Correlations of anticarcinogenic conjugated linoleic acid with other C18 fatty acids in German bovine milk fat

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Abstract

Based on different studies certain conjugated linoleic acids (CLA), in particular the isomer *cis* Δ9,*trans* Δ11-C18:2 (c9t11), are reported to probably have antioxidative, antiatherogenic and especially anticarcinogenic properties. On the other hand, *trans*-C18:1 as well as several *trans*-C18:2 isomers partly are related to negative physiological effects, as e.g. premature atherosclerosis. Knowledge of the variation of c9t11 in milk fat associated with feeding as well as of its formation in the rumen of cows is important to exert influence on the CLA content in milk fat. In different feeding trials it could be demonstrated that the CLA content in milk fat can be increased considerably by means of feeding. But in all cases this also increased the contents of undesirable *trans*-C18:1 and *trans*-C18:2 fatty acids. The high correlation of C18:3 with CLA (r = 0.71) and the smaller inverse correlation of C18:2 with CLA (r = -0.40) suggest the possibility of CLA formation from linoleic acid.

1 Introduction

Due to their probably antioxidative, anticarcinogenic and antiatherogenic properties conjugated linoleic acids (CLA) are of importance [1-6]. Bovine milk fats exhibit higher CLA contents than vegetable fats by far [7]. Having a proportion of at least 90 %, *cis* Δ9,*trans* Δ11-C18:2 (c9t11) represents the predominant CLA isomer in bovine milk fat [7]. Knowledge of the variation of this isomer associated with feeding as well as of its formation in the rumen of cows possibly could allow to exert influence on the CLA content in milk fat. On the other hand, with respect to a possible increase of the CLA contents in milk fat, correlations with further *trans*-C18:2 but also *trans*-C18:1 isomers are of significance, as these, in contrast to CLA, frequently are reported to have negative physiological properties.

In recent years publications on dietary or epidemiological studies caused a Worldwide discussion about potential risks associated with an elevated consumption of *trans* fatty acids (excluding CLA). In this context debates on premature atherosclerosis and cardiovascular diseases were prominent which particularly is supposed to concern *trans*-C18:1 fatty acids [see reviews 8-12]. Moreover, *trans*-C18:2 (excluding CLA) and in particular the isomer t9t12 are regarded as competitive inhibitors for the conversion of linoleic acid to arachidonic acid, partially blocking formation of C20:5 n-3 [13,14]. Thus, the present study is to focus on the relations and correlations of CLA contents with the contents of other *trans* fatty acids as well as linoleic and linolenic acid associated with feeding.

2 Materials and methods

Ca. 2000 bovine milk fats were analysed by gas chromatography (GC) to establish the influence of typical feeding conditions on the content of c9t11. From 1756 milk fats the contents of CLA c9t11, *trans*-C18:1 isomers (sum of t4, t5 to t16) and the *trans*-C18:2 isomers (excluding CLA; sum of t9t12, c9t13, t8c12, t8c13, c9t12, t9c12, t11c15) were calculated by triglyceride formulae derived in a

way described earlier [15]. With this method - based on triglyceride analysis (EU reference method [16]) and calibration with the butterfat reference material CRM 519 prepared in the EU funded BCR project MAT1-CT92007 [17], the different *trans* fatty acids could be determined with high precision.

Furthermore, from 100 of the 2000 milk fat samples, which exhibited a representative range of variation in fat composition, total fatty acids including CLA c9t11 and all *trans*-C18:1 isomers were determined by Ag-TLC/GC of FAME without the use of formulae.

In a further EU funded project (VO-EWG 1001/90-11.3) another trial with a two periods cross-over design was performed to investigate, among other things, the influence of technical treatment of the fed rape-seed (00-sort) on the contents of CLA c9t11 and *trans*-C18:1 in milk fat. Other parameters from this study concerning fat composition or milk fat properties have been published earlier by Frede et al. [18]. In all variants of feeding equal amounts of fat-free wholemeal from extracted rape and extracted soya (3 kg dry matter) as well as variable amounts of wheat were given. The wheat was replaced with rape fat and different manufacturing types being equivalent in net energy. Concentrate variants: NC: negative control without rape fat; 550 g rape oil; 1300 g wholemeal from rape-seed, corresponding to 550 g rape oil; 1450 g whole rape-seed pellets, corresponding to 620 g rape oil. As basic diet all animals obtained maize silage (4.5 kg per day) and whole plant silage from winter wheat ad libitum. Each of these feeding variants was performed with 6 cows and repeated later with another herd of 6 cows. Thus, the investigations were based on 12 cows.

Additionally, milk fat samples were collected weekly during 1 year from 4 great milk collection areas in Germany. These samples were analysed for *trans*-C18:1 fatty acids, CLA (c9t11), linoleic and linolenic acid.

The total fatty acid composition covering ca. 70 fatty acids in the range of C4 to C24 was determined by gas chromatographic analysis of the methyl esters on a 25-m capillary column (CP-Wax 58 CB, equivalent to FFAP, i.d. = 0.25 mm, df = 0.20 μm). On the other hand, after trans-esterification into methyl esters and argentation thin-layer chromatography (Ag-TLC) *trans*-C18:1 as well as C18:2 isomers (excluding CLA) were analysed gas chromatographically on a highly polar 100-m capillary column (CP-Sil 88, i.d. = 0.25 mm, df. = 0.20 μm) at 175°C (*trans*-C18:1) and 150°C (C18:2), respectively. Further details on the calibration and quantitation as well as the Ag-TLC fractionation have been published elsewhere [19,20]. For the GC analysis of CLA isomers CP-Sil 88 capillary columns of 100 and 50 m length were used. Temperature programming was done from 125°C to 240°C with 2°C/min and from 50°C to 240°C with 5°C/min, respectively.

For the identification of *cis*- and *trans*-C18:1 isomers as well as the C18:2 isomers, FAME standards c6, c7, c9, c11, c12, c13, c15, t6, t7, t9, t11, t12, t13, t15 and t9t12, c9t12, t9c12, c9c12 obtained from Sigma (St. Louis, Missouri, USA) were used. All data are given in g/100 g free fatty acids.

Gas chromatographic analyses of triglycerides were performed combining triglycerides with identical acyl-C number and, thus, quantitating C24 to C54. Analytical conditions correspond to those used in the EU-project MAT1-CT92007 [17] and described in another publication [19].

3 Results and discussion

Fig.1 shows chromatograms of two bovine milk fats with the corresponding Ag-TLC fractions as well as of an Ag-TLC fraction from a typical margarine. These partial chromatograms exhibit the range of linoleic acid isomers (excluding CLA) that in case of the unfractionated milk fats interferes with some

cis-/*trans*-C18:1 isomers (c11, c12, c13, c14, c15 and t16) as well as C19. Identification of the peaks was achieved by several standards and particularly on the basis of the findings of Ratnayake and Pelletier [21] as well as of Ulberth and Henninger [22] as described earlier [20].

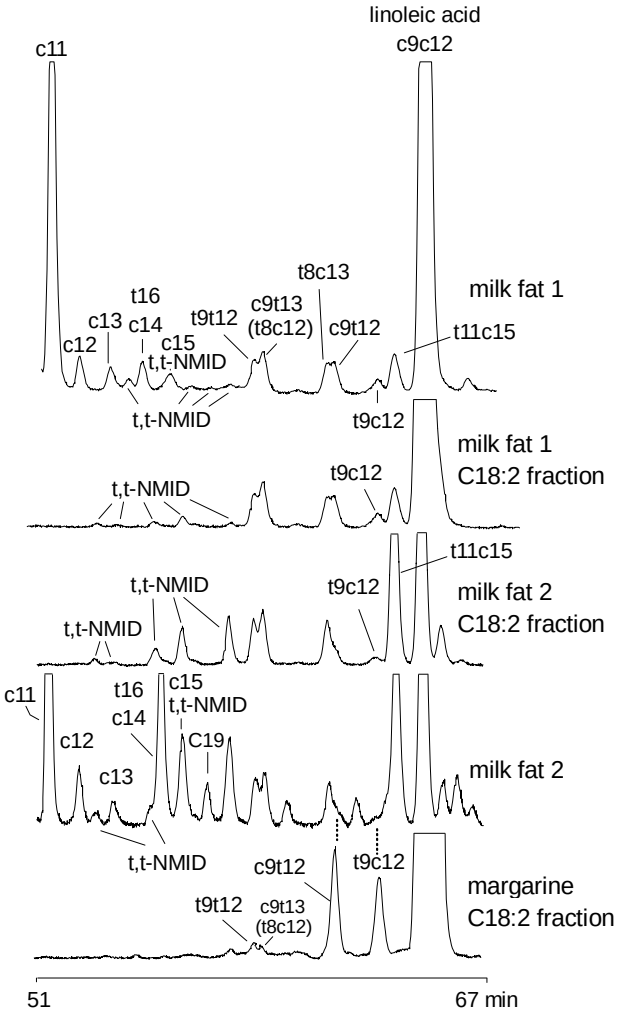

Fig.1: Partial gas chromatograms of two different milk fats and the corresponding Ag-TLC fractions containing C18:2 FAME in comparison to an Ag-TLC fraction from margarine obtained on a CP-Sil 88 capillary column (100 m x 0.25 mm). t = *trans*-bond; c = *cis*-bond; t, t-NMID = unknown non methylene interrupted *trans, trans* dienes; t8c13: tentative identification.

It follows from Fig.1 that the main *trans*-C18:2 isomers in bovine milk fats are t9t12, c9t13+t8c12, t8c13+c9t12 and t11c15. However, the identification of t8c12 and t8c13 has to be considered as tentative. Compared with that margarine contains the main isomers t9t12+c9t13+t8c12, c9t12 and t9c12. Thus, there is only little agreement between milk fat and margarine. In contrast to margarine particularly t9c12 occurs only in trace amounts in milk fat, whereas the main isomer t11c15 of milk fat is not present in margarine. However, the comparison of milk fat 1 and 2 shows that t11c15 contents exhibit a wide variation.

In contrast to these *trans*-C18:2 isomers the group of CLA elutes distinctly later (Fig. 2). Among the conjugated linoleic acids in particular the c9t11 isomer is considered important in terms of anticarcinogenic activity [23]. At the same time c9t11 is the CLA isomer with the highest content in bovine milk fat. Seven further CLA isomers as e.g. t9c11, c10t12 or t10c12 as well as Δ8Δ10 and Δ11Δ13 with all possible *cis* and *trans* configurations were detected in milk fat from French cheese products [24]. In human adipose tissue besides c9t11 the CLA isomers t9t11, c9c11 and t9c11 were found [25].

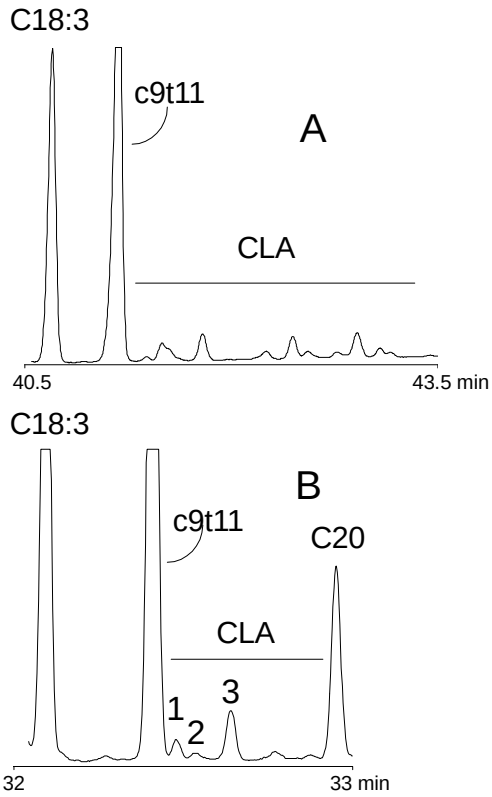

Fig.2: Gas chromatographic patterns of conjugated C18:2 isomers (CLA) eluted on a CP-Sil 88 capillary columns of 100 m length (A: oven programmed from 125°C to 240°C with 2°C/min) and 50 m length (B: oven programmed from 50°C to 240°C with 5°C/min) typically for bovine milk fat

The range of CLA in bovine milk fat obtained after gas chromatographic analysis on a 100-m column as well as on a 50-m column using different temperature programs is presented in Fig.2. With the shorter column C20 eluted within the rear end of CLA, whereas the analytical conditions of the longer column caused the elution of C20 before C18:3 avoiding the interference with CLA isomers. Despite the identical cyanopropyl polysiloxane phase the different column length and temperature programming obviously results in a variable resolution of CLA isomers. The distribution of Fig.2 B particularly corresponds to the pattern of CLA peaks found by Lavillonnière et al. [24] for milk fat from cheese who identified the peaks 1, 2 and 3 as combinations of the CLA t9,c11+c10t12, t10c12 and c8c10+c9c11+c10c12+c11c13. Moreover, small amounts of c8t10 are reported to be included in the c9t11 peak. Compared with that, the longer column (Fig. 2 A) possibly exhibits a further splitting of these numerous peaks. However, also according to Adlof and Lamm [26] peak 1 in Fig.2 B is t9c11. Further, Fritsche et al. [25] identified the corresponding peak as t9c11 in fat from human tissue as we did in our studies on fat from human milk. Apparently, the gas chromatographic conditions used by us at least separate c9t11 from t9c11. However, an overlap with a rather small amount of c8t10 can not be excluded, but possibly this isomer only results from the biohydrogenation in cheese [24].

Information on gas chromatographic conditions, Ag-TLC fractionation and identification of *trans*-C18:1 positional isomers in bovine milk fat have been given by us in several publications [19,27-29] and are not to be explained in detail here. In particular, we pointed out that the literature frequently provides too low *trans*-C18:1 contents, mainly because *trans* isomers partly were masked by *cis* isomers [28,30].

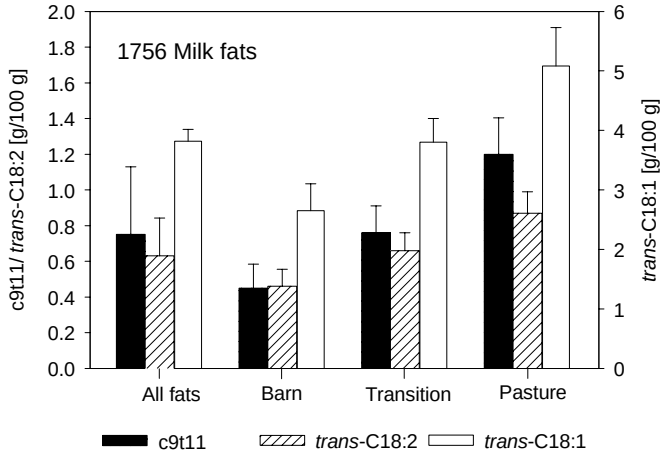

Fig.3: Contents and standard deviation of CLA, *trans*-C18:1 and *trans*-C18:2 (excluding CLA) at typical *barn* feeding (n = 927), during the *transition* period (n = 236), at *pasture* feeding (n = 593) and on average over *all fats* (n = 1756)

Our analyses of 1756 milk fats resulted in an average c9t11 content of 0.75% (range: 0.10-1.89 %). The mean content increased from barn feeding in winter (n=927) to the transition period in spring and late autumn (n = 236) and further to pasture in summer (n = 593) from 0.45% over 0.76% up to 1.20% (Fig.3). The average contents of *trans*-C18:2 (excluding CLA; sum of t9t12, c9t13, t8c12, t8c13, c9t12, t9c12, t11c15) and *trans*-C18:1 (sum of t4, t5 to t16) amounted to 0.63 % and 3.62 %, respectively. Further, the 3 feeding periods exhibited *trans*-C18:2 contents of 0.46, 0.66 and 0.87 % and *trans*-C18:1 contents of 2.65, 3.80 and 5.08 %.

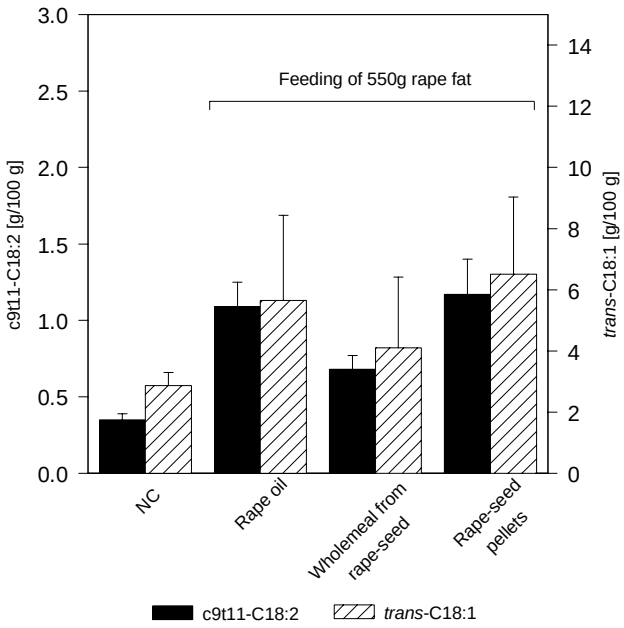

Fig.4: Influence of the technical treatment of fed rape seed or rape oil on CLA (c9t11) and *trans*-C18:1 contents in milk fat.

Further studies with defined feedings of cows exhibited particularly high CLA contents (up to 3 times higher compared to the basic diet NC) after feeding of 550 g rape oil or rape-seed pellets (Fig.4), whereas less high but still elevated CLA contents resulted from feeding of wholemeal from rape-seed. Regarding *trans*-C18:1 (sum of t4, t5 to t16) the four feeding variants shown in Fig.4 led to contents of 2.87, 5.65, 4.10 and 6.51 %.

Our previous studies [29] demonstrated that the changes in CLA contents associated with feeding correspond to changes in the *trans*-C18:1 content affecting almost exclusively vaccenic acid (t11), however. As can be seen from Fig.5 that is based upon 100 milk fats selected for a great variation in composition a strong linear correlation in particular is found between t11 and c9t11 with an extremely high correlation coefficient of r = 0.98 [c9t11 = 0.0476565 + 0.437086 • t11]. A reason for this finding may be that c9t11 is a precursor of in-vivo synthesis of vaccenic acid. In milk fat from different cheeses Lavillonnière et al. [24] found a correlation coefficient between CLA (c9t11) and vaccenic acid of r = 0.96. Regarding this, also Jiang et a. [31] established a correlation with r = 0.78 from feeding trials with milk cows.

Another high correlation with r = 0.95 follows from Fig.5 for CLA and total *trans*-C18:1 (sum of all positional isomers).

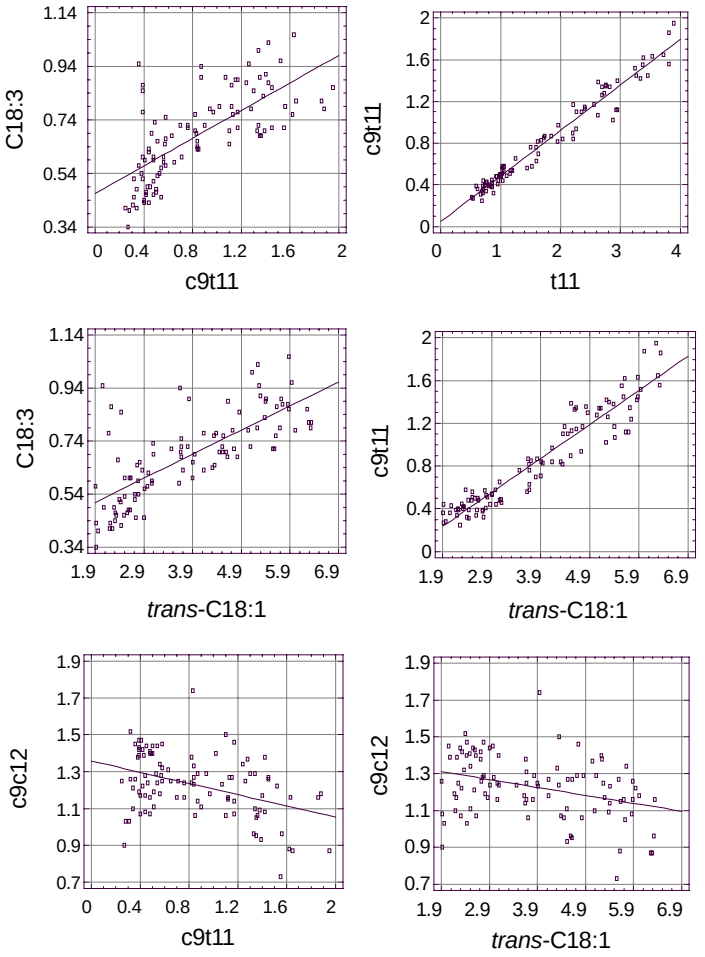

Fig.5: Relations between the contents of conjugated linoleic acid (c9t11), linolenic acid (c9c12c15), *trans*-C18:1 (all positional isomers), vaccenic acid (t11) and linoleic acid (c9c12)

The statistical evaluation of the correlations shown in Fig.5 leads to the following coefficients r:

C18:3 - CLA	:	r = 0.71
C18:3 - *trans*-C18:1	:	r = 0.74
CLA - t11	:	r = 0.98
CLA - *trans*-C18:1	:	r = 0.95
c9c12 - CLA	:	r = - 0.40
c9c12 - *trans*-C18:1	:	r = - 0.34

Fig.6 shows the results obtained weekly for the contents of C18:3 (c9c12c15), *trans*-C18:1, C18:2 (c9c12) and the CLA c9t11 in milk fat from a large German milk collection area during one year. The qualitative course of data points is nearly identical for C18:3, *trans*-C18:1 and CLA. So, linolenic acid contents are higher in summer compared to winter by 50 %, whereas *trans*-C18:1 isomers and CLA contents exhibit a corresponding seasonal rise by two and three times, repectively. Thus, here as well the desirable increase in CLA contents achievable by feeding is associated with a distinct but undesirable increase in *trans*-C18:1 contents, as already follows from Figs.3 and 4.

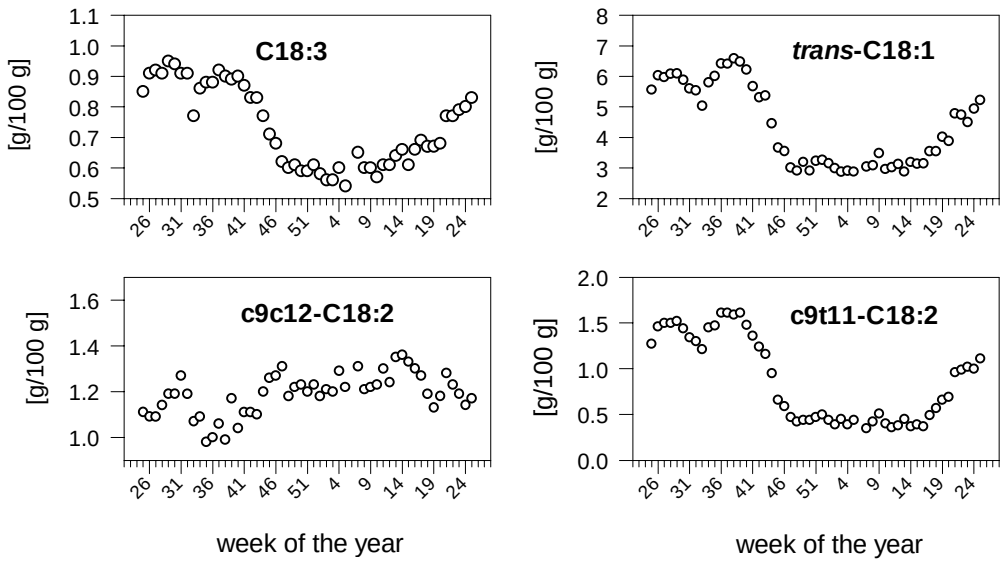

Fig.6: Weekly variation in the contents of C18:3, *trans*-C18:1, C18:2 and CLA c9t11 in milk fat from a large collection area

Further, Fig.6 shows a very good correlation between C18:3 (c9c12c15) and the CLA c9t11 in contrast to C18:2 (c9c12). In Fig.6 there seems to be a small inverse correlation between C18:2 and c9t11 also expressed by the correlation coefficient. On the other hand, in the studies of Lavillonnière et al. [24] the CLA contents in milk fat from different kinds of cheese exhibited a positive correlation (r = 0.57) with C18:2 (c9c12), whereas Lin et al. [32] found an inverse relation again.

In several publications the first step in the biohydrogenation pathway of linoleic acid is reported to be the formation of c9t11 effected by the rumen bacterium Butyrivibrio fibrisolvens. In a second step hydrogenation of the CLA leads to vaccenic acid (t11) [33-36]. When C18:3 was used in biohydrogenation trials it was at first isomerized to c9t11c15 according to Kepler and Tove [37] or to c9c11c15 or c9c13c15 according to Wilde and Dawson [38]. The latter authors found that this trienoic acid was further hydrogenated to a non-conjugated octadecadienoic acid containing at least one *trans* double bond. Regarding the conjugated trienoic acid c9t11c15 Kepler and Tove [37] described a further hydrogenation step to a non-conjugated *cis*,*trans*-dienoic acid but no monoenoic acid. Shorland et al. [39] studied the biohydrogenation of linolenic acid by sheep rumen contents. They found that a Δ11,15-C18:2 acid was the major resulting dienoic acid. This possibly could explain the comparatively high average t11c15-C18:2 content (see Fig.1) of 0.3 % (n = 100) found in our studies corresponding to ca. 27 % of the mean linoleic acid content. Regarding the C18:3 contents being relatively low in summer milk fat as compared to the high amount ingested from the pasture, the question arises how this fatty acid is metabolized in the rumen. In grass samples from different locations Prodöhl [40] found 38.7-68.5 % C18:3 (mean: 50.8 %), 13.4-32.3 % C18:2 (mean: 25.6 %) and 1.4-4.9 % C18:1 (mean: 3.6 %), whereas summer milk fat only exhibits ca. 1% C18:3 (see Fig. 6). Lavillonnière et al. [24] did not rule out the possibility of CLA formation as intermediates at least in the biohydrogenation of C18:3 in cheese, as was also described by Viviani [41] in studies concerning the metabolism of long chain fatty acids in the rumen. Lavillonnière et al. [24] found a positive relation between the α-linolenic acid content and the CLA content in cheeses (r = 0.74).

Because of the high correlation of C18:3 with CLA and the minor correlation of C18:2 with CLA found in our studies and because of the elevated CLA and *trans*-C18:1 contents in milk fat resulting during the extremely high ingestion of C18:3 from pasture, we also do not exclude that CLA and *trans*-C18:1 isomers in bovine milk fat derive to a considerable extent from C18:3. It should be emphasized that feeding trials performed by Wolff et al. [42] exhibited a high correlation of C18:3 with CLA in bovine milk fats as well but in accordance with our studies also no corresponding correlation of C18:2 (c9c12) with CLA (c9t11).

4 Conclusions

The content of the conjugated linoleic acid c9,t11-C18:2 in milk fat can be increased considerably by means of feeding. As shown by all the different feeding trials, this also strongly increases the contents of undesirable *trans*-C18:1 and *trans*-C18:2 fatty acids. A high correlation of C18:3 in pasture and milk fat with CLA as well as a minor correlation of C18:2 with CLA suggest the possibility of a CLA formation from linolenic acid.

Acknowledgement

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References

1. Y. L. Ha, N. K. Grimm and M. W. Pariza, 1987, Carcinogenesis, 8, 1881-1887
2. Y. L. Ha, J. Storkson and M. W. Pariza, 1990, Cancer Res., 50, 1097-1101
3. C. Ip, S. F. Chin, J. A. Scimeca and M. W. Pariza, 1991, Cancer Res., 51, 6118-6124

4. R. J. Nicolosi, K. V. Courtemanche, L. Laitinen, J. A. Scimeca and P. J. Huth, 1993, Circulation 88, Suppl. 2458, 457
5. K. N. Lee, D. Kritchevsky and M. W. Pariza, 1994, Atherosclerosis 108, 19-25
6. C. Ip, J. A. Scimeca and H. J. Thompson, 1994, Cancer Suppl. 74, 1050-1054
7. S. F. Chin, W. Liu, J. M. Storkson, Y. L. Ha, and M. W. Pariza, 1992, J. Food Composition and Analysis, 5, 185-197
8. R. Wood, 1992, ″Biological Effects of Geometrical and Positional Isomers of Monounsaturated Fatty Acids in Humans″, in: Fatty Acids in Foods and Their Health Implication, ed. by C. K. Chow, Marcel Dekker, Inc., New York, 663-688
9. Report of the Expert Panel on *Trans* Fatty Acids and Coronary Heart Disease, *Trans* Fatty Acids and Coronary Heart Disease Risk, 1995, ed. by J. H. Dickson, Am. J. Clin Nutr., 62, 655S-707S
10. M. B. Mensink and P. L. Zock, 1995, Annu. Rev. Nutr., 15, 473-493
11. S. Stender, J. Dyerberg, G. Hølmer, L. Ovesen, and B. Sandström, 1995, ″The Influence of *trans* Fatty Acids on Health: A Report from the Danish Nutrition Council″, Clin. Sci., 88, 375-392
12. D. Precht and J. Molkentin, 1995, Nahrung - Food, 39, 343-374
13. O. S. Privett, F. Phillips, T. Shimasaki, T. Nozawa and E.C. Nickell, 1977, J. Clin. Nutr., 30, 1009-1017
14. E. A. Emken, 1979, ″Utilization and effects of isomeric fatty acids in humans″. In: E. A. Emken and H. J. Dutton, eds. ″Geometrical and positional fatty acid isomers″. Champaign, IL: American Oil Chemists Society, 99-129
15. D. Precht and J. Molkentin, 1997, Nahrung - Food, 41, 330-335
16. Commission Regulation (EC) No 454/95, annex III. Official Journal No L 46 (1995) p. 1 ff.
17. D. Precht and J. Molkentin, 1997, ″The certification of the triglyceride contents of an anhydrous butter fat reference material with additional value for free cholesterol, CRM 519″. European Commission, Community Bureau of Reference, Report EUR 17613 EN, p.1-130
18. E. Frede, D. Precht, K. Pabst and D. Philipczyk, 1992, Milchwissenschaft, 47, 505-510
19. D. Precht and J. Molkentin, 1996, Intern. Dairy J., 6, 791-809
20. D. Precht and J. Molkentin, 1997, Fett / Lipid, 99, 319-326
21. W. M. N. Ratnayake and G. Pelletier, 1992, J. Am. Chem. Soc., 69, 95-105.
22. F. Ulberth and M. Henninger, 1994, J. Dairy Res., 61, 517-527
23. Y. L. Ha, J. Storkson, and M. W. Pariza, 1990, Cancer Res., 50, 1097-1101
24. F. Lavillonnière, J. C. Martin, P. Bougnoux, and J.-L. Sébédio, 1998, J. Am. Oil Chem. Soc., 75, 343-352
25. J. Fritsche, M. M. Mossoba, M. P. Yurawecz, J. A. G. Roach, N. Sehat, Y. Ku and H. Steinhart, 1997, Z. Lebensm. Unters. Forsch., 205, 415-419
26. R. Adlof and T. Lamm, 1998, J. Chromatography A, 799, 329-332
27. J. Molkentin and D. Precht, 1995, Chromatographia, 41, 267-272
28. D. Precht and J. Molkentin, 1997, Kieler Milchwirtsch. Forschungsber., 49, 17-34
29. D. Precht and J. Molkentin, 1997, Milchwissenschaft, 52, 564-568
30. R. L. Wolff, D. Precht and J. Molkentin, 1998, ″*Trans*-18:1 Acid Content and Profile in Human Milk Lipids. Critical Survey of Data in Connection with Analytical Methods″. J. Am. Oil Chem. Soc., 75, (in press)
31. J. Jiang, I. Bjoerck, R. Fondén, and M. Emanuelson, 1996, J. Dairy Sci., 79, 438-445
32. H. Lin, T. D. Boylston, M. J. Chang, L. O. Luedecke and T. D. Shultz, 1995, J. Dairy Sci., 78, 2358-2365
33. C. R. Kepler, K. P. Hirons, J. J. McNneill and S. B. Tove, 1966, J. Biol. Chem., 241, 1350-1354
34. I. S. Rosenfeld and S. B. Tove, 1971, J. Biol. Chem., 246, 5025-5030
35. W. J. Hunter, F. C. Baker, I. S. Rosenfeld, J. B. Keyser and S. B. Tove, 1976, J. Biol. Chem., 251, 2241-2247
36. P. E. Hughes, W. J. Hunter and S. B. Tove, 1982, J. Biol. Chem., 257, 3643-3649
37. C. R. Kepler and S. B. Tove, 1967, J. Biol. Chem., 242, 5686-5692
38. P. F. Wilde and R. M. C. Dawson, 1966, Biochem. J., 98, 469-475
39. F. B. Shorland, R. O. Weenink, A. T. Johns and I. R. C. McDonald, 1957, Biochem. J., 67, 328-333
40. L. Prodöhl, 1994, ″Untersuchungen zur Verbesserung der Fettqualität in der Milcherzeugung″, Thesis, Christian-Albrechts-Universität, Kiel, Germany
41. R. Viviani, 1970, Adv. Lipid Res., 8, 267-346
42. R. L. Wolff, C. C. Bayard and R. J. Fabien, 1995, J. Am. Oil Chem Soc., 72, 1471-1483