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Effect of feeding linseed oil in diets differing in forage to concentrate ratio: 2. Milk lactone profile

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Lactones are important contributors to the flavour and aroma of milk and dairy products. This study was conducted to evaluate the effects of dietary linseed oil (LO) and forage to concentrate ratio on milk lactone profile. Twenty four Holstein cows were used during a 4-week feeding trial in a randomised complete block design. Cows were fed diets containing 30% (LC) or 70% (HC) concentrate, and 0% (NLO) or 3% LO in a 2 × 2 factorial arrangement of treatments. Milk lactone profile was evaluated using the solid phase microextraction technique. The highest levels of δ-lactones (δ-6:0, δ-8:0, δ-10:0, and δ-12:0) were found with the LC/NLO diet. These concentrations were then decreased when cows received either a high level of concentrate or supplemental LO, but these effects were not additive (interaction of LO by concentrate, $P<0{\cdot}01$). An interaction of LO by concentrate ($P<0{\cdot}01$) was also noted on milk γ-12:0 for which the highest concentration was observed when supplementing LO in HC diet, while no effect was apparent when LO was added in LC diet. Moreover, feeding HC increased the level of γ-12:1 in milk as compared with LC, while LO had no effect on this γ-lactone. Finally, γ-12:2 was not detected in any of the milk samples studied. Organoleptic properties of milk were evaluated in a triangle test showing that a significant number of assessors perceived a difference between milk from cows fed LC/NLO as compared with milk from cows fed HC/LO. The sensory evaluation was completed by a ranking test where the intensities of fresh lactic, foreign and global flavours were not different between treatments. In conclusion, feeding LO in HC diet modified milk lactone profile with a shift toward more γ- and less δ-lactones as compared with LC diet not supplemented with LO. A difference was perceived in a triangle test between milk from these two treatments, but the sensory attributes responsible for this difference have not been identified in the current trial.

Keywords: Dairy cow, flaxseed, lactone, forage to concentrate ratio.

Lactones are among the most important groups of aromatic compounds found in milk (Urbach, 1990), where they are recognised to contribute to the unique flavour of butter. Different forms of lactones, which differ by the size of their heterocycle, have been identified. Among them, γ-lactone (4 carbons) and δ-lactone (5 carbons) are the most abundant series reported in ruminant products (Shahidi et al. 1986; Hettinga, 2005). In bovine milk, δ-lactones were found in higher concentrations compared with γ-lactones (Hettinga, 2005).

Although lactones are synthesised by the animal, a part could also be produced during heat treatment of milk fat (Stark et al. 1978; Urbach & Stark, 1978). The precursors of δ- and γ-lactones are 5- and 4-hydroxy acids, respectively (Singh et al. 2003). Triglycerides of freshly produced milk contain low concentrations of hydroxy acids which are released by hydrolysis, and converted to lactones during milk processing. Ruminant milk fat has therefore been studied for its 'lactone potential' e.g., the qualitative and quantitative lactone profiles generated following heat treatments in absence of oxygen (Stark et al. 1978; Urbach & Stark, 1978).

Dimick & Harner (1968) evaluated the effects of different dietary, environmental and animal factors affecting the lactone potential of milk fat, and reported the following observations on the concentration of δ-decalactone (δ-10:0), δ-dodecalactone (δ-12:0) and δ-tetradecalactone (δ-14:0): (i) a seasonal trend, with higher concentrations of lactones

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during winter when cows received indoor diets compared with summer and pasture feeding; (ii) a shift in lactone content in milk which increased from 25–30 ppm following parturition to 170–180 ppm at about 150 d of lactation; (iii) a negative correlation between lactone concentration and milk fat content and yield throughout lactation; (iv) a positive correlation between lactone content and the proportion of fatty acids with chain length between 4 and 14 carbon atoms; and (v) a slightly higher lactone potential in milk fat of Holstein compared with Brown Swiss or Jersey/Guernsey cows.

Moreover, Urbach & Stark (1978) observed an increase in the γ-dodecalactone (γ-12:0) potential when cows were moved from pasture to a ration based on alfalfa hay supplemented with oats (source of starch), but not when they were transferred to alfalfa hay only. The same authors also observed that feeding full-fat sunflower seeds (source of unsaturated fatty acids), but not feeding sunflower meal (defatted), increased the γ-dodecaenolactone (γ-12:1), and decreased the saturated δ-lactone potential of milk fat. In light of these results, it can be concluded that variations in the supply of starch and unsaturated fatty acids modified the γ-lactone potential of milk fat.

More recently, Bendall (2001) compared milk from cows fed pastures and total mixed rations in New Zealand. A total of 12 different lactones were evaluated using Solvent-Assisted Flavour Evaporation, followed by the Nasal Impact Factor procedure. No difference between treatments was observed on the frequency of detection of γ-12:0 and γ-12:1. In contrast, γ-12:2 had a frequency of detection of 50% in milk of cows fed total mixed rations, while it has not been detected in milk of cows on pasture.

According to Joblin & Hudson (1997), in dairy cows, synthesis of γ-12:0 and γ-12:1 is a two-step process where 18-carbon unsaturated FA are first transformed in the rumen by hydration into hydroxy acid intermediates. After absorption, hydroxy acids are shortened by three rounds of β-oxidation, followed by cyclisation to γ-lactones. Dietary oleic and linoleic acids are precursors of γ-12:0 and γ-12:1, through the production of 10-hydroxy-stearic acid and 10-hydroxy-oleic acid, respectively (Urbach, 1990). Following the same series of reactions, α-linolenic acid could potentially be the precursor of γ-12:2, with 10-hydroxy-linoleic acid as intermediate, as it has been shown in the microbial lactonisation of fatty acids of linseed oil (Kim, 2005).

It appears, from this series of observations, that the impacts of dietary factors on milk lactone profile are not fully understood. The objective of this study was to evaluate the effect of feeding linseed oil in diets providing different levels of grains as a source of starch on milk lactone profile.

Materials and methods

Animals and diets

The experimental design is described in detail in a companion paper (Saliba et al. 2014). Briefly, 24 Holstein cows were used in a randomised complete block design based on days in milk, with a 2 × 2 factorial arrangement of treatments. Within each block, cows were fed one of four experimental diets containing LC (30% of dry matter) or HC (70% of dry matter), without LO (NLO), or with LO supplemented at 3% of dry matter. The experimental period was 4 weeks in length. Body weight, dry matter intake and milk yield were recorded, and samples of feed and milk were harvested during the last week of the experimental period. Data on production performance and milk fatty acids profiles were reported by Saliba et al. (2014).

Sampling, measurements and analyses

On day 26 of the experimental period, composite milk samples from individual cows were obtained by mixing samples from a.m. and p.m. milkings in proportion to milk yield. These samples were used for the determination of milk lactone profile. This analysis was performed in triplicate using the solid phase microextraction (SPME) technique. Results from triplicate analysis were averaged for statistical analyses. A milk sample of 9 g and 200 µl of the internal standard solution containing 4 µg/ml of γ-undecalactone (γ-11:0) were placed in a 20-ml glass vials which was sealed with silicon/Teflon septa and magnetic caps. Milk lactones were extracted from the headspace with a 2-cm, 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane SPME fibre (Supelco, Bellefonte, PA). The analysis of lactones was carried out by using a CombiPal autosampler (CTC Analytics, Zwingen, Switzerland) attached to an Agilent 6890N gas chromatograph with 5973 inert mass spectrometry (MS) detection (Agilent Technologies Inc., Santa Clara, CA). The sample was pre-equilibrated at 55 °C for 5 min with agitation at 250 RPM. The fibre was then exposed to the headspace at the same temperature and agitation for 60 min. Once the adsorption time was finished, the volatile compounds extracted with the SPME fibre were thermally desorbed into the gas chromatograph injector in splitless mode at 255 °C during 3 min. Between each use, the fibre was cleaned at 270 °C for 20 min. in the CompilPal fibre conditioning station. Lactone compounds were separated with a DB-FFAP column (30 m × 0·25 mm, film thickness 0·25 µm; Agilent Technologies Inc.). The oven temperature was held at 40 °C for 5 min., and then ramped up at a rate of 5 °C/min. up to 245 °C. At that point, the temperature was held at 245 °C for 25 min. Helium was used as carrier gas at a flow rate of 0·7 ml/min.

The mass spectrometer was operated in the electron impact ionisation mode at 70 eV; the mass range used was m/z 35–350. Scan was used as data acquisition mode. The MS transfer line and the ion source temperatures were 255 and 230 °C, respectively. Compounds were identified by comparison with mass spectra from a library database (NIST 08 Mass Spectral Library, version 2.0, Gaithersburg, MD, USA), and confirmation of the identification of the lactones was achieved by comparing the GC retention times with those of authentic standards spiked into milk. These volatile

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compounds were then quantified using selective ions extracted from the scan data.

Standards of lactones (except γ-12:2) were a provided courtesy of Soda Aromatic Co. Ltd. (Tokyo, Japan). Standard of γ-12:2 was kindly donated by Dr Justin Bendall, Fonterra Research Centre, New Zealand. The relative abundance of each lactone was obtained as the ratio of its peak to the area of γ-11:0 peak used as internal standard from selective ion-monitoring MS.

Also on day 26 of the experimental period, milk from the two consecutive milkings was collected separately from each group of cows and pooled by treatment in 250-l bulk tanks. Once refrigerated at 4 °C, milk was transported in stainless steel milk cans to the Université Laval pilot plant. Each milk batch was standardised to 3·25% fat, homogenised, and pasteurised at 75 °C for 16 s. A sample from each treatment was frozen at − 20 °C for further analysis of lactones. Additional samples of each type of milk were collected and stored at 4 °C to be used for sensory evaluation.

Sensory evaluation

Processed milk samples from d 26 of the experiment were first evaluated by a panel of 30 untrained assessors recruited among laboratory staff and students to perform a triangle test (Meilgaard et al. 2007). Panellists compared the flavour of milk from cows fed LC/NLO with the milk from cows fed HC/LO. This comparison was conducted after the storage of pasteurised milk for 7 d at 4 °C. Milks were served at 7 °C in 3-digit-coded 100-ml amber glass cup. A red light was used in polling booths to hide any information about the colour of milk.

While performing the triangle test, assessors were asked to characterise the nature of the difference between samples using a checklist of twelve attributes. The rating frequencies were as follow: grass (5), hazelnut (2), coconut (1), peach (1), butter (1), vanilla (5), sweet (4), cream (12), foreign flavour (5), oil (2), fat (4), and others (6). These frequencies were used to identify sensory attributes to be evaluated in a subsequent ranking test by a similar panel of 15 instructed assessors. The test was performed with the same sample presentations and environmental conditions. Panellists were asked to rank LC/NLO, LC/LO and HC/LO milk samples in a complete block design for the intensity of fresh lactic flavour (fresh milk, fresh cream, fresh butter), foreign flavour (herbaceous, cardboard, oil, rancidity), and global flavour (fruit, nuts, roasted, vanilla, caramel) on a scale of 1 to 3 (from less to more intense).

Statistical analysis

Data for milk lactone composition were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model included the fixed effects of level of concentrate, oil supplementation, and their interaction, and block as a random effect. Results are reported as least square means and standard errors of the means. Differences between treatments were declared at $P \leqslant 0{\cdot}05$, and tendencies at $0{\cdot}05 < P < 0{\cdot}10$. Statistical analyses for data regarding sensory evaluation were performed with Fizz (Sensory Analysis and Consumer Test Management Software, version 2.0a, Biosystemes, Couternon, France). For the triangle test for difference, data were analysed by counting the number of correct responses (correctly identified 'different' sample) and the number of total responses. These numbers were compared with critical values as described by Meilgaard et al. (2007) to determine significant differences. For the difference test, parameters were defined at $n = 30$, $\alpha = 0{\cdot}05$, $\beta = 0{\cdot}10$, and Pd = 30%. For the ranking test, sums of ranks were calculated for the three selected sensory attributes and were tested using a nonparametric 2-way analysis on ranks (Friedman test, significance level $\alpha = 0{\cdot}05$).

Results

The chemical composition of experimental diets was reported by Saliba et al. (2014). The HC diets contained higher proportions of cracked corn and rolled barley, which increased the proportion of starch by 3-fold, and decreased the proportion of ADF and NDF by about 40% compared with LC diets. Supplementing diets with LO doubled the concentration of total FA in the diet compared with NLO treatments. Moreover, LO diets contained about 3 times more oleic and α-linolenic acids as compared with NLO diets.

Regarding the concentrations of individual δ-lactones in milk, interactions were observed between LO and the level of grains in the diet (Table 1). The highest concentrations of δ-lactones were found with the LC diet not supplemented with LO. These concentrations were then decreased when cows received either a high level of concentrate or supplemental LO, but these effects were not additive. The only exception was observed with milk concentrations of δ-14:0, which decreased when diets were supplemented with LO but was not affected by level of concentrate.

An interaction of LO by concentrate was also noted on milk γ-12:0 for which a significant increase was observed when supplementing LO in HC diet, while no effect was apparent when LO was added in LC diet (Table 1). Moreover, feeding HC increased the level of γ-12:1 in milk as compared with LC, while LO had no effect on this parameter. Finally, γ-12:2 was not detected in any of the milk samples studied.

When expressed as relative proportions, δ- and γ-lactones represented 93·5 and 6·5% of total lactones, respectively, in milk from cows fed the LC diets. A shift toward a higher proportion of γ-lactones was observed when cows were fed with HC diets (88·4 and 11·6%; effect of concentrates: $P < 0{\cdot}01$). Adding LO to the diet also resulted in a shift towards a higher proportion of γ-lactones (NLO: 95·0 and 5·0% for δ- and γ-lactones, respectively, LO: 86·9 and 13·1%; $P < 0{\cdot}01$). Similar variations in the proportions of δ- and γ-lactones were observed in processed milk samples used for sensory evaluation (Fig. 1).

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Table 1. Milk lactone profile in cows fed low (LC) or high (HC) concentrate diets without supplemental oil (NLO), or supplemented with linseed oil (LO)

	Treatment†							
	NLO		LO			*P*-value‡		
Item	LC	HC	LC	HC	SEM	LO	C	LO × C
	Relative abundance§							
δ-hexalactone (δ-6:0)	16·60	5·93	6·81	4·54	2·12	<0·01	<0·01	0·01
δ-octalactone (δ-8:0)	35·94	11·45	12·80	7·43	4·58	<0·01	<0·01	0·02
δ-decalactone (δ-10:0)	254·57	92·03	77·23	81·28	18·84	<0·01	<0·01	<0·01
δ-dodecalactone (δ-12:0)	91·44	56·34	25·06	40·03	10·07	<0·01	0·28	0·02
δ-tetradecalactone (δ-14:0)	6·24	7·62	2·42	4·11	2·02	<0·05	0·37	0·93
γ-octalactone (γ-8:0)	3·76	3·33	3·06	3·46	0·73	0·44	0·97	0·25
γ-decalactone (γ-10:0)	4·74	4·31	5·14	6·35	1·08	0·06	0·51	0·18
γ-dodecalactone (γ-12:0)	3·19	2·92	3·07	14·81	1·87	<0·01	<0·01	<0·01
γ-dodecaenolactone (γ-12:1)	1·59	3·10	1·10	2·51	0·60	0·33	0·02	0·93
γ-dodecadienolactone (γ-12:2)	nd¶	nd	nd	nd	–	–	–	–
Sums								
Total δ-lactones	402·06	170·49	124·31	137·39	31·67	<0·01	<0·01	<0·01
Total γ-lactones	13·47	13·58	12·37	27·13	3·51	0·03	<0·01	0·01
Total lactones	413·09	182·71	136·68	164·52	33·85	<0·01	<0·01	<0·01

† $n = 6$ per treatment
‡ *P*-value for the effect of linseed oil (LO), concentrate (C), and the interaction of linseed oil by concentrate (LO × C)
§ The relative abundance of each lactone was obtained as the ratio of its peak to the area of γ-undecalactone peak used as internal standard
¶ Not detected

Results of the triangle test show that a significant number of assessors (15 correct observations; $n = 30$; $P = 0{\cdot}04$) perceived a difference between milk from cows fed LC/NLO when compared with milk from cows fed HC/LO. The sensory analysis was completed by a sensory ranking test ($n = 15$). The results indicate that the intensity of fresh lactic ($P = 0{\cdot}55$), foreign ($P = 0{\cdot}28$) and global ($P = 0{\cdot}42$) flavours were considered not different between LC/NLO, LC/LO and HC/LO milk samples.

Discussion

Freshly secreted milk is known to contain varying concentrations of lactones which are synthesised in the rumen or in animal tissues (Walker et al. 1968; Joblin & Hudson, 1997), and are identified as 'free lactones'. In several experiments, lactone potential (e.g. free lactones, plus lactones produced from hydroxy acids by heat treatment) was analysed using cold-finger molecular distillation, followed by silicic acid column chromatography, and then gas chromatography of the fractions (Stark et al. 1978). The SPME technique used in the current trial provides a quantification of lactones in headspace over milk samples while heating at 55 °C for 60 min. with agitation. This method estimated the free lactone content, but it cannot be excluded that the release of 4- and 5-hydroxy acids from milk triglycerides, and their respective conversion to γ- and δ-lactones, could have been quantitatively significant during this extraction procedure. In this regards, Stark et al. (1976) have shown that breakdown of lactone precursors of milk fat and their conversion to free lactones occur at temperature as low as 35 °C. It should be noted, however, that the complete generation of lactone potential requires much higher temperature (e.g. 180 °C; Stark et al. 1978).

The interaction between LO and level of concentrate on the level of δ-lactones is consistent with the data reported by Urbach & Stark (1978) showing numerically lower δ-lactone potential in cows fed crushed oats (source of starch), or sunflower seeds (sources of unsaturated fatty acids). However, Bendall (2001) reported no difference in the frequency of detection of δ-lactones (δ-6:0, δ-8:0, δ-10:0, δ-12:0 and δ-14:0) in milk from cows on pasture or fed total mixed rations containing grain supplements. On the contrary, Dimick & Harner (1968) observed higher potential for δ-10:0, δ-12:0 and δ-14:0 when cows received indoor diets as compared with pasture feeding.

According to Walker et al. (1968), even carbon-numbered saturated δ-lactones could be synthesised from acetate in the mammary gland. Diets containing high levels of concentrates are known to produce a lower proportion of acetate in the rumen (Griinari et al. 1998). Moreover, Loor et al. (2005) have shown that shifting from a high forage (65:35 F:C) to a high concentrate (35:65 F:C) diet decreased plasma concentration and the jugular/mammary venous difference of acetate. Less acetate is then available, in the mammary gland, which may explained the lower level of δ-lactones in milk of cows fed high concentrate diets in the current trial. The negative impact of vegetable oil on milk δ-lactone content could also be explained by the same mechanism, as dietary unsaturated fatty acids have also been shown to reduce ruminal production of acetate (Griinari et al. 1998) through the inhibition of fibre digestion.

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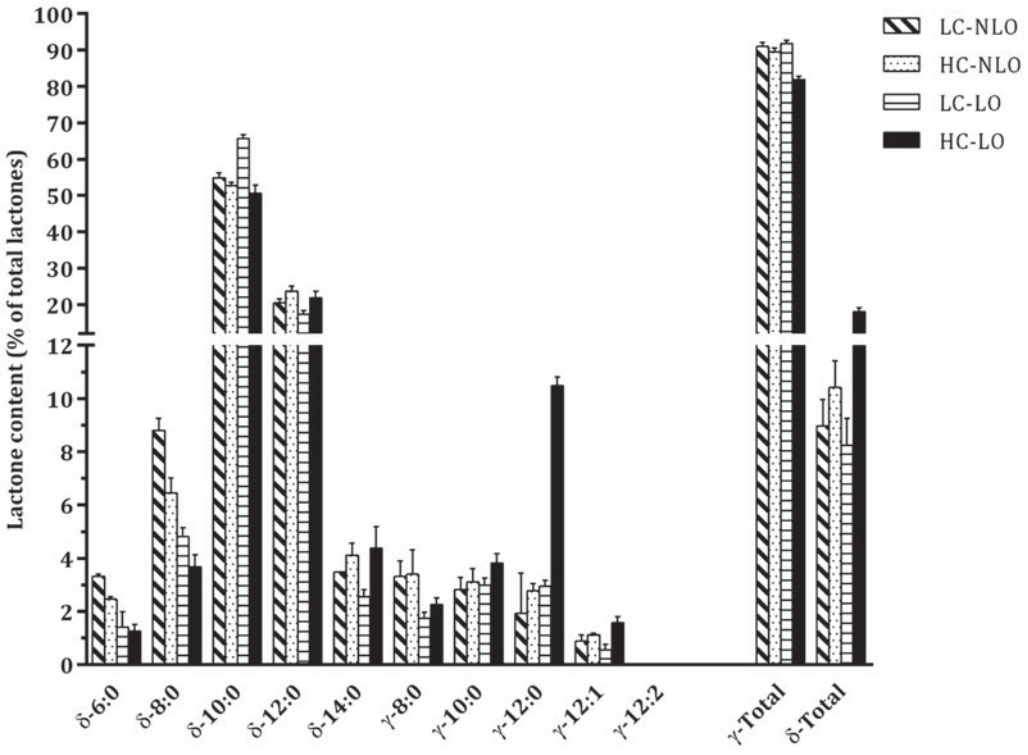

Fig. 1. Lactone profile of processed milk samples from cows fed low (LC) or high (HC) concentrate diets without supplemental oil (NLO), or supplemented with linseed oil (LO). Values represent the average of 3 replicates, with standard deviations shown as error bars.

The interaction observed in the current study on the levels of γ-12:0 for which a substantial increase has been obtained with the combined effects of LO and HC diets is also in line with the experiment conducted by Urbach & Stark (1978). In this latter study, the concentrations of γ-12:0 were increased by both supplemental concentrates (crushed oats) and unsaturated fatty acids (full-fat sunflower seeds).

The γ-12:0 is formed by the hydration and β-oxidation of oleic acid which was found in higher concentrations in diets supplemented with LO (Saliba et al. 2014). In particular, Jenkins et al. (2006) have shown that oleic acid is the ruminal precursor of 10-hydroxy stearic acid whose accumulation in ruminal contents is directly related to the supply of oleic acid. Moreover, Hudson et al. (1995, 2000) have shown that *Selenomonas ruminantium* and *Streptococcus bovis* are among major bacteria capable of hydrating unsaturated fatty acids in the rumen. Khafipour et al. (2009) have shown that the proportion of these latter bacteria increased during mild grain-induced subacute ruminal acidosis; which may explain the higher level of γ-12:0 observed with HC diet supplemented with LO in the current experiment. In contrast with this series of observations, Bendall (2001) reported no difference in the frequency of detection of γ-10:0, γ-12:0, and γ-12:1 in milk of cows on pasture or fed total mixed rations.

No γ-12:2 has been detected using the SPME technique in the current trial, even if dietary supply of α-linolenic acid was increased by feeding LO (Saliba et al. 2014). In contrast, 50% of the sniffers were able to identify the presence γ-12:2 in milk of cows fed total mixed rations in a study conducted by Bendall (2001) when milk volatile compounds were concentrated by the Solvent-Assisted Flavour Evaporation technique (Engel et al. 1999). As described by Kim (2005), γ-12:2 could be produced during the microbial lactonisation of α-linolenic acid, with 10-hydroxy-linoleic acid as intermediate. According to results from the current trial, this reaction does not appear to be significant in dairy cows. We therefore hypothesised that dietary α-linolenic initially undergoes partial biohydrogenation by ruminal microorganisms to produce *trans*-octadecenoic intermediates. This is supported by an increase in *trans*-octadecenoic acid content of milk fat when feeding LO, as reported in a companion paper (Saliba et al. 2014). *Trans*-octadecenoic acids produced in the rumen could be hydrated to yield 10-hydroxy-stearic acid, as it has been shown by McKain et al. (2010). Subsequently, 10-hydroxy-stearic acid is converted to γ-12:0, which was found at higher concentration in milk of cows fed LO in HC diet.

When expressed as relative proportions, δ- and γ-lactones represented 96·7 and 3·3% of total milk lactones, respectively, when cows received LC diet without supplemental oil. The relative proportion of δ- and γ-lactones observed with this treatment is similar to the data reported by Hettinga (2005) when summarising the literature with saturated δ- and γ-lactones constituting 94 and 6% of total milk lactones, respectively. The shift toward higher proportions

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of γ-lactones (16·4% of total lactones) at the expense of δ-lactones (83·6% of total lactones) observed when LO was added in HC diet is in line with the potentially reduced acetate production (substrate for δ-lactone synthesis) and higher hydration of unsaturated fatty acids (initial step in the production of γ-lactones). Processed milk samples from day 26 of the experimental period, which were used for sensory evaluation, showed similar variations with a change for more γ-lactones and less δ-lactones when feeding LO in HC diet (Fig. 1).

Results of the ranking test show that the three samples were classified an equal number of times in the first, second or third position for each sensory attribute. Consequently, there is no difference between treatments in the intensity of fresh lactic flavour with sensory descriptors 'cream' and 'butter', or in the intensity of global flavour with sensory descriptors 'fruit' and 'roasted'. These descriptive sensory characteristics were all previously attributed to lactones (Moio et al. 1993; Bendall, 2001). However, the variations in lactone profile between treatments were not related to any perceived differences in the sensory attributes evaluated by the panel. One may speculate that untrained assessors did not have the expertise to detect subtle changes in the aroma descriptors related to lactones, even if they were able to perceive a difference between samples during the triangle test. It is also possible that the differences between samples detected during the triangle test were related to sensory characteristics different from those proposed in the ranking test. Indeed, sensory properties of fluid milk are affected by the concentrations of highly diversified classes of compounds, some of them presenting lower flavour threshold than lactones (Belitz et al. 2009). It is possible that dietary treatments might have impacted the concentrations of other volatile compounds, the latter being associated with different sensory attributes.

In conclusion, the SPME technique used in the current trial allows for the determination of milk lactone profile which was affected by dietary supply of LO or the level of grains in the ration. An interaction of LO by concentrate was observed for the levels of most δ-lactones for which the highest concentrations were found with the LC/NLO diet. These concentrations were then decreased when cows received either a HC diets or supplemental LO, while these effects were not additive. An interaction of LO by concentrate was also apparent on milk γ-12:0 for which no effect was apparent when LO was added in LC diet, while a higher concentration was observed when supplementing LO in HC diet. Finally, LO had no effect on γ-12:1, while feeding HC diets increased the level of this γ-lactone in milk compared with LC diets. Assessors were able to perceive a difference in a triangle test between milk from these two treatments, but the attributes responsible for this sensory perception have not been identified in the current trial.

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