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Temperature and duration of heating of sunflower oil affect ruminal biohydrogenation of linoleic acid in vitro

F. Privé,*†‡ S. Combes,*†‡ L. Cauquil,*†‡ Y. Farizon,*†‡ F. Enjalbert,*†‡ and A. Troegeler-Meynadier*†‡[1]
*ENVT, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, F-31076 Toulouse Cedex 3, France
†INRA, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, F-31326 Castanet-Tolosan, France
‡Université de Toulouse, INPT ENSAT, UMR1289 Tissus Animaux Nutrition Digestion, F-31326 Castanet-Tolosan, France

ABSTRACT

Sunflower oil heated at 110 or 150°C for 1, 3, or 6 h was incubated with ruminal content in order to investigate the effects of temperature and duration of heating of oil on the ruminal biohydrogenation of linoleic acid in vitro. When increased, these 2 parameters acted together to decrease the disappearance of linoleic acid in the media by inhibiting the isomerization of linoleic acid, which led to a decrease in conjugated linoleic acids and *trans*-C18:1 production. Nevertheless, *trans*-10 isomer production increased with heating temperature, suggesting an activation of Δ^9-isomerization, whereas *trans*-11 isomer production decreased, traducing an inhibition of Δ^{12}-isomerization. The amount of peroxides generated during heating was correlated with the proportions of biohydrogenation intermediates so that they might explain, at least in part, the observed effects. The effects of heating temperature and duration on ruminal bacteria community was assessed using capillary electrophoresis single-strand conformation polymorphism. Ruminal bacterial population significantly differed according to heating temperature, but was not affected by heating duration. Heating of fat affected ruminal biohydrogenation, at least in part because of oxidative products generated during heating, by altering enzymatic reactions and bacterial population.
Key words: ruminal biohydrogenation, conjugated linoleic acid, heat processing, lipid peroxidation

INTRODUCTION

Conjugated linoleic acid (**CLA**) is a collective term for positional and geometrical isomers of linoleic acid (*cis*-9,*cis*-12-C18:2) with conjugated double bonds. Among them, *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA have been reported to prevent some diseases like cancer, obesity, and arteriosclerosis (Pariza et al., 2001). In particular, *cis*-9,*trans*-11-CLA was shown to inhibit the cancer genesis in animal models. However, *trans*-10,*cis*-12-CLA was also reported to have potential adverse effects in humans in some cases, like insulin resistance in obese men with the metabolic syndrome (Riserus et al., 2002). Many foods contain CLA, with dairy products having the highest concentrations.

In dairy cows, milk CLA have 2 origins. The first origin is their formation during microbial ruminal biohydrogenation (**BH**) of *cis*-9,*cis*-12-C18:2, which begins with isomerization, mediated by several bacterial isomerases leading to several CLA isomers (Enjalbert and Troegeler-Meynadier, 2009), such as Δ^{12}-isomerase and Δ^9-isomerase that produce *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA, respectively (Griinari and Bauman, 1999). Thereafter, initial reduction produces different *trans*-octadecenoic acid (*trans*-C18:1) isomers: *trans*-11-C18:1 (also called vaccenic acid) and *trans*-10-C18:1, respectively, for the above-mentioned CLA isomers. Finally, subsequent reduction produces stearic acid (C18:0). Because of its rapid reduction, very little CLA flows out of the rumen, implying that ruminal origin is of minor importance for milk CLA. A more significant origin of milk CLA is the mammary desaturation of *trans*-11-C18:1 into *cis*-9,*trans*-11-CLA (Griinari et al., 2000). *Trans*-11-C18:1 is an intermediate of ruminal BH of both *cis*-9,*cis*-12-C18:2 and α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-C18:3). Thus, milk CLA content could be modulated by factors affecting *cis*-9,*cis*-12-C18:2 and *cis*-9,*cis*-12,*cis*15-C18:3 BH as well as factors affecting desaturation in the mammary gland.

One way to efficiently increase CLA milk concentrations is the addition of a fat supplement to the dairy cow diet, particularly fat rich in *cis*-9,*cis*-12-C18:2 and *cis*-9,*cis*-12,*cis*-15-C18:3. In dairy cow diets, fat is usually sourced from oilseeds, which are often heated (e.g., by roasting or extrusion). Among studies, these processes have been shown to have variable effects on BH that need to be specified and explained. Heated oilseeds often induced a decrease of *cis*-9,*cis*-12-C18:2 and *cis*-9,*cis*-12,*cis*-15-C18:3 BH in vivo (Gonthier et al., 2005), in situ (Chouinard et al., 1997b; Troegeler-Meynadier

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[1]Corresponding author: a.troegeler@envt.fr

et al., 2006b) and in vitro (Reddy et al., 1994); however, this partial protection of PUFA against BH was not always noticed (Chouinard et al., 1997a; Akraim et al., 2006; Neves et al., 2007). Moreover, in most of these studies, heat treatments resulted in an accumulation of BH intermediates, particularly *cis*-9,*trans*-11-CLA, or *trans*-11-C18:1, or both.

Oilseeds heating effects on ruminal lipid digestion could be caused by a modification of the seed coat protection, a reduction of the amount of PUFA subjected to BH, or the production of oxidation products (Reddy et al., 1994). These products could act on BH or on the biohydrogenating ruminal bacteria because anaerobes are known to be sensitive to peroxides (Brioukhanov and Netrusov, 2004). Oil heating can be an efficient model for the study of the effects of heat-induced lipid modifications on ruminal BH.

The purpose of this study was to investigate the effects of 2 main modulators of the heating process, the duration of heating (1 vs. 3 vs. 6 h) and the temperature of heating (110 vs. 150°C), on *cis*-9,*cis*-12-C18:2 BH in ruminal cultures in vitro. The relationship between BH and the level of oil oxidation induced by heating (evaluated by peroxide value) was investigated, and the effects of heating on the structure of the bacterial community was studied using capillary electrophoresis single-strand conformation polymorphism (**CE-SSCP**).

MATERIALS AND METHODS

In Vitro Cultures

The oxidized oil was prepared by heating 20 mL of a commercial sunflower oil at 110 or 150°C for 1, 3, or 6 h in a crystallizing dish (9 cm in diameter). The heated oils were kept 1 h at ambient conditions to mimic cooling conditions after industrial heating processes, and then were frozen at −80°C in 2-mL syringes until use. In parallel, nonheated oil was frozen in syringes to be used as a control for both the efficiency of the BH in our in vitro incubation system and for the global effect of heating.

Ruminal incubations were performed in a water bath rotary shaker (Aquatron, Infors AG, Bottmingen, Germany). Ruminal fluid was obtained from a fistulated, nonlactating Holstein cow receiving an alfalfa hay-based diet (5 kg of alfalfa hay, 2 kg of wheat straw, 2.5 kg of corn meal, and 0.3 kg of a minerals and vitamins supplement, as fed). The cow was allowed to adapt to this diet for 3 wk before the beginning of the experiment. Rumen fluid was taken from the cow with a vacuum pump 30 min after feeding and strained through a metal sieve (1.6-mm mesh). Rumen fluid was transferred quickly to the laboratory in anaerobic conditions at 39°C.

Eighty milliliters of strained ruminal fluid were incubated in a 250-mL Erlenmeyer flask containing 200 mg of oil, 3 g of dehydrated alfalfa, and 80 mL of a bicarbonate buffer solution composed of 19.5 g/L of $Na_2HPO_4 \cdot 2H_2O$, 9.24 g/L of $NaHCO_3$, 0.705 g/L of NaCl, 0.675 g/L of KCl, 0.108 g/L of $CaCl_2 \cdot 2H_2O$, and 0.180 g/L of $MgSO_4 \cdot 7H_2O$. The buffer solution was prewarmed to 39°C and saturated with CO_2. The filled flasks were gassed with CO_2 and placed in the water bath at 39°C. Flasks were then closed with a rubber cap crossed by a plastic tube leading into the water to vent fermentation gas without allowing the entrance of oxygen. Flasks were stirred at 130 rpm and kept safe from the light during the 6-h incubation.

Two replicates of the control and of each heat treatment were incubated during each of the 3 d of culture (i.e., a total of 6 replicates per treatment). Each day, 2 blanks (without fat and not incubated) were prepared to analyze the initial state of media. At the end of the incubation, fermentations were stopped by placing the flasks into ice water, pH was measured, and 2 × 200 μL were sampled for bacterial community analysis. The contents of the flasks were then immediately frozen. Samples were freeze-dried (Virtis Freezemobile 25, Virtis, Gardiner, NY), weighed, ground and homogenized in a ball mill (Dangoumau, Prolabo, Nogent-sur-Marne, France), and kept at −18°C for later analysis.

Fatty Acids and Peroxides Analysis

The degree of oxidation of oils was estimated by the peroxide value (**PV**; AFNOR, 2004), which was expressed as milliequivalents per kilogram (mEq/kg) of oil. The fatty acids of nonheated oils, heated oils, and incubated media were extracted and methylated in situ using the procedure of Park and Goins (1994), except that the solution of 14% of boron trifluoride in methanol was replaced by a solution of methanol-acetylchloride (10:1). Nonadecanoic acid (C19:0) was used as the internal standard at a dose of 0.8 mg. The fatty acid methyl esters were then quantified by gas chromatography (Agilent 6890N, equipped with a model 7683 auto injector, Network GC System, Palo Alto, CA) using a fused silica capillary column (100 m × 0.25 mm i.d., 0.20-μm film thickness; CPSil88,Varian, Middelburg, the Netherlands).

For analysis, the flame ionization detector temperature was maintained at 260°C and the injector at 255°C; the split ratio was 1:50. Hydrogen was the carrier gas, with a constant flow of 1 mL/min. The samples were injected in 1 μL of hexane. The initial temperature of the oven was 60°C, which was held for 2 min; it was then increased by 10°C/min to 150°C, held at 150°C for 12 min, increased by 2°C/min to 175°C, held at

175°C for 20 min, increased by 20°C/min to a final temperature of 225°C, and maintained at 225°C for 10 min. This temperature program allowed the separation of *cis*-15-C18:1 from C19:0, but did not allow the separation of *cis*-9,*cis*-12,*cis*-15-C18:3 from C20:1 and did not correctly separate *trans*-C18:1 isomers. Therefore, a second temperature program was used to separate these coeluted fatty acids. For this analysis, the flame-ionization detector temperature was maintained at 260°C and the injector at 255°C; the split ratio was 1:50. Hydrogen was the carrier gas, with a constant pressure of 160 kPa. The samples were injected in 0.5 μL of hexane. The initial temperature of oven was 60°C, which was held for 3 min; it was then increased by 8°C/min to 190°C, held at 190°C for 13 min, increased by 5°C/min to 225°C, held at 225°C for 10 min, increased by 10°C/min to a final temperature of 230°C, and maintained for 10 min. Peaks were identified and quantified by comparison with commercial standards (Sigma Co., St. Louis, MO), except *trans*-C18:1 other than *trans*-9- and *trans*-11C18:1, which were identified by order of elution. The fatty acid concentrations were expressed as milligrams per kilogram of DM.

Bacterial Community Analysis

Bacterial community analysis was performed as described by Michelland et al. (2009b). Briefly, total DNA was extracted and purified with QIAamp DNA Stool Mini kit (Qiagen Ltd., West Sussex, UK) directly from approximately 0.2 g of sample. The V3 region of the 16S rRNA genes of bacterial species, corresponding to a 205-bp fragment in *Escherichia coli*, were used as a diversity marker by performing PCR using the primers W49 5′-ACGGTCCAGACTCCTACGGG-3′ and 6FAM-labeled W34 5′-TTACCGCGGCGTGCTGGCAC-3′ (Delbès et al., 1998; Zumstein et al., 2000). Then, PCR was carried out in 50-μL reaction mixtures containing 5 μL of 10× PCR buffer (PfuUltra II, Stratagene, La Jolla, CA), 0.2 μ*M* of each primer, 200 μ*M* of each dNTP, 0.25 U of *Pfu* Ultra II Fusion HS DNA polymerase (Stratagene), 25 μg of BSA (Biolabs), and 1 μL of 200× diluted DNA extract. The temperature program consisted of 2 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 61°C, and 30 s at 72°C; and a final extension of 3 min at 72°C. The PCR products were checked for appropriate size by 1% agarose gel electrophoresis.

Samples were then analyzed by the method of CE-SSCP, a capillary electrophoretic method based on the heterogeneity of single-stranded ribotype secondary structure providing different mobility through a gel. An internal standard using a different fluorochrome 6-carboxy-X-rhodamine (Applied Biosystems, Foster City, CA) was analyzed simultaneously. The SSCP mix contained 1 μL of PCR product, 7.8 μL of deionized formamid (Genescan, Applied Biosystems), and 0.2 μL of the internal standard fluorochrome 6-carboxy-X-rhodamine. The mix was denatured at 95°C for 5 min and placed on ice before loading. Then, CE-SSCP was performed on an ABI Prism 3100 Genetic Analyzer using a capillary 36 cm in length and a 7.2% nondenaturing polymer consisting of 80% CAP polymer (Applied Biosystems), 10% glycerol, and 10% 10× Tris-borate EDTA buffer (Applied Biosystems). Electrophoresis was performed at 25°C for 3,500 s at 15 KV and produced chromatograms containing both sample and internal standard signals. Bacterial communities were spread out in about 1,200 scans. The CE-SSCP data processing was computed with StatFingerprints software (Michelland et al., 2009a), and CE-SSCP profiles were aligned using pairwise alignment of their internal standard with the same reference internal standard. Simpson's diversity index (**D′**) was calculated as follows (Rosenzweig, 1995):

$$D' = -\log\Sigma a_i^2,$$

where a_i corresponds to the area of the ith peak.

Calculations

The rates and efficiencies of the *cis*-9,*cis*-12-C18:2 isomerization (**v1** and **E1**, respectively), CLA reduction (**v2** and **E2**, respectively), and *trans*-C18:1 reduction (**v3** and **E3**, respectively) were calculated as described previously by Troegeler-Meynadier et al. (2006a):

$$v1 = ([C18:2]b - [C18:2]e)/6 \text{ and}$$

$$E1 = ([C18:2]b - [C18:2]e)/[C18:2]b,$$

where 6 is the incubation time and [C18:2]b and [C18:2]e represent the concentration of C18:2 at the beginning and at the end of the 6-h incubation, respectively.

$$v2 = ([C18:2]b - [C18:2]e + [CLA]b - [CLA]e)/6 \text{ and}$$

$$E2 = ([C18:2]b - [C18:2]e + [CLA]b - [CLA]e)/([C18:2]b - [C18:2]e + [CLA]b),$$

where [CLA]b and [CLA]e represent the concentration of total CLA isomers measured at the beginning and at the end of the 6-h incubation, respectively.

$$v3 = ([C18:2]b - [C18:2]e + [CLA]b - [CLA]e + [\textit{trans}\text{-}C18:1]b - [\textit{trans}\text{-}C18:1]e)/6 \text{ and}$$

$$E3 = ([C18:2]b - [C18:2]e + [CLA]b - [CLA]e + [\textit{trans}\text{-}C18:1]b - [\textit{trans}\text{-}C18:1]e)/([C18:2]b - [C18:2n\text{-}6]e + [CLA]b - [CLA]e + [\textit{trans}\text{-}C18:1]b),$$

where [*trans*-C18:1]b and [*trans*-C18:1]e represent the concentration of total *trans*-C18:1 isomers measured at the beginning and at the end of the 6-h incubation period, respectively.

For each fatty acid, production or disappearance was calculated as amount in incubated flask − (amount in blank + amount from added oil).

Data Analysis

Fatty acids production or disappearance, rates and efficiencies of BH, and D′ of bacterial community were analyzed using the General Linear Model of SYSTAT (version 9, SPSS Inc., Chicago, IL) and were reported as mean values with standard errors of the mean.

Data were analyzed by ANOVA according to the model

$$\text{variable} = \text{mean} + \text{effect day of incubation} + \text{effect oil treatment} + \varepsilon,$$

with 3 levels for the day of incubation and 7 levels for oil treatment (control or 110 or 150°C for 1, 3, or 6 h), and ε representing the residual error. When significant differences were found, means of heated oils were compared with the control by a pairwise Dunnett's comparison. The effects of temperature, duration of heating, and their interaction were analyzed on data obtained with heated oils; the effect of temperature was analyzed by 1-dimensional contrast, and the effects of duration of heating and temperature × duration interaction were analyzed by multidimensional contrasts.

Linear relationships were investigated between PV and fatty acids involved in *cis*-9,*cis*-12-C18:2 BH (*cis*-9,*cis*-12-C18:2, *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, *trans*-11-C18:1, *trans*-10-C18:1, and C18:0) and between PV and kinetic parameters of BH (v1, E1, v2, E2, v3, and E3).

Linear relationships were then investigated between D′ and PV and between D′ and intermediates and kinetic parameters of *cis*-9,*cis*-12-C18:2 BH. For the latter regression, the intercept was not different from zero so that a zero-intercept regression was computed. Significance was declared at $P \leq 0.05$.

To compare the structure of the bacterial communities, the pairwise Euclidean distances of the 33 CE-SSCP profiles were used. To explore this distance matrix, nonmetric multidimensional scaling was performed using 10,000 random starts. Analysis of similarity (**ANOSIM**) was performed on the distance matrix using 10,000 Monte Carlo permutations. Global ANOSIM was performed to test the fixed effects of oil treatment (all data) and of heating temperature and duration (excluding C data). The ANOSIM R-value indicated the extent to which the groups differed and was between 0 (no separation) and 1 (well-separated groups).

RESULTS

Effects of Duration and Temperature of Heating on Oil

The PV of heated oils are presented in Table 1 with their respective amounts of oleic acid (*cis*-9-C18:1), *cis*-9,*cis*-12-C18:2, and *cis*-9,*cis*-12,*cis*-15-C18:3. Peroxidation of oil increased with duration and temperature of heating. The oil heated at 150°C for 6 h (treatment **150–6**) was characterized by a very high PV compared with the other heated oils. Initial amounts of unsaturated fatty acids weakly varied among treated oils.

Effects of Duration and Temperature of Oil Heating on Fatty Acids Disappearance and Production After the 6-h Incubation with Ruminal Fluid

Among short- and medium-chain fatty acids, the amounts of produced C7:0 and C8:0 increased with heating, and *anteiso*-C13:0 was not produced in the presence of heated oils (Table 2). Quantity and percentage of *cis*-9-C18:1 and *cis*-9,*cis*-12,*cis*-15-C18:3 disappearance were not affected by heat treatments. The amounts and percentages by which *cis*-9,*cis*-12-C18:2 disappeared were decreased by both the longest duration and 150°C heating, acting in synergy and leading to the lowest values for treatment 150–6. These conditions also decreased total CLA and *trans*-C18:1 production. The production of *cis*-11-C18:1 was increased by 150°C heating; the highest value was obtained for treatment 150–6.

Among CLA isomers (Table 3), *cis*-9,*trans*-11-CLA production was significantly affected by heating: the 6-h duration and 150°C temperature acted in synergy to greatly decrease *cis*-9,*trans*-11-CLA production for treatment 150–6. The production of *cis*-9,*cis*-11-CLA

Table 1. Peroxide values (PV; mEq/kg of oil) and unsaturated fatty acids content (g/100 g of oil) of sunflower oils before and after heating at 110 or 150°C for 1, 3, or 6 h

		110°C			150°C		
Item	No heating	1 h	3 h	6 h	1 h	3 h	6 h
PV[1]	4.8[e]	11.1[de]	31.4[cd]	63.5[b]	27.7[d]	54.5[bc]	237.8[a]
cis-9-C18:1[2]	23.3	24.0	23.8	23.7	22.3	24.0	22.9
cis-9,*cis*-12-C18:2[2]	47.7	49.4	48.8	48.1	45.7	48.8	44.1
cis-9,*cis*-12,*cis*-15-C18:3[2]	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[a–e]Means within a row with different superscripts significantly differ ($P < 0.05$).

[1]SEM of PV = 2.1 (n = 21, 3 repetitions per treatment).

[2]Single determination.

was slightly affected by heating duration only, and the production of *trans*-9,*trans*-11-CLA was faintly decreased by 150°C heating. Conversely, *trans*-10,*cis*-12-CLA production was significantly increased at 150°C heating, and a synergy between the 6-h duration and 150°C heating also existed, leading to the highest value for treatment 150–6.

Among *trans*-C18:1 isomers, an increase in the amount of *trans*-12-C18:1 produced was observed with 150°C temperature and the 3-h and 6-h durations of oil heating, leading to the maximum production for those treatments. Such an increase was also noticed for *trans*-9-C18:1, but no interaction was observed between the 2 factors. The production of *trans*-10-C18:1 was increased by 150°C heating but was not affected by duration. The production of *trans*-11-C18:1 was strongly decreased in a synergistic manner by 150°C temperature and the 6-h duration of heating of oil, leading to the lowest value for 150–6. The production of *trans*-16-C18:1 was affected only by 150°C heating; no production of *trans*-16-C18:1 was observed in the media containing the oils heated at 150°C.

An increase in heating temperature led to a significant increase in production of *trans*-10 isomers, whereas the amount of *trans*-11 isomers produced decreased significantly for the 6-h heating duration and 150°C heating. As a result, *trans*-10 production was the highest and *trans*-11 production the lowest for 150–6 (Table 3).

Effects of Temperature and Duration of Oil Heating on Linoleic Acid Biohydrogenation Kinetic Parameters After the 6-h Incubation with Ruminal Fluid

Increase in duration and temperature of heating significantly decreased v1 and E1 (isomerization), and heating temperature and duration acted in a synergic manner, leading to the lowest values for treatment 150–6 (Table 4). The rate of the first reduction, v2, decreased for the 6-h duration and 150°C heating, the lowest value also being for treatment 150–6, and the rate of the second reduction, v3, was weakly affected by heating. The efficiencies of both reductions (E2 and E3) were significantly increased by the 6-h heating duration, but only E2 was affected by temperature.

Effects of Duration and Temperature of Oil Heating on Ruminal Bacterial Community After 6 h of In Vitro Incubation

Duration of heating did not influence bacterial community structure (Table 5; $P = 0.86$; ANOSIM R-value = 0), but bacterial community structure was affected by heating temperature ($P = 0.01$; ANOSIM R-value = 0.21). Accordingly, the 2-dimensional nonmetric multidimensional scaling plot of the 33 CE-SSCP profiles from the ruminal cultures showed that bacterial communities could be separated according to heating temperature (110 vs. 150°C) (Figure 1).

No effect of duration ($P = 0.27$) and temperature ($P = 0.93$) of heating was observed on bacterial community D′. However, this D′ was strongly and linearly related to all BH intermediates and kinetic parameters of *cis*-9,*cis*-12-C18:2 BH (Table 5).

Relationships Between Intermediates and Kinetic Parameters of Linoleic Acid Biohydrogenation, Bacterial Community Diversity, and PV

Among the fatty acids involved in *cis*-9,*cis*-12-C18:2 BH, only C18:0 was not linked to PV (Table 6). The amount of *cis*-9,*cis*-12-C18:2 that disappeared from the media decreased when PV increased. The production of *trans*-10 isomers increased slowly but significantly with the increase of PV values, whereas *trans*-11 isomers were negatively, significantly, and more strongly linked with PV.

Among kinetic parameters of *cis*-9,*cis*-12-C18:2 BH, v1 and E1 (isomerization) were negatively linked with PV, and the efficiencies of both reductions (E2 and

Table 2. Effects of duration and temperature of heating of sunflower oil on production (positive values) or disappearance (negative values) of fatty acids (mg), and percentages of disappearance of oleic, linoleic, and linolenic acids after the 6-h incubation with ruminal fluid

		110°C			150°C				*P*-value		
Item	No heating	1 h	3 h	6 h	1 h	3 h	6 h	SEM	Duration	Temperature	Duration × temperature
Fatty acid balance (mg)											
C6:0	0.87	1.04	1.38	1.73*	1.53*	1.70*	1.53*	0.061	0.10	0.15	0.11
C7:0	0.06	0.08	0.11	0.15*	0.12	0.13*	0.14*	0.006	0.03	0.32	0.42
C8:0	0.02	0.01	0.02	0.04	0.02	0.03	0.10*	0.003	<0.01	<0.01	<0.01
C9:0	0.03	0.04	0.04	0.05	0.04	0.04	0.06	0.003	0.08	1.00	0.81
C10:0	0.03	0.01	0.01	0.02	0.02	0.02	0.02	0.002	0.80	0.46	0.96
C11:0	−0.33	−0.68*	−0.66	−0.58	−0.65	−0.76*	−0.78*	0.034	0.92	0.21	0.47
C12:0	−0.01	0.00	−0.09	−0.03	−0.10	−0.17	−0.13	0.033	0.62	0.22	0.99
C13:0	0.05	0.00	0.03	0.05	0.03	0.04	0.02	0.005	0.36	0.81	0.09
anteiso-C13:0	0.27	−0.01*	0.02*	0.00*	−0.01*	−0.07*	−0.08*	0.011	0.58	0.02	0.29
iso-C13:0	0.03	−0.01	0.00	0.02	0.07	−0.01	0.02	0.006	0.03	0.02	<0.01
C14:0	0.57	−0.03	0.36	0.53	0.29	0.26	0.24	0.060	0.33	0.90	0.15
C15:0	0.65	0.36*	0.45	0.53	0.51	0.40*	0.37*	0.025	0.90	0.77	0.07
anteiso-C15:0	0.69	0.42*	0.55	0.61	0.64	0.57	0.52	0.021	0.85	0.24	0.02
iso-C15:0	0.32	0.16*	0.22	0.29	0.29	0.18*	0.20	0.013	0.48	0.89	<0.01
C16:0	−0.48	−2.85	−1.33	2.17	0.56	−0.31	−0.70	0.326	0.09	0.44	0.01
C16:1	−0.18	−0.17	−0.14	−0.12	−0.13	−0.38	−0.12	0.039	0.35	0.46	0.31
C17:0	0.33	0.23	0.26	0.37	0.30	0.13	0.22	0.024	0.25	0.18	0.14
anteiso-C17:0	0.15	0.12	0.12	0.16	0.16	0.13	0.13	0.007	0.48	0.54	0.11
iso-C17:0	0.12	0.08	0.08	0.11	0.10	0.07	0.07	0.005	0.51	0.66	0.09
cis-10-C17:1	0.03	0.03	0.04	0.04	0.05	0.05	0.03	0.003	0.79	0.58	0.09
C18:0	27.90	29.80	30.82	31.76	35.75	31.77	31.46	0.796	0.90	0.29	0.46
anteiso-C18:0	0.08	0.08	0.10	0.01	0.12	0.11	0.09	0.011	0.18	0.09	0.47
iso-C18:0	0.03	0.01	0.00	−0.01	−0.01	0.01	−0.03*	0.005	0.11	0.25	0.68
cis-9-C18:1	−15.17	−14.91	−15.17	−14.15	−13.16	−15.55	−14.20	0.253	0.05	0.23	0.0
cis-11-C18:1	0.84	0.87	0.84	0.70	0.93	1.08	1.35*	0.037	0.43	<0.01	0.01
cis-15-C18:1	0.03	0.02	0.02	0.00	0.04	0.03	0.03	0.004	0.40	0.02	0.31
Total *trans*-C18:1	21.33	21.41	21.28	20.55	23.51	21.32	12.55*	0.342	<0.01	<0.01	<0.01
cis-9,*cis*-12-C18:2	−53.08	−53.92	−52.36	−48.92	−49.69	−53.14	−41.43*	0.449	<0.01	<0.01	<0.01
Total CLA[1]	5.36	6.13	4.81	4.13	4.96	4.58	1.09*	0.154	<0.01	<0.01	0.02
cis-9,*cis*-12,*cis*-15-C18:3	−4.37	−4.59	−4.31	−3.59	−4.30	−3.83	−4.19	0.119	0.36	0.66	0.15
Disappearance (%)											
cis-9-C18:1	30.17	28.90	29.54	27.78	27.16	30.21	28.80	0.499	0.18	0.68	0.26
cis-9,*cis*-12-C18:2	50.13	49.52	48.54	46.10	48.69	49.39	42.10*	0.421	<0.01	0.05	0.09
cis-9,*cis*-12,*cis*-15-C18:3	45.52	47.50	45.00	38.23	43.56	40.89	44.28	1.122	0.39	0.77	0.16

[1]CLA = conjugated linoleic acid.

*Treatment significantly ($P < 0.05$) differs from no heating (Dunnett's test).

Table 3. Effects of duration and temperature of heating of sunflower oil on produced amounts (mg) of *trans*-C18:1 and CLA[1] isomers after the 6-h incubation with ruminal fluid

		110°C			150°C				*P*-value		
Item	No heating	1 h	3 h	6 h	1 h	3 h	6 h	SEM	Duration	Temperature	Duration × temperature
trans-C18:1 isomer											
trans-4-C18:1	0.15	0.14	0.11	0.08	0.15	0.09	0.09	0.009	0.06	0.89	0.76
trans-5-C18:1	0.11	0.11	0.10	0.08	0.12	0.11	0.09	0.006	0.22	0.63	0.99
trans-6,7,8-C18:1	0.89	0.85	0.80	0.86	1.01	0.94	0.71	0.021	0.06	0.34	0.02
trans-9-C18:1	0.40	0.23	0.27	0.67	0.73	0.79	1.09*	0.050	<0.01	<0.01	0.91
trans-10-C18:1	0.79	0.68	0.79	0.79	1.01	1.08	1.15	0.040	0.47	<0.01	0.96
trans-11-C18:1	16.51	16.72	16.47	15.20	18.36	15.77	7.25*	0.229	<0.01	<0.01	<0.01
trans-12-C18:1	0.77	0.82	0.73	0.84	0.85	1.12*	1.10*	0.020	0.04	<0.01	<0.01
trans-13,14-C18:1	0.90	1.25	1.26	1.13	1.40	1.46	1.10	0.080	0.48	0.57	0.85
trans-15-C18:1	0.27	0.27	0.24	0.57	0.37	0.41	0.43	0.031	0.06	0.49	0.16
trans-16-C18:1	0.54	0.33	0.52	0.33	−0.50*	−0.46*	−0.46*	0.043	0.55	<0.01	0.67
CLA isomer											
trans-10,*cis*-12-CLA	0.14	0.16	0.12	0.12	0.14	0.15	0.19*	0.004	0.15	<0.01	<0.01
cis-9,*trans*-11-CLA	4.11	4.77	3.78	3.29	4.18	3.65	1.05*	0.101	<0.01	<0.01	<0.01
cis-9,*cis*-11-CLA	0.15	0.16	0.10	0.11	0.15	0.11	0.04*	0.009	<0.01	0.22	0.15
trans-9,*trans*-11-CLA	0.96	1.03	0.81	0.61	0.57	0.66	0.31	0.069	0.17	0.04	0.66
Sum											
trans-10 isomers	0.93	0.84	0.91	0.91	1.15	1.23	1.34*	0.041	0.46	<0.01	0.78
trans-11 isomers	21.58	22.53	21.06	19.11	23.12	20.09	8.61*	0.325	<0.01	<0.01	<0.01

[1]CLA = conjugated linoleic acid.

*Treatment significantly ($P < 0.05$) differs from no heating (Dunnett's test).

Table 4. Effects of duration and temperature of heating of sunflower oil on rates (mg/L per hour) and efficiencies (%) of the 3 reactions of linoleic acid biohydrogenation[1] in the 6-h incubation with ruminal fluid

		110°C			150°C				*P*-value		
Item	No heating	1 h	3 h	6 h	1 h	3 h	6 h	SEM	Duration	Temperature	Duration × temperature
v1	55.29	56.16	54.54	50.96	51.76	55.35	43.15*	0.468	<0.01	<0.01	<0.01
E1	50.13	49.52	48.54	46.10	48.69	49.39	42.10*	0.421	<0.01	0.05	0.09
v2	49.71	49.78	49.53	46.66	46.51	50.58	41.49*	0.511	<0.01	<0.01	0.04
E2	88.38	87.81	89.56	90.54	88.73	90.42	94.97*	0.317	<0.01	<0.01	0.05
v3	27.49	27.48	27.36	25.25	22.02	28.37	28.42	0.623	0.07	0.49	0.01
E3	45.66	47.26	46.97	46.18	39.17	48.07	57.43*	0.688	<0.01	0.40	<0.01

[1]v1 and E1 refer to the isomerization of *cis*-9,*cis*-12-C18:2 to conjugated linoleic acid (CLA); v2 and E2 refer to the reduction of CLA to *trans*-C18:1; v3 and E3 refer to the reduction of *trans*-C18:1 to C18:0.

*Treatment significantly ($P < 0.05$) differs from no heating (Dunnett's test).

E3) were positively linked with PV. However, although significant, r^2 values were less than 0.7. Diversity index was not linked to the peroxide content of the media.

DISCUSSION

Effect of Heating on PV of Oils

As expected, PV of oil increased with duration and temperature of heating, with the highest values for 150°C during the 6-h incubation. For treatment 150–6, the values obtained were very high compared with all other combinations of temperatures and durations of heating. The reaction of oxidation probably reached its propagation phase, characterized by an exponential increase of peroxides in the fat.

Heating of Oil Affected Linoleic Acid Biohydrogenation

Increasing duration and temperature of heating of sunflower oil, rich in *cis*-9,*cis*-12-C18:2, strongly affected *cis*-9,*cis*-12-C18:2 BH. The disappearance of *cis*-9,*cis*-12-C18:2 was inhibited, leading to a decreased production of CLA and *trans*-C18:1, whereas disappearances of *cis*-9-C18:1 and *cis*-9,*cis*-12,*cis*-15-C18:3 remained unchanged. This inhibition of *cis*-9,*cis*-12-C18:2 disappearance could, at least partly, explain the protective effect of heat treatment of oilseeds against BH that was observed in some studies (Reddy et al., 1994; Chouinard et al., 1997b; Troegeler-Meynadier et al., 2006b). In these studies, the conditions of extrusion and roasting were often not specified apart from the temperature of heating. In studies where extrusion did not lead to a protection of PUFA against BH, the temperature of extrusion was low, about 120°C (Akraim et al., 2006; Neves et al., 2007) as opposed to 140 to 160°C in studies where a protection was noticed. Duration of extrusion was probably not the same among studies because only 2 studies specified this: 30 s in the study of Neves et al. (2007), who did not observe PUFA protection, and 43 s for Gonthier et al. (2005), who observed PUFA protection. For roasting, duration of heating is longer than for extrusion, which could explain that a protection of PUFA against BH was frequently observed (Reddy et al., 1994; Chouinard et al., 1997a; Troegeler-Meynadier et al., 2006b). The capacity to withstand BH that was observed when using heated oilseeds compared with raw oilseeds could be explained, at least in part, by the effect of duration and temperature of heating on the modulation of isomerization and subsequent disappearance of PUFA.

Heating of Oil Affected CLA and Trans-C18:1 Production

Contrary to the studies where heating of oilseeds resulted in an accumulation of BH intermediates (Chouinard et al., 1997a; Gonthier et al., 2005; Neves et

Table 5. Regression equations between bacterial population diversity index (D′) of incubated ruminal fluid and disappeared or produced amounts (mg) of fatty acids intermediates, rates (mg/L per hour), and efficiencies (%) of the 3 reactions of linoleic acid biohydrogenation,[1] combining results obtained with heated and unheated oils (n = 42)

Regression equation	r^2	*P*-value
cis-9,*cis*-12-C18:2 = −7.79 D′	0.97	<0.01
trans-10,*cis*-12-CLA = 0.02 D′	0.96	<0.01
cis-9,*trans*-11-CLA = 0.52 D′	0.90	<0.01
trans-10-C18:1 = 0.13 D′	0.93	<0.01
trans-11-C18:1 = 2.31 D′	0.94	<0.01
C18:0 = 4.88 D′	0.97	<0.01
v1 = 8.12 D′	0.97	<0.01
E1 = 7.35 D′	0.97	<0.01
v2 = 7.42 D′	0.97	<0.01
E2 = 13.89 D′	0.98	<0.01
v3 = 4.23 D′	0.98	<0.01
E3 = 7.39 D′	0.98	<0.01

[1]v1 and E1 refer to the isomerization of *cis*-9,*cis*-12-C18:2 to conjugated linoleic acid (CLA); v2 and E2 refer to the reduction of CLA to *trans*-C18:1; v3 and E3 refer to the reduction of *trans*-C18:1 to C18:0.

Table 6. Regression equations between peroxide values (PV; mEq/kg of oil) and disappeared or produced amounts (mg) of fatty acids intermediates, rates (v; mg/L per hour) and efficiencies (E; %) of the 3 reactions of linoleic acid biohydrogenation,[1] and bacterial population diversity index (D′) of incubated ruminal fluid, combining results obtained with heated and unheated oils (n = 42)

Regression equation	r^2	*P*-value
cis-9,*cis*-12-C18:2 = 0.05 PV – 53.35	0.41	0.01
trans-10,*cis*-12-CLA = 0.0002 PV + 0.13	0.54	<0.01
cis-9,*trans*-11-CLA = −0.01 PV + 4.41	0.82	<0.01
trans-10-C18:1 = 0.002 PV + 0.80	0.40	<0.01
trans-11-C18:1 = −0.04 PV + 17.76	0.83	<0.01
C18:0 = 0.01 PV + 30.85	0.07	0.66
v1 = −0.05 PV+ 55.57	0.41	0.01
E1 = −0.03 PV + 49.65	0.32	0.04
v2 = −0.03 PV + 49.78	0.28	0.08
E2 = 0.03 PV + 88.31	0.69	<0.01
v3 = 0.00 PV + 26.09	0.10	0.52
E3 = 0.05 PV + 44.06	0.60	<0.01
D′ = 0.00 PV + 5.95	0.23	0.20

[1]v1 and E1 refer to the isomerization of *cis*-9,*cis*-12-C18:2 to conjugated linoleic acid (CLA); v2 and E2 refer to the reduction of CLA to *trans*-C18:1; v3 and E3 refer to the reduction of *trans*-C18:1 to C18:0.

al., 2007), in this study, the heating of oil for 6 h at 150°C induced a significant diminution of total CLA and *trans*-C18:1 production. In the study of Troegeler-Meynadier et al. (2006b), the increase of CLA and *trans*-C18:1 production with extruded compared with raw soybeans was associated with a decrease in the efficiencies of both reductions. Conversely, in the present study, heat treatment of oil increased the efficiencies of both reductions, which means that reduction was not inhibited. Therefore, the low production of CLA and *trans*-C18:1 observed with heated oils could be caused only by the inhibition of isomerization. Indeed, the inhibition of the isomerization led to a lack of substrate for the subsequent reaction, the reduction of CLA, and so to a decrease in the rate of this reaction.

However, the effect was not the same in the production of individual CLA as for *trans*-C18:1 isomers: *trans*-10 isomers were increased and *trans*-11 isomers were decreased by 150°C heating. In fact, the diminution of total CLA production was caused mainly by the inhibition of the production of the main CLA isomer, *cis*-9,*trans*-11-CLA, and to a lesser degree to the inhibition of the production of *cis*-9,*cis*-11-CLA by long heating duration and of *trans*-9,*trans*-11-CLA by high heating temperature. Hence, there was an inhibition of the Δ^{12}-isomerization. In contrast, there was an increase in produced quantities of *trans*-10,*cis*-12-CLA with heating temperature and duration, suggesting an enhancement of the Δ^{9}isomerase activity.

Similarly, the amount of *trans*-11-C18:1 produced was significantly reduced for the 6-h duration and 150°C temperature, which suggested an inhibition of the Δ^{12}-isomerization; the *trans*-10-C18:1 increase for 150°C heating could be linked to a more important Δ^{9}-isomerization. The inhibitory effect of heating duration and temperature on *trans*-11 isomers in ruminal cultures has been previously reported in a preliminary study in vitro (Troegeler-Meynadier et al., 2006c), and *cis*-9,*trans*-11-CLA and *trans*-11-C18:1 percentages in milk were reported to decrease when dairy cows ingested oxidized soybean oil with PV = 240 mEq/kg of oil (Vazquez-Anon et al., 2008). Conversely, studies with heated oilseeds (Chouinard et al., 1997a; Troegeler-Meynadier et al., 2006b; Neves et al., 2007) reported an accumulation of *cis*-9,*trans*-11-CLA isomers, *trans*-11-C18:1 isomers, or both. The nature, or the amount, or both, of oxidized products generated during heating may depend on the form of fat (oil vs. seed) and on the heating process (extrusion, roasting, and so on), which could explain the differences among these studies.

Effect of heating on *trans*-10 isomer production has not yet been reported. Nevertheless, the increase of *trans*-10,*cis*-12-CLA in cultures with heated oil observed in this study could explain the decrease of milk fat content noticed in some in vivo studies with the use of extruded soybeans compared with raw soybeans (Guillaume et al., 1991; Neves et al., 2007) because this isomer causes a decrease in milk fat content (Baumgard et al., 2000).

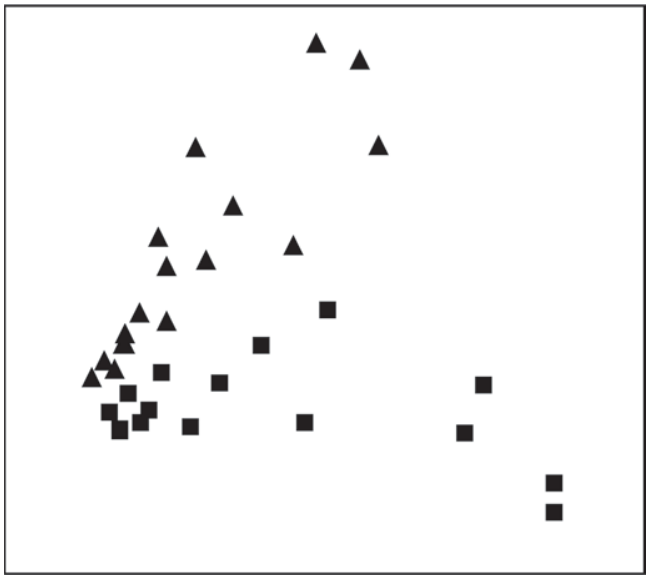

Figure 1. Two-dimensional nonmetric multidimensional scaling plot of the 33 capillary electrophoresis single-strand conformation polymorphism profiles from the ruminal cultures incubated for 6 h with oil heated at 110°C (▲) or 150°C (■) regardless of the duration of heating. Analysis of similarity R-value = 0.21; $P < 0.01$.

Heated Oils Could Act Directly on Biohydrogenation Reactions and on Ruminal Bacteria

The action of heating duration and temperature of oil occurred principally on the isomerization process, but an additional action on lipolysis could not be excluded. The heated oils could act either on the reaction or on isomerizating bacteria. The isomerizations of *cis*-9,*cis*-12-C18:2 to *trans*-10,*cis*-12-CLA and *cis*-9,*trans*-11-CLA do not follow the same pathways (Wallace et al., 2007) and are not performed by all bacteria; fibrolytic bacteria, belonging to the *Butyrivibrio* genus, produce only *trans*-11 isomers (Maia et al., 2007; Wallace et al., 2007), and some lactate-consuming bacteria predominantly produce *trans*-10 isomers (Verhulst et al., 1987; Kim et al., 2002; Wallace et al., 2007). As a consequence, the effects of heated oils on each pathway could be different: activation of Δ^9-isomerization versus inhibition of Δ^{12}-isomerization.

The Δ^{12}-isomerization is a radical reaction, possibly initiated by hydrogen abstraction on C-11 catalyzed by a radical intermediate enzyme (Wallace et al., 2007). The *cis*-9,*cis*-12-C18:2 oxidation is also a radical reaction that also begins by an hydrogen abstraction on C-11 (Spiteller et al., 2001), so that some common intermediates or some structural analogs generated during oxidation could interfere with the Δ^{12}-isomerization. This explanation cannot apply to the activation of Δ^9-isomerization, which is not a radical intermediate reaction but rather an ionic reaction with a carbocation as an intermediate (Liavonchanka et al., 2006; Wallace et al., 2007).

These 2 pathways of *cis*-9,*cis*-12-C18:2 BH are realized by different bacteria: *trans*-11 isomers are produced mostly by fibrolytic bacteria (mainly *Butyrivibrio fibrisolvens* and to a lesser extent *Butyrivibrio proteoclasticus*, formerly *Clostridium proteoclasticum*; Moon et al., 2008), whereas the *trans*-10 isomers are produced by lactate-consuming bacteria (*Propionibacterium acnes*, or *Megasphaera elsdenii*, or both; Kim et al., 2002; Maia et al., 2007; Wallace et al., 2007). Heated oils could contain products that inhibit the growth or activity of fibrolytic bacteria and favor those of *P. acnes* and *M. elsdenii*. This hypothesis is strengthened by other modifications in the production of fatty acids. In our experiment, heating increased the production of *trans*10,*trans*12-CLA and *trans*12-C18:1, and *P. acnes* is known to produce *trans*-10,*trans*-12-CLA (Wallace et al., 2007), which is partly reduced to *trans*-12-C18:1 (Shingfield et al., 2008). Similar results were obtained by Choi et al. (2005) studying the effect of oxygen on CLA production by mixed ruminal bacteria. They showed that in aerobic conditions, more *trans*-10,*cis*-12-CLA accumulated than *cis*-9,*trans*-11-CLA and concluded that bacteria producing *trans*-10,*cis*-12-CLA were more aero tolerant than bacteria producing *cis*-9,*trans*-11-CLA. This tolerance could be because of the production of antioxidant enzymes like superoxide dismutase and glutathione peroxidase by ruminal bacteria, as shown on *Streptococcus bovis* and *Selemonas ruminantium* (Lenartova et al. 1998). On the other hand, Hino et al. (1993) demonstrated that vitamin E and β-carotene increased growth of fibrolytic bacteria, possibly by decreasing the noxious effect of oxidative compounds. As a consequence, oxidative compounds would be more noxious for fibrolytic bacteria than for lactate consumers and therefore increase the participation of bacteria producing *trans*-10,*cis*-12-CLA relative to that of bacteria producing *cis*-9,*trans*-11-CLA.

In the present work, we studied the whole bacterial community dynamics using CE-SSCP (Hori et al., 2006; Michelland et al., 2009b). Temperature of oil heating modulated the structure of the ruminal bacterial population. Heating at 150°C could generate 1 or several oxidative products that could alter the microbial population. This hypothesis was supported by *cis*-9,*cis*-12-C18:2 BH and its intermediates being very closely linked to the bacterial community D′ (Table 5). Estimating D′ consists of summarizing a complex community represented by a molecular fingerprint pattern in a single value by taking into account the number of species (number of peaks) and their relative abundance (area under each peak; Michelland et al., 2009b). The lowest value of D′ is obtained when the profile contains only 1 peak and the highest value of D′ obtained when the profile contains many overlapping peaks of equal abundance. Thus, the positive relationship between C18:2 BH process and D′ indicated (Table 5) that the lowering effect of heated oils on the diversity of the bacterial community makes the BH process less efficient.

The heating of oils generates many different oxidative products that could act on the reaction of BH or directly on the bacteria. The parallel evolution between PV and *cis*-9,*cis*-12-C18:2, CLA, and *trans*-C18:1 intermediates of *cis*-9,*cis*-12-C18:2 BH, underlined by significant linear relationships and a major effect of the 150–6 oil treatment (highest PV), confirmed their possible effect on BH. Therefore, fatty acid peroxidation might explain, at least in part, our results. In this first approach, PV was chosen to estimate the oxidative status of the oils; however, it cannot allow a precise estimation of the different products generated by oxidation. Because heating duration did not modify bacterial population while affecting BH, and heating temperature modified bacterial population, we hypothesized that at least 2 or more oxidative compounds were involved: first(s) accumulated for long-duration heating, acting directly on the Δ^{12}isomerase, and other(s), identical or not,

produced at high temperature and able to act on the microorganisms. This is supported by the strong negative linear relationship that existed between *trans*-11 isomers and PV because this isomerization may be affected by these 2 actions; bacteria producing *trans*-11 were probably more affected by peroxides and so produced fewer *trans*-11 isomers, and Δ^{12}isomerase was inhibited by these compounds (Table 6). Conversely, *trans*-10 isomers were positively, and to a lesser, extent linked to the PV, showing that the appearance of these isomers would not be directly linked to the presence of peroxides but rather probably was a result of the higher resistance of the bacteria producing *trans*-10 to these compounds. The nature of these oxidative products was not determined here, and few data exist in the literature about the effect of the different oxidative products on BH. Contrary to our results, Lee et al. (2007) observed a stimulation of BH and therefore an increase of the production of CLA and *trans*-11-C18:1, and a decrease of *trans*-10,*cis*-12-CLA and *trans*-10-C18:1, when using pure *trans*-2-decenal, which is an end product of *cis*-9,*cis*-12-C18:2 oxidation (Spiteller et al., 2001). In the present study, oil was rapidly frozen at −80°C so that radical intermediates and hydroperoxides, which are formed at the beginning of oxidation, were probably the dominant oxidative products. Terminal oxidation products need more time to accumulate. They probably act differently on BH compared with peroxides. This underlines the importance of distinguishing oxidative products and their different capacity to modulate BH.

CONCLUSIONS

Increasing the duration and temperature of heating of sunflower oil had a synergistic action on *cis*-9,*cis*-12-C18:2 BH. An overall decrease in *cis*-9,*cis*-12-C18:2 disappearance occurred, leading to an overall decrease in CLA and *trans*-C18:1 production because of a decreased production of *cis*-9,*trans*-11-CLA and *trans*-11-C18:1. In contrast, there was an increase in the production of *trans*-10 isomers, mainly because of high heating temperature. Our results suggest that heating at high temperature would act via a modulation of the bacterial community, whereas increasing heating duration would not. Oxidative compounds produced during oil heating could explain, at least in part, these effects. They could act on BH reactions by generating substances directly affecting isomerases, or by stimulating or inhibiting specific bacterial communities, or both. Oxidative compound(s) accumulated during heating would act directly on the isomerization step, and oxidative compound(s), identical or not, generated only at high temperature would be able to affect bacterial population equilibrium. In this study, PV was used as a proxy for *cis*-9,*cis*-12-C18:2 oxidation but did not reflect the production of other oxidative products (i.e., triglycerides oxipolymers, ketones, aldehydes). Further studies are therefore necessary to identify precisely which oxidative products affect BH.

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