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Designing milk fat to improve healthfulness and functional properties of dairy products: from feeding strategies to a genetic approach

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ABSTRACT - The present review, in the first part, deals with the most effective feeding strategies applied to dairy ruminants in order to enhance the healthfulness of milk fat. The largest changes in milk fatty acid (FA) composition have been obtained either by changing the amounts and the nature of forages in the diets of ruminants, particularly pasture, or by adding plant or marine oils to the diet. Alpine and legume based pastures are associated with high levels of conjugated linoleic acid (CLA), omega-3 FA and with low levels of saturated FA, but further studies are needed with the aim to better explain the effect of some plant species on milk FA composition. Linseed, soybeans safflower and sunflower are the most effective sources of unsaturated plant lipids used to enhance CLA and unsaturated FA content in milk fat. Among animal sources, marine oil is more effective than plant oils for enhancing CLA, vaccenic acid (VA) and omega-3 FA in milk fat, especially when fish oil is fed in combination with oil supplements rich in linoleic acid. In the second part of the review the potential contribute of genetic improvement to modifying milk FA composition is discussed. Recent studies have suggested that the genetic improvement of the nutritional quality of milk based on FA profile may be possible. At this aim, genetic parameters of milk FA composition have been estimated in Dutch, US and Italian Holstein populations and in a Belgian multiple breed population of dairy cows. In dairy sheep and goat there is still a lack of knowledge about genetic parameters and correlations of milk FA. The develop of specific selection indexes aimed to improve the nutritional properties of milk could be supported by the new insight about potential candidate genes able to affect a significant quote of the milk FA variability. Increasing evidences indicate the Stearoyl-CoA Desaturase (SCD) and Diacylglycerol acyltransferase (DGAT-1) genes as possible sources of FA variation in milk. Evidences from quantitative and molecular studies suggest a potential future animal selection, but some question about the kind of selection mechanism and the economic incentive for the improvement should be preliminarily answered.

Introduction – Evidences from human feeding and epidemiological studies together results from clinical trials suggest a potential for alteration in FA composition of the diet to reduce coronary heart disease (CHD) risk. In particular, saturated FA (SFA) and *trans* FA are indicated as the main dietary factor risk, whereas unsaturated FA decrease the risk. Since milk and dairy products are key sources of total fat and the single largest source of SFA in many European countries (Givens, 2008), a simply strategy to decrease the intake of SFA would be to reduce the consumption of dairy products. This approach however is not likely to produce benefits, because milk and dairy products are key sources of essential nutrients (calcium, vitamin B_{12}, etc.), easy digestible proteins with a balanced amino acid profile and also supply up to approximately 30% of dietary *cis*-monounsaturated FA (MUFA) in a number of European countries (Hulshof *et al*., 1999). Therefore, optimization of the FA composition of milk and dairy products could have major public health benefits. As a matter of fact, the FA pattern of milk is often considered not well balanced for human health, in view of the demonstrated serum LDL-raising properties of medium chain SFA (MCFA) and *trans* FA (Hornstra, 1999). Moreover, since milk fat contains low level of n-3 polyunsaturated FA, dairy products may weakly contribute to the recommended lowering of the n-6/n-3 ratio in the western country diets to values such as 1:1 or similar (Simopuolos, 1999). On the other hand, cohort studies provide no convincing evidence that milk is harmful. While there still could be residual confounding from unidentified factors, the studies, taken together, suggest that milk drinking may be associated with a small but worthwhile reduction in heart disease and stroke risk (Elwood *et al*., 2004). Moreover, more recently, German *et al*. (2009) reviewed the impact of dairy foods and milk fat on cardiovascular disease risk and concluded that recommendations to reduce dairy food consumption irrespective of the nature of the dairy product should be made with caution. In fact, despite the contribution of dairy products to the SFA composition of the diet, milk fat contains several components that may play an important role in human health (Parodi, 1999; Sofi *et al*., 2009). This involves designing milk fat to improve its healthfulness and functional properties. In particular, reducing the content of MCFA because of their hypercholesterolaemic effects and enhancing the content of conjugated linoleic acid (*cis*-9, *trans*-11 C18:2, CLA), vaccenic acid (*trans*-11 C18:1, VA), omega-3 FA, branched-chain FA, butyric acid (C4:0) and sphingolipids because of their recognised beneficial properties for human health (Steijns, 2008). The present review, in the first part, deals with the most effective feeding strategies applied to dairy ruminants in order to enhance the healthfulness of milk fat, subsequently the potential contribute of genetic improvement is discussed.

Modifying milk FA composition by feeding strategies. Nutrition is the main environmental factor regulating milk fat synthesis and FA composition in ruminants (Chilliard *et al*., 2007). The largest changes in milk FA composition have been reached either by changing the amounts and the nature of forages in the diets of ruminants, particularly pasture, or by adding plant or marine oils to the diet (Dewhurst *et al*., 2006; Chilliard *et al*., 2007). Generally speaking, when the pasture percentage increases in the diet, linear increases in C18:3 n-3 (ALA), VA and CLA and linear decreases in C10:0–C16:0 have been observed (Couvreur *et al*., 2006). The very consistent effects of pasture on milk fat content of ALA, VA and CLA are related to the high content of ALA in most pastures, which is partly biohydrogenated into VA and partly absorbed intact in the gut and secreted into milk. Moreover, in mammary gland, the amount of VA which is converted in CLA by the action of Stearoyl-CoA desaturase enzyme accounts for 24-29% and represents from 80 to 90% of total CLA in milk (Shingfield *et al*., 2007; Tyburczy *et al*., 2008). However differences in the FA composition of pasture milk have been reported according to the botanical composition of pasture and, within plant species, among grazing seasons and phenological stages of plants. For example, polyunsaturated FA (PUFA) and CLA contents in milk fat were highest during spring and autumn with declines during summer as a consequence of declining quality and quantity of pasture (Dewhurst *et al*., 2006; Nudda *et al*., 2005). In dairy cow, milk from Alpine pasture resulted in higher concentration of CLA, and ALA and lower content of saturated MCFA than milk from lowlands (Collomb *et al*., 2002). Kraft *et al*., (2003) reported an increase of

880% and 254% for CLA and ALA content, respectively, in milk fat from cows grazing alpine pasture in comparison to cows fed silage and concentrates. Leiber *et al*. (2005) reported that milk from cows grazing alpine pastures contained higher amount of ALA than milk from cows grazing lowland pasture, although *Lolium perenne* and *Trifolium repens*, two of the dominant species in the lowland pastures, contain far higher contents of ALA than many other grasses and legumes. Several studies reported that, under certain grazing condition, the increase of PUFA in milk fat is not related to the intake of C18:2 n-6 and ALA. Although the metabolic mechanisms which drive this phenomenon are not yet completely understood, some authors have recently proposed that specific secondary plant constituents (such as polyphenols, terpenoids, tannins), are one of the major reasons affecting CLA and ALA milk contents, by inhibiting hydrogenating microorganisms in the rumen (Leiber *et al*., 2005; Vasta *et al*., 2008). According to this theory, increasing sheep grazing time on Sulla (a tanniferous plant), when the plants were at reproductive phenological stage, determined a greater increase in C18:3 n-3 and a stronger decrease of VA and CLA in milk than that caused by sheep grazing on young Sulla or ryegrass (Cabiddu *et al*., 2005). Moreover, FA composition of ewe's milk may be conveniently modified by botanical pasture composition and by pasture management. In general milk from sheep grazed legumes had higher content of CLA, VA and ALA than milk from sheep grazed grass, probably due to the higher content of ALA and linoleic acid in legumes (Cabiddu *et al*., 2005). Similar results have been reported also for grazing cows (Dewhurst *et al*., 2006). Moreover Addis *et al*. (2005) reported that feeding lactating sheep fresh forage of *Chrysanthemum coronarium* allowed to increase VA and CLA concentration in milk fat, in similar way or to a higher extent than feeding sheep with fresh legumes.

A great number of studies have been published on the effect of different animal and vegetal lipid source on milk FA composition and, recently, Glasser *et al*., (2008) reported results of a meta-analysis about the effect of oilseed supplements on FA composition of cow milk. The addition of adequate lipid sources (more than 3% of dry matter intake) to ruminant feeds can change the FA composition of milk, whose profile reflects that of the diet, as affected by rumen biohydrogenation and microbial activity (Chilliard *et al*., 2007). In general, PUFA lipid sources are able to induce a decrease of MCFA and branched chain FA content in milk and an increase of C18 FA, resulting in a general improvement of milk fat quality, because the ratio saturated:unsaturated FA decreases and the content of unsaturated C18 FA increases. On the contrary, butyric acid percentage in milk fat is rarely significantly affected by lipid supplements, probably because this FA is partly synthesized by metabolic pathways different to acetyl-CoA carboxylase (Chillard *et al*., 2007). Linseed, rapeseed, soybean, sunflower and safflower are the main sources of unsaturated plant lipids (as seeds, oil or protected form) tested in the diet of lactating ruminants (Glasser *et al*., 2008). Among animal sources, fish oil is more effective than plant oils for enhancing CLA, VA and omega-3 FA in milk fat, especially when fish oil is fed in combination with oil supplements rich in linoleic acid (Shingfield *et al*., 2005). Feeding dairy cows with a ration containing 45g/kg (on dry mater basis) of a mixture (1:2 wt/wt) of fish oil and sunflower oil resulted in an increase in milk fat of CLA, VA and long chain omega-3 FA by 810, 1294 and 158%, respectively (Jones *et al*., 2005). In dairy goat, adding 1.1% of fish oil in the diet doubled the omega-3 FA content of milk (Cattaneo *et al*., 2006). Similar results were obtained also in dairy ewe (Kitessa *et al*., 2003). Among plant oils rich in 18:2 n-6, soybean is the source more frequently adopted in published studies, followed by sunflower and safflower oils.

These lipid sources markedly increase milk CLA, VA and linoleic acid content, expecially when they were included in the diet as unprotected oil (Chilliard *et al.*, 2007; Mele *et al.*, 2006; Mele *et al.*, 2008). The dietary inclusion of linseed (as oil or seed), a source of ALA, resulted in an increase of CLA, VA and ALA and a decrease of MCFA in milk. The levels of CLA in milk linearly increased when the amounts of vegetable oil in the diet increase, but differences have been reported according to the nature of the basal diet (hay, silage, grasses, legumes) and to the forage:concentrate ratio of the diet (Chilliard *et al.*, 2007). In general, when the amount of concentrate did not exceed 50% of total dry matter intake, the inclusion of unsaturated vegetable oils in the diet favoured the rumen accumulation of VA, with an increasing secretion of VA and CLA in milk. Higher intake of concentrate, especially when associated with maize silage based diet, are associated with a shift in rumen biohydrogenation toward *trans*-10, *cis*-12 C18:2 and *trans*-10 C18:1 (Chilliard *et al.*, 2007). In this case, a depression of milk fat yield has been frequently reported in dairy cow, due to the negative effect of *trans*-10, *cis*-12 C18:2 isomer (and probably of other *trans* isomers of C18:1, C18:2 and C18:3) on mammary lipogenic enzymes (Harvatine *et al.*, 2009).

Modifying milk FA composition: the potential contribute of genetic improvement. Recent studies have suggested that the genetic improvement of the nutritional quality of milk based on FA profile may be possible (Mele *et al.*, 2007; Soyeurt *et al.*, 2007; Carta *et al.*, 2008; Stoop *et al.*, 2008; Mele *et al.*, 2009). Possibilities of changing milk FA composition by genetically altering proportion of FA are therefore of interest, but effecting genetic improvement requires genetic variation, a mechanism of selection and an economic incentive for the improvement. Regarding the first point, in the last 15 years breed variability in milk FA composition have been documented in cow and sheep (Beaulieu and Palmquist, 1995; Secchiari *et al.*, 2001; White *et al.*, 2001; Secchiari *et al.*, 2003; Tsiplakou *et al.*, 2006; Signorelli *et al.*, 2008). Fewer evidences are available in dairy goat (Zan *et al.*, 2006). In dairy cows, large individual variability of milk FA composition (expecially for CLA content) has been observed on cows fed the same diet (Bauman *et al.*, 2001). Recent studies reported that the CV values of FA in cow milk ranged from 8 to 37%, with lower values for short chain FA and MCFA and higher values for CLA and other unsaturated FA (Stoop *et al.*, 2008; Mele *et al.*, 2009). In an experimental Sardinian x Lacaune back-cross population, Carta *et al.* (2008) reported a large individual variability of FA composition in sheep milk: the CV ranged from 9.1% for butyric acid to 27.9% for CLA. Thus, when feeding strategies able to improve milk FA composition are applied, an important role of the genetic background of animals may be hypothesized. Therefore, the genetic variability of milk FA should be used, in order to develop a selection index and to transmit from generation to generation the improvement of milk FA composition. At this aim, the estimation of genetic parameters for milk FA composition is the first step. In dairy cow, the first estimates of milk FA genetic parameters have been reported by Edwards (1973), Renner and Kosmack (1974) and Karijord *et al.* (1982) but these estimates were affected by bias due to the low number of analysed samples or by poor accurate statistic methodologies to estimate variance components (Arnould and Soyeurt, 2009). More recent studies using animal model methodologies reported more accurate estimation of genetic parameters of milk FA in Dutch, US and Italian Holstein populations and in a Belgian multiple breed population of dairy cows (Soyeurt *et al.*, 2007; Bobe *et al.*, 2008; Stoop *et al.*, 2008; Mele *et al.*, 2009). FA heritabilities ranged from low (below 0.1) to moderate (from 0.3 to 0.5), with higher values for neo synthesized FA and lower values for unsaturated FA. Heritability of milk CLA content ranged from 0.12 in Italian Holstein to 0.21 in Dutch Holstein population (Stoop *et al*., 2008; Mele *et al*., 2009). The variability among heritabilities values of the cited studies is due to differences in 1) the methodology used for the milk FA analysis (Soyeurt group used mid-infrared spectrometry, while the other authors used gas-cromatography), 2) the statistic models used (multi-trait or single-trait models), 3) the amount of data available, and 4) the range of DIM of cows. Nevertheless, some observation may be shared among the cited studies: the existence of a genetic variability of milk FA content is confirmed and it suggests a potential future animal selection. More studies are needed in order to improve the accuracy of the genetic parameter estimate, by using larger data set that include repeated milk samples. This aspect, however, put in evidence that lab facilities to obtain FA data should be easier to use and cheaper than gas-chromatography. Mid-infrared spectrometry seems to be a good alternative, but more studies are needed in order to improve the accuracy of this methodology, especially if a large number of labs will be involved (Arnould and Soyeurt, 2009). These evidences suggest that it is possible to create a large database of milk FA composition in order to develop a selection index aimed to improve the nutritional quality of milk fat. Nevertheless some aspects should be preliminarily taken into consideration. In milk there is a large number of FA (more than 400) and in some cases strong genetic correlations have been reported among individual FA (Soyeurt *et al*., 2007; Stoop *et al*., 2008; Mele *et al*., 2009), as a consequence of common metabolic production pathways. The use of synthetic variables such as the FA ratios that reflect the ability of mammary gland to produce MUFA and CLA should be a first step in order to simplify the phenotypic pattern. Moreover, the application of multivariate analysis techniques should be useful, in order to extract synthetic indicators that reflect the most important information contained in the milk FA variability. An other question is related to the genetic correlation among milk FA and milk production traits. Recent studies reported that CLA and other unsaturated FA are negatively correlated with milk fat and protein content, whereas saturated MCFA are positively related (Soyeurt *et al*., 2007; Stoop *et al*., 2008; Mele *et al*., 2009). Selection objectives aimed to increase milk fat nutrition properties necessarily lead to a decrease of milk fat and protein content, but actual procedures of milk payment are based on milk fat and protein content. In order to avoid a negative economic impact for farmers, new procedure of milk payments should be developed.

What above reported for dairy cow could be in large part extended also to sheep and goat, but for these species there is still a lack of knowledge about genetic parameters and correlations of milk FA. In dairy sheep, Carta *et al*., (2008) analysing milk FA composition of an experimental Sardinian x Lacaune back-cross population, reported that a certain amount of additive genetic variance is available for selection purpose for most FA. In fact, the values of sire variance, which represents the proportion of variability due to genetic differences among sires, ranged from 5% to 20%. However further studies are needed in order to implement the above information also in other dairy sheep populations.

The develop of specific selection indexes aimed to improve the nutritional properties of milk could be supported by the new insight about potential candidate genes able to affect a significant quote of the milk FA variability. Increasing evidences indicate the Stearoyl-CoA Desaturase (SCD) gene as one of the possible sources of FA variation in milk. SCD gene codes for the homonymous enzyme that plays a key role in mammary lipid metabolism, because it introduces a double bond at the $\Delta 9$-position in a large spectrum of FA (Ntambi, 1999). Its most important substrates are acyl-CoA of C14, C16, C18, and *trans*-11 C18:1, which are converted into C14:1 n-5, C16:1 n-7, *cis*-9 C18:1, and *cis*-9, *trans*-11 CLA. More than 70% of the *cis*-9, *trans*-11 CLA of ruminant's milk is produced in the mammary tissue by the activity of SCD (Bauman *et al*., 2006). In bovine, the SCD locus has been mapped on the chromosome 26. Three single nucleotide polymorphisms (SNP) in complete linkage disequilibrium and that result in 2 haplotypes have been detected in the fifth exon. The third SNP causes the substitution of valine (allele V) with alanine (allele A) on the 293rd residue. Because valine is highly conserved across mammals, it is considered the ancestral amino acid in that position (Taniguchi *et al*., 2004). The SCD polymorphism has been found in Holstein Friesian, Jersey, Brown Swiss, Piedmontese, Valdostana and Japanese Black cattle breeds (Medrano *et al*., 1999; Taniguchi *et al*., 2004; Mele *et al*., 2007; Moioli *et al*., 2007; Schennink *et al*., 2008; Kgwatalala *et al*., 2009). Recent evidences reported a significant effect of Stearoyl Co-A Desaturase (SCD) gene polymorphisms on MUFA, CLA and FA ratios, which represent an index of FA mammary desaturation, both in dairy cattle and ewes (Mele *et al*., 2007; Moioli *et al*., 2007; Schennink *et al*., 2008; Kgwatalala *et al*., 2009; Miari *et al*., 2009). In ewe, SCD locus has been mapped on the chromosome 22 and, recently, Garcia-Fernadez *et al*., (2009) reported the complete coding sequence of SCD gene. No polymorphism were found within the coding region of the SCD gene, whereas the analysis of non coding region revealed 4 SNP located in the promoter region, intron 2, and intron 3. Interestingly, the most polymorphic SNP was that in the promoter region of the gene and preliminary results seemed to indicate a significant effect of the SNP polymorphisms on milk FA composition in Assaf and Churra breeds (Garcia-Fernadez *et al*., 2009). Miari *et al*., (2009) sequencing a large part of the SCD gene in an experimental Sardinian x Lacaune back-cross population found only one SNP at the intron 4 (3295 C>T), that was recovered only in one family with a significant high content of CLA in milk fat. Since the found SNP was in a non coding region of the SCD gene, the authors suggested that the found mutation could be in strong linkage with the causative one. In goat, only the sequence of cDNA has been published and the SCD locus has been mapped on the chromosome 26. The complete sequencing of the cDNA revealed that the coding region is followed by an unusually long 3' UTR sequence, deriving from a single exon, in which a polymorphism, due to a deletion of a nucleotide triplet (TGT), was detected (Bernard *et al*., 2001).

Another gene involved in milk FA variability is Diacylglycerol acyltransferase (DGAT-1). This gene codes for the homonymous enzyme able to catalyse the transfer of fatty acyl CoA to diacylglycerols in order to complete the milk fat triglycerides. In bovine, DGAT-1 locus is located on the chromosome 14. At position 232 (K232A) of the exon 8 of the DGAT-1 gene, a lysine to alanine substitution has been observed and this mutation is associated with a larger fraction of C16:0 in milk fat, but less C14:0, unsaturated C18:0 and CLA (Schennink *et al*., 2008).

Conclusions – The application of feeding strategies is undoubtedly the most efficient way to modifying milk FA composition in order to enhance the healthfulness of dairy products and a plenty of literature concurred to define the most efficient strategies. Nevertheless, results from recent studies suggest that proper selection indexes may also be developed, in order to transmit from generation to generation the improvement of milk FA composition. Moreover, evidences from molecular studies showed that the polymorphism of some major genes could complement the quantitative approach and may concur to explain why the response to the same feeding strategy may significantly vary according to the animal genotype. Therefore, future strategies could take into consideration animal selection in order to obtain an optimal response to feeding strategies aimed to enhance the healthfulness of dairy products. At this aim further researches about the biochemical and genetic regulation of lipid metabolism pathways and the interaction among nutrients and genotype are needed.

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