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Effect of nanoemulsified oils addition on rumen fermentation and fatty acid proportion in a rumen simulation technique

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ABSTRACT. The present study was carried out to investigate the potential of different oils nanoemulsions on the modulation of fatty acid proportions and their effect on selected ruminal bacteria using four-fermenter RUSITEC units of 1 l capacity each. Four treatments were investigated: 1. control group (11 g of dried total mixed ration), 2. the control plus soyabean oil (5% on dry matter basis), 3. the control plus fish oil (5% on dry matter basis), and 4. the control plus soyabean-fish oils blend (1:1 v/v; 5% on dry matter basis). All oils were in nanoemulsified form and were added directly to the RUSITEC fermenters during the 10-day-feeding process. The obtained results indicated that the use of the nanoemulsified oils didn't affect total bacterial count; however, the nanoemulsified fish and soyabean-fish oil blend treatments decreased ($P < 0.002$) the relative proportions of both *Butyrivibrio fibrisolvens* and *Ruminococcus albus*. A significant decrease ($P = 0.035$) in *Butyrivibrio proteoclasticus* was only noticed after the nanoemulsified soyabean-fish oil blend addition. Regarding the fatty acids in the fermentation fluid, the nanoemulsified oils increased significantly ($P < 0.001$) the proportions of oleic, linoleic and linolenic acids. In conclusion, nanoemulsified soyabean oil modulates the polyunsaturated fatty acids in ruminal cultures without the negative effect on rumen fermentation parameters.

Introduction

Modulation of fatty acid profile in the rumen is assigned to several factors including diet. Feeds supplemented with polyunsaturated fatty acid (PUFA) rich oils of either plant or marine origin were widely investigated in the last decades. It was estimated that they have an ability to change positively the rumen fatty acid proportions by affecting the activity of rumen microorganisms (AbuGhazaleh and Ishlak, 2014; Boerman and Lock, 2014). Further works illustrated the ability of fish oil supplemented up to 4.17 g · l^{-1} to inhibit the rumen biohydrogenation of linoleic acid and linolenic acid in a ruminal culture which consequently increases the accumulation of *trans*-11 C18:1 (Wąsowska et al., 2006). However, oil supplementation has some dietary limitations due to the possible negative impact on

rumen fermentation (Martínez Marín et al., 2013; Ishlak et al., 2014). On the other hand, rumen lipolysis and biohydrogenation act as barriers that prevent an easy transfer of the dietary PUFA to milk (Lanier and Corl, 2015). Rumen bacteria biohydrogenate toxic dietary unsaturated fatty acids (UFA) to saturated fatty acid (SFA) in order to protect their cellular construction. This mechanism results in a higher outflow of SFA to the small intestine for digestion and absorption (Beam et al., 2000; Boerman and Lock, 2014). So, it is desirable to find other forms of supplemented oils that could preserve PUFA from being affected by rumen lipolysis and biohydrogenation, and could not have the negative effect on the rumen fermentation and the cellular construction of the rumen microorganisms at the same time.

Recently, nanotechnology has found innumerable applications in many different areas. Delivery of bioactive components using nanoscale technology has been documented not only in pharmaceutics but also in the cosmetic and food sciences (Fathi et al., 2012; Ghosh et al., 2014; Zhang et al., 2014). Nanoemulsion is one of the most important nanotechnology applications with a wide usage in several scientific and practical fields. Nanoemulsion is defined as multiphase colloidal dispersions formed by a mixture of one liquid that is dispersed as nanoscale droplets in another immiscible liquid. Physical share-induced rupturing leads to a droplet's diameter that is less than 100 nm (Mason et al., 2006). In a previous investigation (El-Sherbiny et al., 2016) nanoemulsified form of edible oil blends preserved higher proportion of UFA from being hydrogenated in ruminal batch cultures compared to the same level of raw oils addition. In the present study, we hypothesized that nanoscale droplets of oil blends rich in PUFA, added directly to the rumen fermentation culture in the RUSITEC system, would probably inhibit the microbial reactions, i.e. lipolysis and biohydrogenation without affecting the total microbial population. This inhibition could preserve more UFA from being lost during those processes. Therefore, the main objective is to investigate the effects of different nanoemulsified oils rich in UFA (mainly PUFA) as a novel dietary component on the modulation of rumen fatty acid and the rumen microbial population in a long-term ruminal fermentation culture (RUSITEC).

Material and methods

Nanoemulsified oils preparation

The oil-in-water nanoemulsion was prepared using a HIELSCHER UP50H ultrasonic processor (80% amplitude for 20 min; Hielscher Ultrasonics, Teltow, Germany) as described by Lakalayeh et al. (2012). Soyabean oil, fish oil and their 1:1 (v/v) blend were used as the inner phase, Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) was used as the only surfactant. The oil-in-water emulsion formulation was composed of 15% oil, 5.6% Tween 80 and 79.4% deionized water as was suggested by Kentish et al. (2008).

Equipment and experimental design

The study was carried out using rumen simulation technique equipment (RUSITEC) as developed by Czerkawski and Breckenridge (1977). The RUSITEC system consisted of four fermenters, each of 1 l capacity placed in a water bath maintained at 39 °C throughout the experiment. The rumen inoculum was obtained from 3 ruminal cannulated Polish Holstein-Friesian dairy cows (body weight of 600 ± 25 kg, month 4 of lactation) 3 h after the morning feeding. Rumen donor cows were fed 20.5 kg of dry matter (DM) per day of total mixed ration (TMR), similar to the diet used in the *in vitro* experiment. The ruminal content was collected from the top, bottom and middle part of the rumen of each cow separately. The ruminal contents from all cows were equally blended, strained through four layers of gauze into a Schott Duran® bottle (SCHOTT North America, Inc., Elmsford, NY, USA), and immediately transported to the laboratory in a 39 °C preheated water bath. On the first day of each experimental run, each fermenter was filled with 900 ml of strained rumen fluid and 100 ml of pre-warmed McDougall buffer (McDougall, 1948) achieving a final pH of the incubation mixture between 6.9 and 7.1. Moreover, two nylon bags: one filled with 11 g of solid rumen content and the other filled with 11 g of the control diet on DM basis, were put in a perforated feed container and placed in the respective fermenter. The nylon bags (70 mm × 140 mm) of a 100 µm pore size as cited by Soliva and Hess (2007) were used in the study. After 24 h of incubation, the nylon bag containing solid rumen content was replaced with a nylon bag containing the control diet. Each feed bag was therefore incubated for 48 h. To immediately re-establish the anaerobic conditions in the gaseous phase of the fermenters, gaseous nitrogen was flushed through the incubation units for 3 min (3 l · min^{-1}), each time after closing the system. During the experimental runs, artificial saliva (McDougall, 1948) was infused continuously into every fermenter at an average buffer flow rate of 500 ml per day. To guarantee a constant buffer flow an electronic peristaltic pump (Miniplus 3; Gilson, Inc., Middleton, WI, USA) was used. Liquid effluent was automatically transferred

through an overflow tube to the respective effluent vessels containing 10 ml of 6 N HCl to stop the fermentation process.

The experiment was arranged in a completely randomized block design with 4 treatments and 3 repetitions (10 days each), using a four-fermenter RUSITEC system. The experimental groups were as follows: 1. CON – control group (11 g of dried total mixed ration), 2. NES – the control + soyabean oil (5% on DM basis), 3. NEF – the control + fish oil (5% on DM basis), and 4. NEB – the control + soyabean-fish oils blend (1:1 v/v; 5% on DM basis). The oils were used in nanoemulsified form. The substrate used in the experiment was similar to the diet offered to the ruminal cannulated dairy cows (rumen fluid donors). All ingredients were dried and then milled separately. A homogenous mixture of the experimental substrate was made on DM basis by mixing together the following amounts of the dried ingredients (g · kg^{-1} DM): maize silage 396, lucerne silage 71, grass silage 104, beet pulp 113, brewer's grain 85, extracted rapeseed meal 42, commercial concentrate containing 18% of crude protein 185, and a mineral mixture 4. The nanoemulsified oils (daily prepared) were calculated on DM basis and added directly to the RUSITEC fermenters during the 10-day-feeding process. The prepared nanoemulsified oils were intentionally added directly to the incubation fluids. The mixing it with the feed was avoided to simulate our initial idea of adding the nanoemulsion to the drinking water given to dairy cattle.

Sampling and chemical analysis

In 3 RUSITEC runs (10 days each) the data from the first 5 days was not considered in the statistical evaluation since the microbes in the RUSITEC system need 4 – 5 days for adaptation (Soliva and Hess, 2007). The following 5 days represent the sampling period, where fermentation fluid samples were collected at each day 3 h before the new nylon feed bag addition. Collected fermentation fluid was analysed for pH value using a pH meter (CP-104; Elmetron, Zabrze, Poland) and ammonia concentration according to the colorimetric Nessler method modified by Szumacher-Strabel et al. (2002). The total protozoan counts were determined according to the method described by Michalowski et al. (1986). Total bacterial counts were quantified by the method cited by Ericsson et al. (2000) using a Thoma counting chamber (Blau Brand®, Wertheim, Germany). The volatile fatty acids (VFA) in fermentation culture samples were determined by gas chromatography technique (GC Varian CP 3380, Sugarland, TX, USA) according to Tangerman and Nagengast (1996), with some modifications. Briefly, 3.6 ml of the immediately collected rumen fluid sample was stabilized with 0.4 ml of a 46 mM $HgCl_2$ solution and frozen (–20 °C) until analysis by gas chromatograph fitted with flame ionization detector (FID) and capillary column 30 m × 0.25 mm (19091N-133; Agilent HP-Innowax, Agilent Technologies, Santa Clara, CA, USA). The qualitative and quantitative identification of VFA peaks was made by mixing individual VFA purchased from Fluka (Sigma Aldrich, St. Louis, MO, USA) using MS Work Station 5.0 (Agilent Technologies, Santa Clara, CA, USA).

Fatty acid methyl esters in the total mixed ration, nanoemulsions and fermentation fluid samples (Table 1) were extracted and analysed according to Cieślak et al. (2015) with some modifications. Briefly, 2500 µl of rumen fluid was suspended in 3 ml of 2 M NaOH and incubated in a block heater at 90 °C for 40 min. After cooling to room temperature, 1.7 ml of 4 M HCl was added to lower the pH below 2. Before extraction, 2 ml of distilled diethyl ether was added to each sample. The tubes were vigorously shaken for 10 min and then centrifuged

Table 1. Fatty acid (FA) proportion in total mixed ration and supplemented nanoemulsions, g · 100 g FA

Fatty acid	CON[1]	Supplement[2]		
		NES	NEF	NEB
C12:0	0.31	0.32	0.26	0.40
C14:0	0.62	0.26	2.37	1.28
C16:0	20.8	9.82	9.36	9.99
C18:0	2.41	5.41	2.97	5.19
cis-9 C18:1	19.5	28.8	43.2	34.6
cis-9 *cis*-12 C18:2	40.7	42.0	12.3	26.5
cis-9 *cis*-12 *cis*-15 C18:3	7.26	6.41	4.54	5.30
C20:5n-3	nd	nd	0.25	0.14
C22:6n-3	nd	nd	4.32	1.93
SFA[3]	26.6	17.0	16.5	18.3
UFA[4]	73.4	82.9	83.5	81.7
MUFA[5]	49.9	50.7	31.5	40.9
PUFA[6]	23.4	32.2	51.9	40.7
n-6	42.4	43.2	17.8	30.6
n-3	7.53	6.55	11.4	8.83
n-6/n-3	5.62	6.59	1.57	3.48

[1] CON – control treatment consisted of total mixed ration used as the main substrate; [2] supplements: NES – nanoemulsified soyabean oil; NEF – nanoemulsified fish oil; NEB – nanoemulsified soyabean – fish oil 1:1 blend; [3] SFA – sum of saturated fatty acids (C6, C8, C10, C12, C14, C15, C16, C17, C18, C19, C20, C22, C24); [4] UFA – sum of unsaturated fatty acids (C10:1, C14:1, C15:1, C16:1, C17:1, c9C18:1, c11C18:1, c12C18:1, c9c12C18:2, c9c15C18:2, c9c12c15C18:3, C20:1n-9, C20:3n-3, C20:4n-6, C20:5n-3, C22:1n-9, C22:2, C22:5n-3, C22:6n-3, C24:1); [5] PUFA – sum of polyunsaturated fatty acids (c9c12C18:2, c9c15C18:2, c9c12c15C18:3, C20:3n-3, C20:4n-6, C20:5n-3, C22:2, C22:5n-3, C22:6n-3); [6] MUFA – sum of monounsaturated fatty acids (C10:1, C14:1, C16:1, C17:1, c9C18:1, c11C18:1, c12C18:1, C20:1n-9, C22:1n-9, C24:1); nd – not detected

at 6160 *g* for 1 min at 20 °C. The extraction procedure was repeated 3 times, and the supernatant was finally evaporated at 30 °C for 10 min under a flux of nitrogen using a Techne Dri-Block heater Model DB-3 (Bibby Scientific Ltd., Staffordshire, UK). The preparation of the fatty acid methyl esters (FAME) was carried out according to the IUPAC (1987) method 2.301 with slight modification. The extract was boiled with 2 ml of NaOH in methanol (0.5 M) for 3 min, then 3 ml of boron trifluoride-methanol (1.3 M; Fluka – Sigma Aldrich, St. Louis, MO, USA) was added to the solution. As a final step, the solution was reheated for 4 min, and then the reaction was terminated by adding 7 ml of NaCl (0.34 M) and 1 ml of hexane. This mixture was shaken vigorously. The organic phase containing the FAME was used for gas chromatographic analyses using a GC-BRUKER SCION-456-GC (Bruker Corporation, Billerica, MA, USA), equipped with FID and a fused-silica capillary column Chrompack CP7420 (length 100 m, inner diameter 0.25 mm, film thickness 0.25 μm; Agilent HP, Agilent Technologies, Santa Clara, CA, USA). Hydrogen was used as the carrier gas at a flow rate 1.3 ml · min^{-1}. Injector and detector temperatures were 200 °C and 250 °C, respectively. The oven temperature programmes: initially 120 °C for 7 min, then increased by 7 °C · min^{-1} to 140 °C, holding for 10 min and then increased by 4 °C · min^{-1} to 240 °C. Sample (1 μl) was injected into the column. Fatty acids were identified based on their retention times and were expressed as a g · 100 g^{-1} FA. Fatty acid peaks were identified by comparison with the standard retention times (37 FAME Mix, Supelco, Poole, England) and *cis*-9 *trans*-11 C18:2 (Matreya, Pleasant Gap, PA, USA).

The quantities of some selected rumen bacteria (*Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticus* and *Ruminococcus albus*) were determined by DNA isolation from rumen fluid according to Yu and Morrison (2004). Briefly, total DNA from rumen fluid was extracted with a Mini Bead-Beater (BioSpec Products, Bartlesville, OK, USA) for cell lysis. Rumen fluid in the amount of 4 ml was used for DNA extraction and an additional DNA purification step was included (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany). DNA concentration was measured with NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA used for this experiment has to possess an A260:A280 ratio higher than 1.8. The primer pairs for *B. fibrisolvens* (F: ACACACCGCCCGTCACA R: CCTTAC GGTTGGGTCACAGA), *B. proteoclasticus* (F: TCCTAGTGTAGCGGTGAAATG R: TTAGCGACGGCACTGAATGCCTA) and for *R. albus* (F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA) were described by Li et al. (2009), Potu et al. (2011) and Wang et al. (1997), respectively. The primer pairs for total bacteria (F: GTGSTGCAYGGYTGTCGTCA R: GAGGAAGGTGKGGAYGACGT) were described by Maeda et al. (2003). The specificity of primers was confirmed using the BLAST programme in the GenBank Database. The starting DNA concentration for detection of selected ruminal bacteria was 10 ng per 25 μl. The quantification of each bacterial species DNA and total bacteria in total rumen DNA was performed with a QuantStudio 12 Flex PCR system (Life Technologies – Thermo Fisher Scientific, Waltham, MA, USA). The Power SYBR GREEN PCR Master mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR amplification. The reaction mixture in 20 μl of the final volume contained 5 μl of the 2 × Mastermix, 10 ng of template DNA and 0.5 μM of each primer. Amplification involved one cycle at 95 °C for 10 min for initial denaturation and then 45 cycles of 95 °C for 15 s followed by annealing at the temperatures (depends on analysed bacteria) for 5 s and then at 62 °C for 67 s. Detection of the fluorescent product was set at the last step of each cycle. To determine the specificity of amplification, analysis of product melting was performed after single amplification (0.1 °C · s^{-1} increment from 65 °C to 95 °C with fluorescence collection at 0.1 °C intervals). Additional product size verification by gel electrophoresis of samples after the PCR run was included. Dilution of purified genomic DNA from control strains was used to construct species-specific calibration curves. Calibration curve was used for calculation of the species-specific DNA concentration in total rumen DNA preparations (number of DNA copies). The relative level of DNA copy of each bacteria species was calculated using the formula $2^{-\Delta\Delta Ct}$, as a reference total bacteria DNA level was used.

Statistical analysis

Measurements (e.g., rumen microbial population, parameters of rumen fermentation) were obtained from the RUSITEC from days 6 to 10. Each run was considered a random block and for all variables the vessel was considered to be the experimental unit. Data was analysed using a mixed model procedure by SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA), which included the fixed effects of treatment, run and their interactions. Differences among treatments were tested using the Tukey's post hock test. Data was accepted as statistically different if $P < 0.05$. All values are shown as group means with pooled standard errors of means.

Results

Rumen fermentation parameters

Total bacterial count, *Entodiniomorpha* and *Holotricha* protozoan counts were not affected by any of the nanoemulsified oils addition (Table 2). The used nanoemulsified oils treatments also did not exert influence on fermentation culture pH and ammonia concentration in comparison to the control diet. However, the inclusion of NEB to the fermentation culture decreased ($P < 0.01$) *in vitro* dry matter digestibility (IVDMD) and the total VFA content when compared to NES and the control diet. It was accompanied by the decrease ($P < 0.02$) in the molar proportions of both acetate and valerate by NEB supplementation.

Interestingly, NES addition had no effect ($P > 0.05$) on the relative proportions of *Butyrivibrio fibrisolvens, Butyrivibrio proteoclasticus* and

Table 2. Effect of nanoemulsified oils supplementation on rumen basic parameters, volatile fatty acids and relative proportions of selected rumen bacteria in RUSITEC system

Indices	Treatment[1]				SEM	*P*
	CON	NES	NEF	NEB		
pH	6.81	6.82	6.82	6.84	0.008	0.538
Ammonia, mM	8.39	8.68	9.23	8.80	0.245	0.385
IVDMD[2]	56.9^{a}	56.5^{a}	55.4^{ab}	53.5^{b}	0.330	0.004
VFA[3], mM						
total VFA	91.3^{a}	94.4^{a}	90.5^{ab}	86.7^{b}	0.767	0.003
acetate	53.6^{ab}	56.1^{a}	53.1^{ab}	51.2^{b}	0.549	0.019
propionate	15.6	15.8	15.6	14.9	0.132	0.117
isobutyrate	1.37	1.52	1.25	1.29	0.035	0.163
butyrate	13.9	14.0	13.8	13.1	0.186	0.366
isovalerate	1.71	1.68	1.57	1.48	0.035	0.144
valerate	5.12^{ab}	5.35^{a}	5.01^{ab}	4.67^{b}	0.070	0.009
Acetate/propionate	3.46	3.57	3.43	3.43	0.038	0.273
Rumen microorganisms enumeration[4]						
Entodiniomorpha, cell × 10^{3} · ml^{-1}	3.50	3.84	3.91	3.68	0.180	0.737
Holotricha, cell × 10^{2} · ml^{-1}	1.07	1.07	1.09	1.16	0.050	0.949
Total bacteria, cell × 10^{8} · ml^{-1}	7.11	6.96	6.41	6.76	0.200	0.432
Selected rumen bacteria[5]						
Butyrivibrio fibrisolvens	0.024^{a}	0.028^{a}	0.017^{b}	0.016^{b}	0.0004	0.040
Butyrivibrio proteoclasticus	0.020^{a}	0.014^{a}	0.013^{a}	0.011^{b}	0.0012	0.035
Ruminococcus albus	0.039^{a}	0.033^{a}	0.026^{b}	0.023^{b}	0.0018	0.003
Total bacteria, arbitrary units	1.00	0.98	1.01	1.03	0.0350	0.530

[1] see Table 1; [2] IVDMD – *in vitro* dry matter digestibility; [3] VFA – volatile fatty acid; [4] total count of selected rumen microorganisms using light microscope; [5] effect of diets on relative proportions (% of total bacteria) of each population; [abc] – means with different superscripts within a row are significantly different ($P < 0.05$)

Table 3. Effect of nanoemulsified oils supplementation on rumen fatty acid (FA) proportion in RUSITEC system, g · 100 g^{-1} FA; $P < 0.001$

Fatty acid	Treatment[1]				SEM
	CON	NES	NEF	NEB	
C8:0	1.04^{a}	0.15^{b}	0.15^{b}	0.16^{b}	0.036
C10:0	0.69^{a}	0.09^{b}	0.09^{b}	0.09^{b}	0.027
C12:0	3.37^{a}	0.38^{b}	0.48^{b}	0.39^{b}	0.119
C14:0	2.71^{a}	0.56^{c}	2.09^{a}	1.35^{b}	0.076
cis-9 C14:1	1.04^{a}	0.13^{c}	0.19^{b}	0.13^{c}	0.036
C16:0	18.5^{a}	11.1^{b}	11.0^{b}	11.0^{b}	0.268
cis-9 C16:1	1.02^{c}	0.32^{b}	1.85^{a}	1.13^{b}	0.053
C18:0	28.1^{a}	16.2^{b}	14.3^{b}	15.9^{b}	0.534
trans-10 C18:1	1.96^{a}	0.29^{c}	0.39^{b}	0.35^{bc}	0.058
trans-11 C18:1	2.03^{a}	0.43^{c}	0.69^{b}	0.56^{b}	0.052
cis-9 C18:1	5.10^{d}	22.3^{c}	30.9^{a}	26.5^{b}	0.814
cis-9 *cis*-12 C18:2	2.65^{d}	32.5^{a}	11.5^{c}	21.8^{b}	0.928
cis-9 *cis*-12 *cis*-15 C18:3	1.25^{d}	5.00^{a}	3.60^{c}	4.31^{b}	0.116
cis-9 *trans*-11 C18:2	0.56^{a}	0.47^{ab}	0.27^{c}	0.33^{b}	0.024
trans-10 *cis*-12 C18:2	0.55^{a}	0.29^{ab}	0.26^{b}	0.18^{c}	0.022
C20:5n-3	nd	nd	0.18^{a}	0.11^{b}	0.006
C22:5n-3	nd	nd	1.16^{a}	0.62^{b}	0.039
C22:6n-3	nd	nd	3.13^{a}	1.62^{b}	0.105
SFA[2]	71.2^{a}	32.5^{b}	31.0^{b}	32.7^{b}	1.396
UFA[3]	28.8^{b}	67.5^{a}	68.9^{a}	67.3^{a}	1.396
MUFA[4]	18.9^{d}	27.2^{c}	40.7^{a}	33.2^{b}	0.713
PUFA[5]	9.91^{d}	40.3^{a}	28.2^{c}	34.1^{b}	0.932
UFA/SFA	0.42^{b}	2.12^{a}	2.95^{a}	2.14^{a}	0.167
Total *trans* C18:1	4.73^{a}	2.59^{c}	3.36^{b}	2.72^{b}	0.452
MCFA[6]	28.8^{a}	13.1^{d}	16.7^{b}	14.7^{c}	0.511
LCFA[7]	57.7^{c}	83.8^{a}	80.6^{b}	82.2^{a}	0.887
n-6	6.53^{c}	33.9^{a}	17.4^{b}	25.5^{a}	0.842
n-3	2.65^{d}	5.51^{c}	9.58^{a}	7.61^{b}	0.224
n-6/n-3	3.01^{b}	6.31^{a}	1.85^{c}	3.35^{b}	0.145

[1] see Table 1; [2] SFA – sum of saturated fatty acids (C6, C8, C10, C12, C12*iso*, C12*aiso*, C13*iso*, C14, C14*iso*, C14*aiso*, C15, C15*iso*, C16, C16*iso*, C16*aiso*; C17, C17*iso*, C17*aiso*, C18,C19, C20, C22, C24); [3] UFA – sum of unsaturated fatty acids (C10:1, C14:1, C15:1, C16:1, C17:1, t5C18:1, t6-8C18:1, t9C8:1, t10C18:1, t11C18:1, t12C18:1, t15C18:1, c9C18:1, c11C18:1, c12C18:1, c13C18:1, c14C18:1, c15C18:1, t10c12C18:2, c9t12C18:2, c9c12C18:2, c9c15C18:2, c9c12c15C18:3, C20:1n-9, C20:3n-3, C20:4n-6, C20:5n-3, C22:1n-9, C22:2, C22:5n-3, C22:6n-3, C24:1); [4] MUFA – sum of monounsaturated fatty acids (C10:1, C14:1, C15:1, C16:1, C17:1, t5C18:1, t6-8C18:1, t9C18:1, t10C18:1, t11C18:1, t12C18:1, t15C18:1, c9C18:1, c11C18:1, c12C18:1, c13C18:1, c14C18:1, c15C18:1, C20:1n-9, C22:1n-9, C24:1); [5] PUFA – sum of polyunsaturated fatty acids (t10c12C18:2, c9t12C18:2, c9c12C18:2, c9c15C18:2, c9c12c15C18:3, C20:3n-3, C20:4n-6, C20:5n-3, C22:2, C22:5n-3, C22:6n-3); [6] MCFA = sum of medium chain fatty acids (C12, C12*iso*, C12*aiso*, C13*iso*, C14, C14*iso* , C14*aiso*, C14:1, C15, C15*iso*, C15:1, C16, C16*iso*, C16*aiso*, C16:1); [7] LCFA = sum of long chain fatty acids (C17, C17*iso*, C17*aiso*, C17:1, C18, t5C18:1, t6-8C18:1, t9C8:1, t10C18:1, t11C18:1, t12C18:1, t15C18:1, c9C18:1, c11C18:1, c12C18:1, c13C18:1, c14C18:1, c15C18:1, t10c12C18:2, c9t12C18:2, c9c12C18:2, c9c15C18:2, c9c12c15C18:3, C19, C20, C20:1n-9, C20:3n-3, C20:4n-6, C20:5n-3, C22, C22:1n-9, C22:2, C22:5n-3, C22:6n-3, C24, C24:1); [abc] – means with different superscripts within a row are significantly different ($P < 0.05$); nd – not detected

Ruminococcus albus compared to the control treatment. NEF and NEB supplementation decreased ($P < 0.05$) the relative proportions of both *B. fibrisolvens* and *R. albus*. A significant decrease ($P = 0.035$) in *B. proteoclasticus* was only noticed with NEB addition.

Rumen fatty acids proportion

Supplementing nanoemulsified oils in RUSITEC fermentation culture decreased ($P < 0.001$) vaccenic acid (*trans*-11 C18:1) proportion, which was severely reduced when nanoemulsified soyabean oil was added (Table 3). Similarly, *cis*-9 *trans*-11 C18:2 proportion was decreased significantly ($P < 0.001$) when the fish oil and oils blend nanoemulsion were added in comparison to the control treatment.

Regarding C18 UFA, supplementation of the nanoemulsified oils contributed in a vast significant increase in oleic acid (*cis*-9 C18:1), linoleic acid (*cis*-9 *cis*-12 C18:2) and linolenic acid (*cis*-9 *cis*-12 *cis*-15 C18:3) proportions as compared to the control group. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) proportions were significantly higher ($P < 0.001$) with NEF compared to the NEB additions. Nanoemulsified oils reduced particular UFA to a lesser extent showing a higher proportion ($P < 0.001$) of UFA, especially PUFA, when compared to the proportion of SFA, which decreased ($P < 0.001$) by all types of nanoemulsified oils. The highest proportions of preserved PUFA, long chain FA and n-6 fatty acids were found when NES was added. In line with the preservation of more UFA on the expenses of SFA, a significant increase ($P < 0.001$) of UFA/SFA ratio was observed when nanoemulsified oils were applied.

Discussion

In the last decades, several studies were conducted mainly to illustrate the role of oil supplementation on the rumen biohydrogenation. Most of the results positively highlighted the role of supplemented oils on different rumen parameters; however, some negative impacts like decreased fibre digestion and milk fat depression were noted as well (Hellwing et al., 2012; Storlien et al., 2012; Patra and Yu, 2013; Morsy et al., 2015). In the present study, nanoemulsified oils were introduced as possibly having less impact on the rumen fermentation characteristics with better effect on preserving PUFA in the biohydrogenation environment.

In a previous investigation (El-Sherbiny et al., 2016) nanoemulsified oil blends (soyabean-fish oil blend or rapeseed-fish oil blend) tended to favourably increase the proportion of the preserved *in vitro* UFA, especially PUFA, as compared to the same level of raw oils blend addition. Such findings suggested that this alternative form of edible oils has the ability to preserve higher proportions of PUFA, which would be available for absorption regardless of the oil type used. However, not enough data was available to suggest a proper mode of action of oils blend nanoemulsion or single oil nanoemulsion (oil used for the blend preparation) and their effect on the different fermentation parameters. Precisely for these reasons, the present study was performed in the long-term *in vitro* experiment and the experimental treatments were based on the results obtained in the previous study by El-Sherbiny et al. (2016), who observed the addition of nanoemulsified soyabean-fish oil blend at the level of 5% of the substrate DM as the most beneficial in rumen nutrition.

Several studies suggested that fish oil could have a minor effect on rumen fermentation when supplemented in a small amount (AbuGhazaleh and Ishlak, 2014; Cieślak et al., 2015). However, above a certain threshold, fish oil supplementation could severely affect the rumen fermentation especially the relative proportions of gluconeogenic and acetogenic fermentation end products (Shingfield et al., 2012). The fish oil inclusion of different fatty acid composition as cited by Shingfield et al. (2010) could lead to a decrease in the molar concentration of the acetate toward the increased concentration of propionate. Propionate-producing gram-negative bacteria are not significantly inhibited by the fatty acids (O'Brien et al., 2014), which could explain the lack of effect of the nanoemulsions containing fish oil on the molar proportions of propionate in the current study. The possible decrease in molar concentration of acetate caused by fish oil fatty acid could explain the decrease observed in total volatile fatty acid count by NEF and NEB supplementation. In spite of the decrease, this effect seems to be significant ($P < 0.01$) only when NEB was added, which was probably due to the accompanied decrease in the *in vitro* dry matter digestibility and valerate molar proportions by NEB supplementation. Generally, it is suggested in the literature that the impact of fish oil on rumen fermentation is related mainly to several factors like the source and inclusion rate of fish oil, the intake potential, and composition of the diet (Shingfield et al., 2010). In the present study, the changes obtained in the relative proportions of the selected rumen bacteria are mostly related to the presence of fish oil which is rich in DHA inhibiting the activity of microorganisms (AbuGhazaleh and Ishlak, 2014). Reduced populations of *B. fibrisolvens*

and *R. albus* in NEF group, and *B. fibrisolvens*, *B. proteoclasticus* and *R. albus* in NEB group were influenced mainly by the presence of long chain FA, especially EPA and DHA. Maia et al. (2010) examined the effect of added PUFA (linoleic acid, EPA and DHA) on the growth of biohydrogenation bacteria. In this study EPA and DHA were effective in decreasing the growth of *B. fibrosolvens* JW11. Moreover, Wąsowska et al. (2006) evaluated the effect of fish oil, EPA and DHA in ruminal cultures on the linoleic and linolenic acids disappearance, and examined the growth and isomerase activity of *B. fibrisolvens*. Their findings showed that fish oil inhibited the biohydrogenation of both linoleic and linolenic acids, causing the accumulation of a number of intermediates. They also suggested that both non-esterified EPA and DHA, but not fish oil, inhibited the growth and linoleic acid isomerase activity of *B. fibrisolvens*. Based on Wąsowska et al. (2006) and Maia et al. (2010) findings, EPA and DHA were more toxic than linoleic acid. This could explain the lack of effect in the case of NES addition in the present study. In spite of the obtained decrease in *B. fibrisolvens* relative proportion, in the current study with NEF and NEB, the inhibition of biohydrogenation was not applicable in case of resulted intermediates (e.g., vaccenic acid, *cis*-9 *trans*-11 C18:2), which suggest that the form of the supplemented oils could interfere with the obtained fatty acid proportion.

Khiaosa-ard et al. (2010) performed *in vitro* lipid emulsification to improve fatty acid distribution in biohydrogenation. They demonstrated that only 3 min dispersing linoleic acid in an ultrasonic bath could severely bias the resulting fatty acid proportion. They suggested that the small fatty acid droplets formed in the stable emulsions with sonication tended to stay in the liquid phase rather than attach to feed particles, which could lower the occurrence of both lipolysis and biohydrogenation in the fermentation fluid. In the present study the aims were different though; we initially aimed to disperse oils rich in PUFA in water, which would increase the possibility of using this supplement in the drinking water given to dairy cattle, and successively provide nanoemulsified oils during the day. That is why in the current experiment performed with the use of RUSITEC system, we intentionally added the prepared nanoemulsified oil blend directly to the incubation fluid instead of mixing it with the feed. This was done to simulate our initial idea of adding the nanoemulsion to the drinking water for dairy cattle. In the present study, the nanoscale droplets of the oil blend negatively affected the biohydrogenation intermediates proportion (vaccenic acid and conjugated linoleic acid) without affecting the total bacterial population. Generally, the toxicity of PUFA rich oils on rumen microorganisms is a physicochemical process. Fatty acids form adsorption layers around the bacterial cells which result in altered cell permeability and decreased nutrient uptake. The findings of Khiaosa-ard et al. (2010) as well as the unnoticed effect of the nanoemulsified oils on total bacteria in the present study could suggest that the nanoscale diameter of the oil blend droplets inhibited the bacterial chemical actions without affecting the cellular structure of the ruminal bacteria. However, in NEF and NEB treatments the toxicity of PUFA on rumen bacteria was noticed with the changes in the relative proportions of some rumen bacterial species. This modulation of the relative proportions of some rumen bacteria assumed that some oils in the form of nanoemulsions could maintain their inner potential toxic effect of decreasing ruminal bacterial populations. Anyway, rumen microorganisms' activity is affected by several factors, showing a non-uniform response toward supplementation of oils rich in UFA in the *in vitro* conditions (Cieślak et al., 2013).

As presented it seems that nanoemulsion technology helps to preserve higher proportions of PUFA in comparison to the control diet. This finding could be due to the direct inhibition of ruminal lipolysis and/or biohydrogenation which consequently preserves a high proportion of PUFA from being saturated under the biohydrogenation condition. However, according to Bauchart et al. (1990), two different metabolic activities of the biohydrogenation bacteria towards UFA, especially linoleic acid, should be highlighted: firstly, the extensive biohydrogenation of UFA, and secondly, the protection of these UFA from biohydrogenation by the uptake and incorporation into cellular free fatty acids. This lead us to another assumption; due to the fact that nanoemulsified form of used oil is in nanodroplets size, the permeability or uptake of this fatty acid by the bacterial cell, and, consequently a preservation of higher proportions of UFA from being hydrogenated to SFA, could be increased.

Conclusions

Nanoemulsified oils have the ability to preserve polyunsaturated fatty acids from being saturated in the biohydrogenation environment, without affecting the total bacterial or protozoan count. However, in the present study, a negative effect of nanoemulsions on the relative proportion of the biohydrogena-

tion bacteria at the presence of fish oil suggests that nanodroplets size of the added oil did not prohibit the toxicity of long chain unsaturated fatty acids (UFA). Conversely, the nanoemulsified form of soyabean oil (5% on dry matter basis) positively modulated the UFA proportion of ruminal culture without negatively affecting the rumen fermentation and rumen microorganisms. These findings suggest that the nanoemulsion process decreased the possible toxicity of the soyabean oil supplemented at high level on rumen microorganisms, which, in our opinion, could represent a promising supplement in dairy cattle nutrition that requires further investigation. Moreover, additional research is needed mainly to highlight the different effects of the prepared nanoemulsions on the rumen fermentation and fatty acid modulation under farm conditions.

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