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Fatty acid composition of sheep milk from a backcross Sarda × Lacaune resource population: Preliminary QTL detection for CLA content

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SUMMARY – Fatty acid composition of milk fat of 847 Sarda × Lacaune backcross ewes was determined. Main attention was given to fatty acids that could play a positive role on human health such as butyric acid, oleic acid, C18 to C22 polyunsaturated acids and conjugated linoleic acid (CLA). Preliminary phenotypic analysis was carried out for QTL detection on the whole genome and, mainly focusing on CLA, to study the level of expression of some enzymes involved in the mammary bio-synthesis of fatty acids. A preliminary QTL analysis on the OAR 22, where the SCD gene encoding *delta* 9-desaturase is located, allowed a putative QTL to be detected for the ratio between CLA and vaccenic acid segregating in families 1 and 4. Therefore an SCD candidate gene approach will be implemented by quantification of the mRNA of the SCD gene in the milk of around one hundred ewes chosen in the high and low tails for the CLA/vaccenic acid ratio of families 1 and 4. Simultaneously a QTL detection to the whole genome is being undertaken.

Key words: Fatty acids, CLA, sheep milk, QTL, candidate gene.

RESUME – *"Composition en acides gras du lait de brebis backcross Sarde × Lacaune comme population ressource : Détection préliminaire des QTL liés à la teneur en ACL". La composition en acides gras du lait de 847 brebis backcross Sarde × Lacaune a été déterminée. Une attention particulière a été portée aux acides gras susceptibles de jouer un rôle positif en santé humaine, tels que l'acide butyrique, l'acide oléique, les acides polyinsaturés C18 à C22, et l'acide conjugué linoléique (ACL). Une analyse phénotypique préliminaire a été réalisée dans le but de conduire la détection de QTL sur l'ensemble du génome et, focalisant principalement sur le CLA, d'étudier le niveau d'expression de certaines enzymes impliquées dans la bio-synthèse mammaire d'acides gras du lait. Une analyse préliminaire de détection de QTL sur le chromosome 22 ovin, où est localisé le gène SDC codant pour la* delta *9-désaturase, a permis de détecter un QTL putatif pour le ratio CLA/acide vaccénique dans les familles 1 et 4. Une approche de détection du gène candidat SCD sera donc mise en œuvre par quantification de l'expression de son ARN messager dans le lait de quelque 100 brebis choisies dans les queues de distribution pour le ratio CLA/acide vaccénique dans les familles 1 et 4. Simultanément la détection de QTL sur l'ensemble du génome sera entreprise.*

Mots-clés : *Acides gras, ACL, lait de brebis, QTL, gène candidat.*

Introduction

The dairy sheep industry in Western European countries is mainly based on the production of high quality PDO cheeses. Thus, dairy sheep selection has been oriented towards milk yield and milk composition (protein and fat content) to maintain competitiveness of this production. Due to the evolution of the EU agricultural policy and consumers demand, more attention has been now given to traits related to food safety, health and nutritional value. In this framework, the milk fat content and composition in fatty acids (FA) due to its impact on human health, plays an important role.

On one hand, FA composition influences milk fat quality contributing to its physical (crystallisation and fractionation of the fat, hardness and melting point of the butter), and sensorial (free short chain FA, oxidation products) properties (Chilliard *et al.*, 2000). Moreover, nutritional aspects have been assuming an increasing importance. In particular, short and medium chain saturated, branched, mono and polyunsaturated and *cis* and *trans* conjugated FA are potentially involved, as positive or negative predisposing factors for human health (Kinsella *et al.*, 1990; Willet *et al.*, 1993; Wolff, 1995; Molkentin, 1999; Parodi, 1999; Sebedio *et al.*, 1999; Williams, 2000).

Recently, research focused on a group of FA, present in milk and dairy products, with 18 carbon atoms and 2 conjugated double bonds, named conjugated linoleic acid (CLA). In *in vivo* experimental models, CLA showed positive effects about the protection against cancer and atherosclerosis, stimulation of immune functions, reduction of body fat and normalization of impaired glucose tolerance in diabetes (Banni and Martin, 1998; Pariza and Cook, 1998; Pariza, 1999). Consequently, there is an increasing interest to achieve an enrichment of CLA in milk to obtain dairy products with improved nutritional value.

Among CLA isomers, the *cis* 9 *trans* 11 form (rumenic acid) is the most abundant in ruminant meat and milk. It originates in the ruminal biohydrogenation of linoleic acid (C18:2). A portion of the so formed CLA escapes further biohydrogenation and is absorbed in the digestive tract (Griinari and Bauman, 1999). The extent of that process is presumed minimal, whereas the *trans* 11-octadecenoic acid (*trans* 11 C18:1; vaccenic acid), the intermediate product of the CLA biohydrogenation, accumulates (Harfoot and Hazelwood, 1988). In the tissues, and principally in the mammary gland, the vaccenic acid is desaturated by *delta* 9-desaturase to produce the rumenic acid (CLA). The *delta* 9-desaturase can use different FA as substrate, so it can influence the amount of several unsaturated fatty acids (UFA). Previous research showed that dietary factors such as the nature of forages, including pasture, and the supplementation of dairy rations with protected or unprotected vegetable or fish oils can substantially increase CLA content in milk of ruminants acting either on substrates for formation of rumenic and vaccenic acids in the rumen or on the microbial activity associated to ruminal biohydrogenation (Chilliard *et al*., 2001). In particular, recent studies on Sarda sheep showed that a feeding regimen based on grazing ryegrass swards increases CLA content in milk fat as compared to a legume *Hedysarum coronarium* (Cabiddu *et al*., 2001; Piredda *et al*., 2001). On the whole, there is evidence that milk rumenic acid essentially comes from mammary synthesis by action of *delta* 9-desaturase on the vaccenic acid provided by the rumen (Griinari and Bauman, 1999). Thus, differences in ruminant species (Jarheis *et al*., 1999), cattle breeds (Lowless *et al*., 1998) and between individual cows (Kelly *et al*., 1998) even if they were fed with the same diet can be probably explained by different activities of *delta* 9-desaturase.

This paper presents the fatty acid composition of the milk fat of a Sarda × Lacaune backcross resource population created in the framework of an EU granted research project (Barillet *et al*., this volume, p. 13) aimed to detect QTL affecting traits related to the reduction of production costs (milkability, functional traits), health (resistance to mastitis or parasitic diseases) and food quality (milk content in fatty acids related to human health). As far as the CLA is concerned, it was foreseen to measure CLA content in about 900 ewes with the aim to detect QTL combined with a candidate gene approach corresponding to the study of the expression of SCD gene encoding for *delta* 9-desaturase using a RT-PCR process to quantify mRNA. The preliminary results of the QTL detection for CLA content in ovine chromosome 22 (OAR 22), where SCD gene is located, are also presented.

Materials and methods

Resource population

In 1998, 14 elite Lacaune rams from France were mated in Italy by AI to 100 Sarda ewes to produce F1 rams. Among those, 10 sons of different Lacaune sires were mated to 3000 Sarda ewes to procreate 967 backcross females born in 1999. These ewes have been bred in an experimental farm in Sardinia and in 2002 they are in third lactation. The farm is located in the South of Sardinia with a semi-arid Mediterranean climate. Ewes are submitted to the same feeding regimen based on 4-5 hours of grazing irrigated mixed swards of ryegrass and berseem clover with important supplementations of lucerne hay, maize silage and concentrates particularly in winter and late spring.

On 27 March 2001, 847 individual milk samples were collected at the morning milking in the middle of the 2nd lactation for fat extraction and fatty acid composition determination. For all the sampled ewes the milk yield, the protein content, the fat content and the somatic cell count were available at the same test date. The ewes were bred in four groups according to the lambing period. The average suckling period was 21 days and ewes were milked twice a day by machine milking. Since two weeks before the test date, the ewes were fed with 0.7 kg lucerne hay, 2.0 kg barley fresh forage, 0.7 kg commercial concentrate and 6 hours grazing on Italian ryegrass sward. Respect to the standard feeding described above, this diet was characterized by a higher level of Graminaceae (ryegrass and fresh barley forage) respect to Legume that would have increased the allowance of CLA precursors (Cabiddu *et al*., 2001).

Fatty acid content determination

The analytical determination of CLA content involved the following steps: fat separation by centrifugation at low temperature, storage of individual cream at –20°C, oil separation by thermal shock and centrifugation, acid trans-methilation. Fatty acids methyl esters (FAME) were determined by gas chromatography using a VARIAN GC 3600 equipped with FID and a fused silica capillary column (SP 2560 Supelco), 100 m × 0.25 mm i.d., film thickness 0.20 μm. Helium was used as the carrier gas at a flow of 1 ml/min. The split ratio was 1:100. The oven temperature was programmed at 75°C and held for 1.50 min, then increased to 190°C at a rate of 8°C/min, held for 25 min, increased to 230°C at 15°C/min, held for 4.47 min. The temperatures of the injector and of the detector were set at 290°C.

QTL detection and candidate gene approach

The SCD gene encoding for *delta* 9-desaturase is located on OAR 22. A preliminary QTL detection was carried out on that chromosome in order to provide information for the candidate gene approach. On that chromosome three markers BMS651; BM4505 and BMS882 were available, located at 0, 42 and 65 cM respectively. With respect to the total length of OAR 22 (85 cM), the length of the analyses linkage group represented 75% of the chromosome. The average number of informative meioses was 55%, 81% and 73% for BMS651, BM4505 and BMS882 respectively. Two traits were analysed: the CLA content in the milk fat and, as indicator of the *delta* 9-desaturase activity, the ratio between CLA and vaccenic acid.

Prior to the QTL analysis, phenotypes of both traits were adjusted for the fixed effect group (4 levels on the basis of the lambing period) and the random effect sire (ten levels). Single trait QTL analysis was carried out according to the methodology proposed by Knott *et al.* (1996) and Elsen *et al.* (1999) by within-sire linear regression using the following model:

$$Y_{ij} = s_i + (2p_{ij}–1)a_i + e_{ij}$$

where Y_{ij} was the individual phenotype adjusted as described above, s_i is the sire, p_{ij} was the probability of inheriting one defined QTL allele from sire i for the daughter j given the marker information, a_i was half the substitution effect of the putative QTL carried by the sire i, and e_{ij} was the residual assumed to be normally distributed with a zero expectation and a heterogeneous variance σ^2_{ei}. The probability for each possible phase of the sires was estimated from progeny marker information. The most likely phase was retained and the probability that each progeny received one or the other chromosomal segment was estimated at every position, given this phase, using a 2 cM step. The rejection thresholds were estimated by within-family permutations as proposed by Churchill and Doerge (1994), for each trait using 10,000 permutations.

Results and discussion

Phenotypic analysis

In Table1 are reported the average fatty acids contents in the milk fat of the Sarda × Lacaune resource population.

It has to be pointed out that the monounsaturated fatty acids (MUFA) presented in the table are the *cis* 9 isomers partially or totally produced by the *delta* 9-desaturase activity and the *trans* 11 C18:1 (vaccenic acid) which is directly absorbed by duodenum. On the whole, the contents reported in the table agree with previous results in sheep (Sevi *et al.*, 1998; Perea *et al.*, 2000) and confirms that sheep show higher contents in short (C4-C10, especially C10), similar in medium (C12-C16) and lower in long chain FA compared to cattle. Finally, the short chain FA represent 18% of the total FA and the UFA 31.3%. The CLA content is consistent with previous results in Sarda sheep (Piredda *et al.*, 2001; Secchiari *et al.*, 2001). Higher values of CLA (32.85 mg/g of fat) were found in Sarda sheep grazing during the spring (Delogu *et al.*, 2000).

Table 1. Average content in analysed FA in milk fat

Fatty acid	Means (mg/fat g)	Sd (mg/fat g)
C4	36.05	3.63
C6	21.03	2.22
C8	15.03	2.24
C10	77.03	14.10
C12	36.00	5.93
C14	91.78	8.66
cis 9 C14:1	1.80	0.46
C15	10.40	1.12
C16	183.21	12.91
cis 9 C16:1	6.99	1.22
C17	6.01	1.04
C18	91.25	9.00
cis 9 C18:1	206.55	29.25
trans 11 C18:1 (vaccenic acid)	19.41	3.61
cis 9 *cis* 12 C18:2	19.60	2.61
cis 9 *trans* 11 C18:2 (rumenic acid – CLA)	10.44	2.58
cis 9 *cis* 12 *cis* 15 C18:3	9.80	1.56

The Table 2 reports the correlations between CLA and other fatty acids and milk yield, protein and fat contents. The correlation between CLA and vaccenic acid is quite high and confirms the strong biochemical link between them (Griinari and Bauman, 1999). A positive relationship between linolenic acid (C18:3) and CLA was already pointed out by Cabiddu *et al.* (2001) in sheep fed with fresh forages. This relationship is expected since linolenic acid is also a precursor of CLA in the rumen, although with a biochemical pathway different from that described above (Griinari and Bauman, 1999). The quite important negative correlation between C17 and CLA could be due to the fact that the rumen conditions, which promote the concentration of the propionic acid, involved in the synthesis of odd chain FA, are opposite to those favourable to vaccenic acid and CLA production (Kucuk *et al.*, 2001). The only milk yield trait with a significant positive correlation coefficient with CLA is the protein content.

Table 2. Correlations between CLA, other FA and milk yield traits

Trait	r	P<
C4	0.01	0.8300
C6	0.02	0.5000
C8	0.07	0.0600
C10	0.05	0.1100
C12	0.11	0.0010
C14	0.09	0.0070
cis 9 C14:1	0.24	0.0001
C15	0.34	0.0001
C16	–0.19	0.0001
cis 9 C16:1	–0.18	0.0001
C17	–0.52	0.0001
C18	–0.26	0.0001
cis 9 C18:1	–0.23	0.0001
trans 11 C18:1 (vaccenic acid)	0.72	0.0001
cis 9 *cis* 12 C18:2	0.08	0.0200
cis 9 *cis* 12 *cis* 15 C18:3	0.40	0.0001
Milk yield	0.00	0.9500
Protein content	0.27	0.0001
Fat yield	0.06	0.0600

Further on, ratios of *cis* 9 UFA produced by *delta* 9-desaturase and their precursors were calculated. Quite high relationships were found between CLA/vaccenic acid ratio and C14:1/C14 (r = 0.45) and C18:1/C18 (r = 0.31) ratios.

The Table 3 reports the means and the residual standard deviations (rsd) per family of the CLA content and of the ratio between CLA and vaccenic acid.

Table 3. Average and residual standard deviations (rsd) within family for both CLA content in fat and ratio CLA/vaccenic acid

Sire	No. of daughters	CLA mean	CLA rsd	CLA/vacc. ac. mean	CLA/vacc. ac. rsd
1	88	10.01	2.01	0.54	0.077
2	82	10.09	1.70	0.56	0.084
3	110	11.00	1.71	0.57	0.088
4	98	10.79	2.10	0.53	0.078
5	74	9.14	1.77	0.51	0.070
6	97	10.83	1.71	0.53	0.075
7	73	10.86	1.87	0.54	0.090
8	81	10.06	1.75	0.52	0.072
9	75	10.55	1.96	0.54	0.100
10	69	10.80	1.71	0.53	0.068
Overall	847	10.44	1.83	0.54	0.080

There is evidence for a remarkable variability in CLA content between families. Indeed, the sire variance estimated with the model used to produce phenotypes for QTL analysis, represented 7.7% of the total phenotypic variance. The ratio between CLA and vaccenic acid ranges from 0.51 and 0.57 with quite large variability (although lower than CLA) between families and a more important heterogeneity of residual variances. The sire component of the phenotypic variance represented 3.8%.

QTL detection

Across family analysis was performed to test for evidence of different location effects in different families. All locations close to significance at α_c chromosome-wise level of 0.05 (i.e. with (26 * 2 * 0.05) = 3 type I errors expected by chance under the null hypothesis) were retained. Although no significant across family location effect was found for CLA, an important within family likelihood ratio test (LRT) (7.18) was found in family 4.

Nevertheless, a chromosome-wise significant QTL (LRT 28.9 with LRT estimated by permutations at the 0.05 threshold of 24.69) was detected for the ratio CLA/vaccenic acid with the most probable location between BM4505 and BMS882 markers. The families 1 and 4 showed the highest within family LRT (6.43 and 7.43) respectively, while other four families showed LRT around the threshold value of 3.

Conclusions

This first analysis of fatty acids content in the milk fat of the Sarda × Lacaune backcross population generally agrees with previous studies on dairy sheep. Further analyses are needed to better explain relationships between FA mainly taking into account individual intakes and variations in weight and body condition scores.

The preliminary QTL analysis on the OAR 22, where the SCD gene is located, allowed to detect a putative QTL for the ratio between CLA and vaccenic acid. This result can be considered as a first step suggesting that the SCD gene polymorphism is actually related to the *delta* 9-desaturase expression level and affects the quantity of CLA produced in the mammary tissues from the vaccenic

acid. The next step will be the quantification of the mRNA of the SCD gene in around one hundred ewes chosen in the high and low tails for CLA/vaccenic acid ratio of families 1 and 4 where a QTL on this trait was detected. The mRNA will be taken from individual milk somatic cells including mammary epithelial cells. As far QTL detection is concerned, the next step will be the extension of the QTL analysis to the whole genome. Moreover in order to confirm the results obtained from the 2nd lactation data, the backcross Sarda × Lacaune ewes have been re-sampled in the middle of the 3rd lactation for FA content determination.

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