microbial BH, for in-vitro BH studies investigating either non-emulsified 9*c*12*c*-18:2 [5] or 9*c*12*c*-18:2 emulsified with Tween® 80 [6]. In addition, about the same dosage of non-emulsified 9*c*12*c*-18:2 showed no effects on microbial fermentation patterns [7]. In contrast, sonicated 9*c*12*c*-18:2 at a concentration of 50 μg ml^{-1} of incubation liquid revealed an inhibitory action on the growth of *Clostridium proteoclasticum*, a ruminal stearate producer [8]. The third emulsifying method, ethanol, was shown to affect the mixed ruminal microbes at a dosage of 34 ml l^{-1} by increasing total short-chain fatty acid and acetate formation, as well as methane production, in vitro [9]. These results emphasize the importance of not only choosing suitable emulsifying methods when investigating specific fatty acid–related aspects of BH in vitro, but also considering possible systematic artifacts induced by the emulsifying method chosen, as these might affect the ruminal microbes.

The aim of this in-vitro study was to investigate and compare the three emulsifying methods, each of which has been used individually for emulsification in previous fatty acid-related in-vitro studies. In this way, the study assessed their possible side effects on fatty acid BH profiles when they have been incubated with mixed ruminal microbes. The emulsification methods chosen for testing in the present study were sonication, Tween® 80, and the most frequently used emulsifier, ethanol. The present study should give enough information for determining the most suitable emulsification method when carrying out ruminal BH studies in vitro.

Experimental Procedure

In-Vitro System and Experimental Design

Two experimental runs were carried out using the static in-vitro gas test system (Hohenheim gas test, [10]). As a carbohydrate source for the ruminal microbes, hemicellulose (xylan from oat spelt containing ≥70% xylose, ≤10% arabinose, and ≤15% glucose after hydrolysis; Sigma-Aldrich, MO, USA) was incubated as a single feed source in the amount of 300 mg dry matter (DM) without or with 15 mg (50 g kg^{-1} of feed DM) of 9*c*12*c*-18:2 (non-esterified, ≥99% purity, Sigma-Aldrich GmbH, Buchs, Switzerland). The fatty acid 9*c*12*c*-18:2 was either directly added into the incubation liquid serving as control or pretreated with one of the three different emulsifying methods. The three emulsifying methods for distributing 9*c*12*c*-18:2 in incubation liquid were applied as follows: (1) Tween® 80 (polyoxyethylene sorbitan mono-oleate, containing 10% total fatty acids consisting of 71.8% 9*c*-18:1, 0.17% 9*c*12*c*-18:2 in the fatty acid composition; Sigma-Aldrich, Saint Louis, MO, USA) was used as an aqueous Tween® 80 solution (1% Tween® 80, v/v) and 9*c*12*c*-18:2 was added at an amount of 50 mg ml^{-1} Tween® 80 solution following [2]; (2) an ethanol-9*c*12*c*-18:2 emulsion was prepared by dissolving 50 mg of 9*c*12*c*-18:2 in 1 ml of 96% ethanol, as described in [1]; (3) finally, 50 mg of 9*c*12*c*-18:2 was dispersed with 10 ml of deionized water in an ultrasonic bath (TEC-25, Telsonic AG, Bronschhofen, Switzerland) for 3 min, applying a method slightly modified from Fellner et al. [11] and Wallace et al. [3]. Further treatments consisted of incubations where the emulsifying methods were applied without 9*c*12*c*-18:2 (the same amounts of Tween® 80, ethanol, and deionized water added to the incubation liquid already containing hemicellulose). Another treatment containing neither 9*c*12*c*-18:2 nor any emulsifying agent was also included. The four treatments containing no 9*c*12*c*-18:2 were subsequently used only as functional treatments for the calculation of result for the 9c12c-18:2-containing experimental treatments.

Incubations were carried out for 4 and 24 h, with each treatment being incubated in four replicates at an incubation temperature of 39 °C following the protocol outlined in Soliva and Hess [10]. Briefly, ruminal fluid was collected before morning feeding from a non-lactating Brown-Swiss cow receiving hay ad libitum and 1 kg of concentrate per day. The cow was handled according to the Swiss guidelines for animal welfare. Ruminal fluid was then filtered through four layers of medicinal gauze (1,000 μm pore size, Type 17; MedPro Novamed AG, Flawil, Switzerland) and mixed with pre-warmed buffer solution (1:2; v/v). Then, 30 ml of the ruminal fluid/buffer mixture (hence "incubation liquid") was dispensed anaerobically into the incubation units already containing the feed substrate hemicellulose. Once they were filled with incubation liquid, 15 mg of non-emulsified or emulsified 9*c*12*c*-18:2, or only the emulsifier itself, was introduced into the incubation units and the liquid volume was recorded. When the incubation was halted after 4 or 24 h, the incubation liquid was stored at −20 °C until being analyzed for its fatty acids profiles and recovery. Incubation liquid samples of the treatments with and without 9*c*12*c*-18:2 at 0 h (not incubated) were also collected for analysis.

Fatty Acid Analysis

The incubation liquid samples were thawed at refrigerator temperature overnight. Then, 0.3 ml of an internal standard, 19:0 (Sigma-Aldrich GmbH, Buchs, Switzerland, prepared as 1 mg ml^{-1} in dichloromethane), was added to 10 ml of the incubation liquid samples. The lipids were

extracted from the samples using a non-chlorinated extraction technique [12], i.e., by adding 8 parts of propan-2-ol and 10 parts of cyclohexane to 11 parts of incubation liquid (v/v/v). For the second extraction step, a propan-2-ol to cyclohexane mixture of 1.3:10 (v/v) was used. The organic phase was collected and the solvents were evaporated using a Rota-Vap (Heidolph VV2000, Heidolph Elektro & Co., KG, Kelheim, Germany). The lipids were dissolved with 2 ml of chloroform that was later evaporated under a nitrogen gas stream. Subsequently, the free fatty acids were methylated by adding 1 ml of a toluene: methanol mixture (1:2; v/v) and 0.1 ml of trimethylsilyl-diazomethane (2 M in hexane), as recommended for samples containing conjugated fatty acids [13]. The methylation process, which was carried out at 40 °C for 10 min, was terminated by adding a drop of glacial acetic acid. Spare solvent and trimethylsilyl-diazomethane were removed by flushing with nitrogen gas. Lipid residues, including the fatty acid methyl esters (FAME), were then resolved with 200 µl of hexane and subsequently cleaned using thin layer chromatography [14]. The identification of the individual FAME was accomplished by carrying out two separate runs on a gas chromatograph (model HP 6890, Agilent Technologies Inc., Wilmington, DE, USA) equipped with a flame ionization detector. For the first run, a 30 m × 0.32 mm Supelcowax-10^{TM} capillary column (Supelco Inc., Bellefonte, PA, USA) was applied. A mixed FAME standard (Supelco 37 Component, Bellefonte, PA, USA) was used for the identification of the individual fatty acids. For detailed *cis*- and *trans*-18:1 isomer identification, a second run using a 200 m × 0.25 mm CP7421 capillary column (Varian Inc., Lake Forest, CA, USA) was performed. The identification of 18:1 isomers was achieved using reference *cis*- and *trans*-18:1 fatty acids (Sigma-Aldrich GmbH, Buchs, Switzerland) and the guidance provided by Kramer et al. [15]. Details of the conditions of both gas chromatograph procedures are described in Khiaosa-Ard et al. [16].

Calculations and Statistical Analysis

The fatty acid content in the incubation liquid was calculated from the known amount of the internal standard. The fatty acid recovery was calculated from the amount of fatty acid recovered after 4 or 24 h of incubation, compared to 0 h using the values of the functional treatments without 9*c*12*c*-18:2 as covariance. For the calculation of the results of the fatty acid profile, and prior to the calculation of the extent of BH, the respective amounts of fatty acids found in the incubation liquid for the functional treatments containing no 9*c*12*c*-18:2 were subtracted from the respective experimental treatments containing 9*c*12*c*-18:2. Thus, in the results, only fatty acids derived from the BH-pathway of 9*c*12*c*-18:2 were considered. The proportion of 9*c*12*c*-18:2 that was apparently biohydrogenated (%; 9*c*12*c*-18:2-BH) was quantified using an equation adapted from Li and Meng [17]: 100× (proportionate 9*c*12*c*-18:2 in total C18 FAME at 0 h—proportionate 9*c*12*c*-18:2 in total C18 FAME after 4 or 24 h of incubation)/proportionate 9*c*12*c*-18:2 in total C18 FAME at 0 h. This was done under the assumption, substantiated by findings of Moate et al. [6], that the concentration of 9*c*12*c*-18:2 (0.5 mg ml^{-1}) used had no inhibitory influence on the BH of the fatty acids. Total C18 unsaturated fatty acids were used to calculate the overall apparent extent of BH extent (%) in the same manner as for 9*c*12*c*-18:2.

Means of all results obtained were subjected to analysis of variance applying the GLM procedure of SAS (version 9.1, SAS Institute Inc., Cary, NC, USA), with emulsifying treatment and incubation time considered fixed effects, and the experimental run considered a blocking factor. Multiple comparisons among means were performed for all statistical evaluations using Tukey's method.

Results

The Emulsifying Methods' Distribution Quality and Effects on Fatty Acid Profile and Recovery

By visual observation, it was found that distribution of 9*c*12*c*-18:2 in the emulsion was better when ethanol (clear solution) was used in comparison to sonication (milky suspension) or Tween® 80 (some droplets aggregated on the surface). All emulsifying methods tested showed a better visual distribution in the emulsion and in the incubation liquid compared to non-emulsified 9*c*12*c*-18:2, where all of the 9*c*12*c*-18:2 aggregated on the liquid's surface.

The C18 fatty acid fraction of the 9*c*12*c*-18:2-supplemented incubation liquid made up 95.6% of total FAME prior to incubation (0 h). Related to total FAME, the C18 fraction consisted mainly of 9*c*12*c*-18:2 (89.9%), while 2.3% were made up of 9*c*11*t*-18:2 and 18:0, and 1.1% consisted of various C18 fatty acid isomers.

Recovery of total, as well as C18, fatty acids after 4 h of incubation was generally low, but clearly higher ($P < 0.001$) with the sonication and ethanol treatments compared to Tween® 80 and non-emulsified 9*c*12*c*-18:2 (control; Table 1). The extent of apparent 9*c*12*c*-18:2-BH was higher ($P < 0.001$) with Tween® 80 and sonication compared to the control and ethanol. By contrast, the extent of apparent overall BH was smaller ($P < 0.001$) with the sonication and ethanol method compared to the Tween® 80 method and the control. The emulsifying methods generally influenced most of the individual C18

Table 1 C18 fatty acid isomer profile and recovery in incubation liquid (g/100 g C18 fatty acids) after 4 and 24 h of ruminal linoleic acid (LNA, 9*c*12*c*-18:2) biohydrogenation in vitro ($n = 4$)

Emulsifying method	4 h of incubation				*P* level	24 h of incubation				*P* level	Time effect (*P*)
	None	Tween® 80	Sonication	Ethanol		None	Tween® 80	Sonication	Ethanol		
C18 FA in total FAME (%)	90.6	89.8	92.6	90.8	0.145	91.3	93.3	91.3	90.3	0.286	0.363
C18 FA recovery[A] (%)	33.0^{b}	39.8^{b}	57.9^{a}	65.2^{a}	0.001	59.4^{ab}	49.7^{b}	51.3^{b}	65.0^{a}	0.009	0.264
FAME recovery[A] (%)	29.3^{b}	38.6^{ab}	56.0^{a}	68.7^{a}	<0.001	65.0	53.0	54.6	65.2	0.060	0.915
LNA biohydrogenated, %[B]	47.5^{b}	81.2^{a}	85.4^{a}	62.2^{b}	<0.001	92.9^{ab}	97.0^{ab}	99.0^{a}	90.6^{b}	0.041	<0.001
Over all BH extent, %[C]	23.12^{a}	31.11^{a}	4.55^{b}	2.13^{b}	<0.001	55.0^{a}	52.5^{a}	18.8^{b}	14.4^{b}	<0.001	<0.001
18:0	24.80^{a}	32.60^{a}	6.64^{b}	4.13^{b}	<0.001	56.0^{a}	53.5^{a}	20.6^{b}	16.3^{b}	<0.001	<0.001
4*t*-18:1	0.06^{b}	0.22^{a}	0.02^{b}	0.01^{b}	<0.001	0.19^{ab}	0.28^{a}	0.10^{b}	0.07^{b}	0.001	<0.001
5*t*-18:1	0.06^{b}	0.22^{a}	0.02^{b}	0.01^{c}	<0.001	0.13^{b}	0.21^{a}	0.07^{b}	0.07^{b}	0.009	0.003
6*t*-8-18:1	0.75^{b}	2.84^{a}	0.21^{c}	0.15^{c}	<0.001	1.39^{b}	2.48^{a}	0.71^{b}	0.69^{b}	<0.001	0.014
9*t*-18:1	0.52^{b}	1.92^{a}	0.18^{bc}	0.09^{c}	<0.001	0.70^{b}	1.42^{a}	0.45^{bc}	0.30^{c}	<0.001	0.556
10*t*-18:1	2.40^{b}	9.49^{a}	1.75^{b}	1.53^{b}	<0.001	3.15^{b}	7.78^{a}	3.61^{b}	1.54^{b}	<0.001	0.565
11*t*-18:1	8.64	11.4	30.8	22.9	0.053	20.5^{b}	15.2^{b}	63.1^{a}	34.2^{b}	0.001	0.010
12*t*-18:1	0.89^{b}	3.28^{a}	0.34^{b}	0.23^{b}	<0.001	1.52^{b}	2.71^{a}	1.06^{bc}	0.79^{b}	<0.001	0.040
13/14*t*-, 6-8*c*-18:1	1.40^{b}	4.91^{a}	0.45^{bc}	0.28^{c}	<0.001	2.44^{b}	4.26^{a}	1.18^{c}	1.03^{c}	<0.001	0.063
16*t*-18:1	0.56^{b}	1.54^{a}	0.15^{c}	0.24^{bc}	<0.001	1.25^{b}	1.90^{a}	0.56^{c}	0.71^{c}	<0.001	<0.001
9*c*/15*t*-18:1	1.14^{b}	3.01^{a}	1.00^{b}	0.96^{b}	<0.001	1.17^{b}	2.07^{a}	0.77^{c}	1.00^{bc}	<0.001	0.008
10*c*-18:1	0.05^{ab}	0.16^{b}	0.06^{ab}	0.03^{b}	0.028	0.10	0.11	0.31	0.14	0.152	0.028
11*c*-18:1	0.22^{b}	0.44^{a}	0.27^{b}	0.27^{b}	0.005	0.24	0.29	1.07	0.61	0.090	0.053
12*c*-18:1	1.15^{b}	3.98^{a}	1.32^{b}	1.23^{b}	<0.001	0.77^{b}	2.14^{a}	0.95^{b}	0.73^{b}	<0.001	0.002
13*c*-18:1	0.06^{b}	0.19^{a}	0.02^{b}	0.04^{b}	<0.001	0.06^{b}	0.13^{a}	0.03^{b}	0.07^{ab}	0.007	0.641
15*c*-18:1	0.10^{b}	0.31^{a}	0.06^{b}	0.08^{b}	<0.001	0.11^{b}	0.21^{a}	0.07^{b}	0.07^{b}	<0.001	0.147
16*c*-18:1	0.05^{b}	0.23^{a}	0.06^{b}	0.05^{b}	<0.001	0.01^{b}	0.06^{a}	0.04^{ab}	0.03^{ab}	0.017	<0.001
9*c*12*c*-18:2	49.4^{a}	17.7^{b}	13.8^{b}	35.5^{a}	0.018	6.68^{ab}	2.85^{ab}	0.99^{b}	8.83^{a}	0.041	<0.001
Other non-conjugated 18:2	0.55^{b}	0.70^{a}	0.37^{c}	0.45^{bc}	0.001	0.12	0.17	0.18	0.26	0.080	<0.001
9*c*11*t*-18:2	5.91^{b}	3.16^{b}	37.63^{a}	27.41^{a}	<0.001	2.44^{b}	1.31^{b}	3.14^{b}	24.74^{a}	0.035	0.011
Other conjugated 18:2	1.24^{b}	1.59^{b}	4.80^{a}	4.27^{a}	<0.001	0.99^{b}	0.87^{b}	1.05^{b}	7.54^{a}	0.037	0.731

LNA added to the incubation liquid accounted for 94% of LNA in the total C18 fatty acid profile of the incubation liquid at 0 h incubation time

Within each subclass (i.e. row for 4 and 24 h of incubation), mean values followed by different letters are significantly different at $P < 0.05$

[A] Statistically analyzed using the positive controls as covariance

[B] Percentage of LNA apparently hydrogenated during 4 and 24 h of incubation, respectively, compared to LNA at 0 h incubation

[C] Referring to apparent completeness of biohydrogenation

fatty acid isomers differently ($P < 0.05$), except for 11*t*-18:1. The ethanol and sonication treatments had higher concentrations of 9*c*11*t*-18:2 by 4.64 and 6.37 times ($P < 0.001$), as well as higher 11*t*-18:1 by 2.65 and 3.56 times ($P = 0.053$), compared to non-emulsified 9*c*12*c*-18:2, respectively. Regarding these isomers, as well as 18:0, Tween® 80 was found to be in the same range as non-emulsified 9*c*12*c*-18:2. Both fatty acids, 9*c*11*t*-18:2 and 11*t*-18:1, were the most prevalent BH intermediate isomers in the profile in all treatments except for Tween® 80, which showed a higher 10*t*-18:1 proportion ($P < 0.05$) after 4 h of incubation compared to the other three treatments.

Total fatty acid recovery of the control was markedly higher after 24 h than after 4 h of incubation, and numerical treatment differences were not significant ($P > 0.05$). In contrast, the C18 fatty acid recovery showed a clear treatment effect ($P < 0.01$) and was highest for the ethanol treatment and lowest for the Tween® 80 method, with the other treatments being intermediate. The extent of overall apparent BH after 24 h of incubation was generally higher ($P < 0.001$), but showed the same trend as after 4 h of

incubation, with the sonication and ethanol treatments having lower BH than the other two treatments ($P < 0.001$). There was a significant incubation time effect regarding apparent 9*c*12*c*-18:2-BH ($P < 0.001$) as, after 24 h of incubation, 9*c*12*c*-18:2 (90–99%) had been biohydrogenated to a greater extent. The proportions of most C18 isomers, including 11*t*-18:1, 9*c*11*t*-18:1, and 9*c*12*c*-18:2 and 18:0, increased after 24 h compared to 4 h of incubation. The proportion of 9*c*11*t*-18:2 after 24 h was less in all treatments except ethanol. Compared to the other treatments, the proportion of conjugated linoleic acids, other than 9*c*11*t*-18:2, was higher ($P < 0.05$), with the ethanol method exhibiting about 7.5 times the initial amount. The BH intermediate 11*t*-18:1 and the BH end product 18:0 were more abundant in the incubation liquid after 24 h compared to after 4 h of incubation. Regarding 11*t*-18:1, higher ($P < 0.05$) proportions were found with the sonication method compared to the other treatments. The highest proportions of 18:0 in total C18 fatty acid occurred with non-emulsified 9*c*12*c*-18:2 and the Tween® 80 method ($P < 0.001$). There was a clear incubation time effect on almost all fatty acid parameters, except for the fatty acid isomers 9*t*-18:1, 10*t*-18:1, 13*c*-18:1, 15*c*-18:1, and conjugated 18:2 (9*c*11*t*-18:2 excluded).

Discussion

In the present in-vitro study, three common emulsifying methods were investigated and compared with each other and with non-emulsification with the aim of identifying possible side effects of this processes on ruminal lipid BH. This is important research because such effects would appear as artifacts and therefore bias the results in fatty acid-related ruminal in-vitro studies. Therefore, when planning an in-vitro experiment where lipid administration is required that necessitates the help of emulsification, these aspects are of high relevance.

Effects of the Emulsification Methods on Fatty Acid Recovery and BH of Linoleic Acid

The recovery of fatty acids was generally low in the present study, even with the stable emulsion treatments ethanol and sonication, which had been expected to improve fatty acid recovery. However, any system, either in vitro or in vivo, results in a basic loss of fatty acids to some extent [11, 18, 19]. During in-vitro incubation, lipids may be lost in several ways. For example, some lipids may attach to the incubation devices and, as a result, will not be retrieved in the sampling procedure. In the context of the present study, this might be due to the rather large glass surface of the incubation vessels and small amounts of incubation liquid. As expected, there was a higher fatty acid loss in the non-emulsified 9*c*12*c*-18:2, as well as in the emulsion prepared with the Tween® 80 method; this was more particularly the case at the short incubation time (4 h). Thus, for short-term in-vitro studies, to improve the recovery of added fatty acids, only sonication or the use of ethanol can be recommended. However, the clear time effect on the fatty acid profile and the terminal BH product, 18:0, found in general, as well as the different responses with time when using different emulsification methods, showed that results obtained after short-time incubation are not reliable unless they are used for kinetic evaluations in the context of repeated measurements [6]. The increase of fatty acid recovery found in the non-emulsified 9*c*12*c*-18:2 group with a time restriction of 4 h cannot be explained; however, it occurred repeatedly in each of the two experimental runs ($n = 4$).

Adhesion properties are a function of the chemical and physical nature of the fats, and therefore the possibility that the various BH products adsorbed onto the incubation vessels to different degrees cannot be excluded. However, when comparing the BH profile of the non-emulsified 9*c*12*c*-18:2 with profiles obtained in previous in-vitro studies [4, 6], as well as in an in-vivo trial [20], the pattern is comparable, i.e. the transient production of 9*c*11*t*-18:2 followed by a large accumulation of 11*t*-18:1. This supports the assumption that although the fatty acid recovery was limited in the present study, the actual fat recovered was of the same composition as the fat not recovered, and therefore the validity of the fatty acid profile was ensured.

The emulsifier Tween® 80 apparently induced alternative BH-pathways, which differed from the main cascade [21]. Thus, Tween® 80 produced artifacts in the BH-pathway of 9*c*12*c*-18:2 that have to be considered when comparing the results with those found in other studies. However, the main fatty acids, indicative of the extent of ruminal BH, did not substantially differ between non-emulsified 9*c*12*c*-18:2 and the Tween® 80–treated 9*c*12*c*-18:2 in the present study. Thus, provided the proportions of 18:1 isomers are interpreted with caution, Tween® 80 appears to be a suitable emulsification method in cases where the ruminal BH is to be tested in vitro.

In contrast, sonication and the addition of ethanol resulted in severe biases in the BH-pathway, rendering them unsuitable for appropriately simulating ruminal BH processes in vivo. It has been shown that the presence of feed particles promotes lipid BH by providing a site for lipids to adsorb and allowing exposure to BH processes [22]. Interestingly, a study by Harfoot et al. [23] found that low lipolysis and BH of trilinolein (0.3 mg ml^{-1} rumen content) occurred when only small amounts of the lipids were adsorbed to the plant particles. In contrast, in a second experiment in the same study, when a high proportion of

trilinolein was associated with the feed particle fraction, lipolysis and BH were high; this resulted in large amounts of free 18:0 in the feed particle fraction. Related to the results of the present study, this would mean that the small 9*c*12*c*-18:2 droplets formed in the stable 9*c*12*c*-18:2 emulsions with ethanol and sonication tended to stay in the liquid phase rather than being attached to feed particles. The unstable 9*c*12*c*-18:2 emulsion with Tween® 80 and no emulsification, however, might have attached to the feed particles to a greater extent, which would explain their higher apparent BH.

Using different emulsification methods to enhance the distribution quality of 9*c*12*c*-18:2 in the incubation liquid might cause toxicity of the fatty acid due to different bacterial species responsible for BH. This could result in different BH fatty acid profiles. The fact that the final step of the BH-pathway appeared to be specifically and severely impaired by sonicated 9*c*12*c*-18:2, resulting in an accumulation of 11*t*-18:1 and correspondingly lower concentrations of 18:0, is consistent with Maia et al. [8]. They showed that sonicated 9*c*12*c*-18:2 particularly inhibited some rumen bacterial species, including stearate-producing bacteria such as *C. proteoclasticum*. A significant increase in the 9*c*11*t*-18:2 and 11*t*-18:1 proportion observed with ethanol treatment indicates that there are inhibitory effects being generated in the second-last and last step of 9*c*12*c*-18:2 BH, which signified the inhibition of the double-bond hydrogenating steps, including *cis*-9 double-bond BH. This is a step that many bacterial species are capable of performing [21]. In the incubation study of Caldwell and Murray [24], which used higher ethanol concentrations than were employed in the present study, a general toxicity to ruminal bacteria was found. This was not the case in the present experiment, where no changes in total bacterial count (data not shown) were observed with the ethanol treatment. However, this does not exclude the possibility that specific rumen bacterial species and their activity were affected by the ethanol treatment.

Conclusion

The present study demonstrated that using emulsification methods to improve lipid distribution in incubation liquid in investigations of ruminal BH in vitro may bias the resulting fatty acid profile. Thus, when carrying out such studies, pretreatment of the lipids with any of the emulsification methods tested cannot be recommended. For cases when the emulsification of lipids seems necessary, Tween® 80 was the emulsification method that exhibited the least interference with the treatment effects, but the results might still be biased by some minor 18:1 isomers in this method. Sonication and ethanol were shown to severely inhibit ruminal BH when used as the administration method for 9*c*12*c*-18:2.

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