Changes in conjugated linoleic acid and C18:1 isomers profile during the ripening of Pecorino Toscano cheese produced with raw milk

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Abstract

The aim of the present study was to investigate the evolution of the fatty acid profile, with particular attention to conjugated linoleic acid (CLA) and to C18:1 isomers, during ripening of sheep cheese (Pecorino Toscano) produced with raw milk. After 60 days of ripening the total concentration of C18:1 isomers and that of CLA pool content decreased. In particular, if isomers profile is considered, the percentage of trans11 C18:1, trans10 C18:1 and cis9, trans11 CLA decreased as consequence of hydrogenation or of double bonds isomerisation, while the concentration of trans10, cis12 CLA increased.

Materials and methods

Introduction

Conjugated linoleic acid (CLA) is a pool of geometrical and positional isomers of linoleic acid (LA) with two conjugated double bonds located from carbon 6 and carbon 14. Several authors demonstrated, mainly in animal model and in vitro studies, that CLA has potential beneficial effects for human health (Benjamin and Spener, 2009; Solli et al., 2010; Pintus et al., 2012). The most biologically active isomer is Rumenic Acid (RA), the cis9 trans11 isomer, synthesised in ruminants partly in rumen, by isomerization of LA, and partly in the mammary gland by Δ2 desaturation of vaccenic acid (VA) (Griniari and Bauman, 1999). Sheep milk is rich in CLA, since it has a higher fat content than that from cows or goats and because sheep feeding regimen is often based on pasture (Antongiovanni et al., 2003; Mele, 2009). The production of sheep cheese plays an important economic role in the Mediterranean area where dairy sheep is present since ancient times. Many Italian cheeses made from sheep milk are called Pecorino and some varieties, such as Pecorino Toscano, have the Protected Designation of Origin (P.D.O., EC regulation 306/2010) status, ruled by strict standards with reference to animal feeding, milk quality, kind of curd and of starter used to fortify the milk, the temperature and the time of clotting.

Previous papers have reported that in Pecorino Toscano cheese from pasteurized milk, the total content of CLA did not change during the whole time of ripening, but a rearrangement among the isomers, characterized by the increase of RA, may occur (Antongiovanni et al., 2005; Buccioni et al., 2007; 2010). As the cheese making procedure for Pecorino Toscano allows the use of raw or pasteurized milk, the resulting cheeses may differ for the microflora composition; in fact, in raw milk usually either lactic acid bacteria and other microorganisms such as enterobacteria, yeasts, and moulds occur and the content or the composition of such no-starter microorganisms depend on the environment where milk has been produced and processed. In this study fatty acid profile changes were evaluated during the ripening of Pecorino Toscano cheese produced with raw milk in order to study the temporal changes of CLA and C18:1 isomers content.

Cheese making procedure and sampling

The cheese (raw milk cheese, RMC) was obtained from raw milk fortified with the follow starter culture: Lyofast SBS 044 and 045 (Sacco srl, Cadorago, CO, Italy) which were composed of Lactococcus lactis and of Streptococcus thermophilus strains (Bizzarro et al., 2000); these starter cultures were officially included in Pecorino Toscano P.D.O. disciplinary. In this case, the microflora of milk was formed by native microorganisms plus microorganisms from starter culture; the addiction of the starter culture in raw milk is a common practice used in Pecorino Toscano P.D.O protocol with the aim of promoting the lactic acid bacteria presence, useful during the ripening.

The cheese making procedure was based on the following steps: milk heated to 35°C and rennet added to curdle the milk. Then, the starter cultures were added. After the milk had clotted (30 min), the curd was cut to the size of a hazelnut and the vat temperature was kept at 35°C for entire the procedure. Curds were placed into 2 kg molds. Molds, located in a thermostatic chamber at 30°C for 6h, were turned five times. Then, after 24 h at 20°C, cheeses were plunged in a salt solution (NaCl, 19% w/v) at 15°C for 24 h and then transferred to a ripening room where they maintained at temperature of 10°C for 60 days. The cheese was produced using milk from a dairy sheep farm located in the North-Western Tuscany and equipped with an on-farm dairy processing plant. The feeding regimen of the flock was based on oat pasture (6 h/day), 500 g/dew of a grain mixture composed by oat and vicia faba.
The loaves of RMC, produced using the same batch of milk, were collected at 0, 30, 60 days from the transfer to the ripening room (9 samples of cheese at each time).

Proximate analysis

The method of thioarbituric acid (TBARS) was adopted to evaluate the oxidative status of cheese samples (Patton and Kruzic, 1951). Milk and cheese fat content was analysed according to Gerber and Gerber-Van Gulik (ISO, 1975), respectively. Dry matter (DM) and crude protein (CP) contents were determined according to AOAC (1990).

Fatty acids analysis

Milk fat, was extracted according to the Roese-Gottlieb method (AOAC, 1990) modified as described by Mele et al. (2008). Cheese fat was extracted according to Folin et al. (1957). The fatty acid methyl esters (FAME) were prepared with a base catalyzed trans esterification, according to Christie (1982). The FAME were separated by a GC equipped with an FID detector and a capillary column (CP-Select CB for FAME Varian, Middelburg, The Netherlands: 100 m × 0.25 mm i.d.; film thickness 0.20 μm) and quantified using nonadecanoic acid (C19:0) methyl ester (Sigma Chemical Co., St. Louis, MO, USA) as the internal standard. The injector and FID detector temperatures were respectively 270°C and 300°C. The oven programmed temperature was 40°C for 4 min, increased to 120°C at a rate of 10°C min⁻¹, maintained at 120°C for 1 min, increased to 180°C at a rate of 5°C min⁻¹, maintained at 180°C for 18 min, increased to 200°C at a rate of 2°C min⁻¹, maintained at 200°C for 1 min, increased to 230°C at a rate of 2°C min⁻¹ and maintained at this last temperature for 19 min. The split ratio was 1:100 and helium was the carrier gas with a flux of 1 mL min⁻¹. Individual FAME were identified by comparison of the relative retention times of FAME peaks from samples, with those of the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA, USA). Individual trans9 C18:1, trans11 C18:1, trans12 C18:1, trans13 C18:1 (Supelco), individual cis9, trans11 and trans10, cis12 C18:2 (Matreya Inc.), CLA mix standard (Sigma Chemical Co.) and published isomeric profile (Griinari et al., 1998; Kramer et al., 2004) were used to identify trans CLA isomers of interest.

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Results and discussion

Fat and protein content (means ± SE, n=3; mg g⁻¹ of milk) and pH values (means ± SE; n=3) of raw milk were 54.2±0.3, 33.4±0.2 and 6.39±0.03 respectively. During the ripening of RMC, DM increased from 494.9 (g kg⁻¹ of cheese) at 0 day to 639.1 (g kg⁻¹ of cheese) at 60 days (P<0.05; SEM 0.28) and fat content increased from 448.6 (mg g⁻¹ on DM) at 0 day to 500.6 (mg g⁻¹ on DM) at 60 days (P<0.05; SEM 0.19). Considering the whole time of cheese ripening, no significant differences were found for the oxidative status: 2.02 M at 0 days vs 2.13 at 60 days (SEM 0.08, P>0.05).

The data related to TBARS showed a very low oxidation activity during cheese ripening. In fact, the presence of oxygen inside the cheese matrix was presumably very low, and the microorganisms operated in anaerobic conditions. This fact induced a low oxidation of the polyunsaturated fatty acids present in the cheese. The fatty acid profile of cheeses at time 0 reflected that of milk used for cheese making, and for the sake of simplicity, only the fatty acid profile of cheese fat at time 0 was reported (Table 1). This result agreed with Nudda et al. (2005), Buccioni et al. (2010) and Mele et al. (2011) that showed a quantitative transfer of fatty acids from milk to sheep cheese.

The most important changes regarded the profile of C18 fatty acids. Stearic acid (SA, C18:0) percentage significantly increased (P<0.05) with time of ripening (Table 1) and the total content of C18:1 and CLA decreased. In particular, the content of all cis and trans C18:1 decreased after 60 days of ripening, with the exception of C18:1 cis9 (0A), which increased. Both C18:1 trans10 and VA decreased respectively by 6.6 and 2.5 mg g⁻¹ of lipids (Table 2). The total content of CLA in lipid fraction decreased by 19.6% and, in particular, the content of RA decreased by 2% (Tables 1 and 3). However, considering that the fat content is enhanced during the ripening, CLA increased from 2.30 g/kg to 3.20 g/kg of wet cheese. Previous nutritional trials in human demonstrated that the intake of cheese with similar content of CLA has had healthful effects (Sofi et al., 2010; Pintus et al., 2012). A similar behaviour was observed also for C16 fatty acids. In fact, while C16:1 decreased, C16:0 percentage increased. The content of LA and linolenic acid (LNA, C18.3 cis9 cis12 cis15) did not vary during the ripening (Table 1). According to this pattern, a partial conversion of CLA and some C18:1 isomers to SA may be hypothesized. Luna et al. (2007) reported that cheese microorganisms may use peptides or other low molecular weight protein compounds as hydrogen donors, in order to modify the CLA isomer composition in Mahon cheese. Since the presence in Pecorino Toscano cheese of peptides as results of proteolytic activity of microorganisms during the ripening has been previously reported in literature (Cogan and Hill, 1993), a bio-hydrogenation of some C18:1 and CLA isomers by cheese microorganisms could be supposed. However, at our knowledge, this is the first time that a rearrangement of C18:1 isomers is reported during the ripening of cheese. More studies are needed in order to...
better elucidate the role of native microorganisms in the milk lipids changes during ripening.

Interestingly, in RMC samples the content of OA increased during ripening (about 2%); in literature little information are available about C18:1 isomerisation in cheeses, but certainly, OA is the isomer thermodynamically more stable than others as consequence of bound position and the geometry. As regard CLA isomer composition, also a rearrangement of the isomer profile was observed. In fact, the content of trans13 cis15, trans9 cis11, trans9 trans11, trans10 cis12, trans10 trans12, and trans8 trans10 CLA increased, whereas the content of RA and trans7 cis9 CLA decreased (Table 3). These data confirm what recently reported by Buccioni et al. (2010) who demonstrated that changes in CLA isomers profile during the ripening of Pecorino Toscano cheese can occur. However, in literature controversial observations are reported. Chin et al. (1992) put in evidence substantial differences in CLA content in several common dairy food. In particular, these authors reported that the CLA content was higher in fresh cheese because, in cured cheese, the free conjugated fatty acids, which origin from lipolysis, were easily susceptible to microbial oxidation. In contrast, Luna et al. (2005, 2007) reported that the ripening did not substantially modify CLA content in several types of Spanish sheep cheeses. Likewise Gnädig et al. (2004) and Lin et al. (1999) found no differences in cheddar cheese at two times of ripening (three and six months) or in Emmental cheese. However, in the above reported studies, only the total content of CLA was considered. Moreover, in these trials cheese samples were often directly acquired on the market, without any information about the origin and the composition of the milk, the cheese technology and the type of ripening applied. As consequence, endogenous enzyme of milk, lipase and esterase activities in cheeses were different. All that make a comparison of results among different studies difficult. Several authors, cited by Sieber et al. (2004) in his review, demonstrated that many strains of Lactobacilli, Lactococci and Streptococci, used in Pecorino cheese as starter culture, are able to produce CLA from LA in skim or whole milk. In particular, Kim and Liu (2002), after several studies on lactic acid bacteria isolated from milk, showed that some of these microorganisms were able to produce CLA in anaerobic condition by free LA isomerisation.

Considering that in a previous trial Buccioni et al. (2010) has found an increase of RA percentage during the ripening of Pecorino P.O.D. produced with pasteurized milk added with the

| Table 1. Selected fatty acid composition during the ripening of raw milk cheese (mg g⁻¹ of lipids). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Fatty acid                      | 0 days n=9      | 30 days n=9     | 60 days n=9     | SEM             |
| C10:0                           | 55.5⁺           | 50.5⁺           | 49.2⁺           | 0.5             |
| C12:0                           | 33.9⁺           | 29.2⁺           | 28.9⁺           | 0.2             |
| C12:1                           | 0.3             | 0.1             | 0.2             | 0.1             |
| C13 iso                         | 0.1             | 0.2             | 0.1             | 0.1             |
| C13 ante                        | 0.1             | 0.0             | 0.0             | 0.1             |
| C13:0                           | 0.7             | 0.6             | 0.6             | 0.1             |
| C14 iso                         | 1.2             | 1.1             | 1               | 0.1             |
| C16:0                           | 227.1⁺          | 227.5⁺          | 229.7⁺          | 0.5             |
| C16:1                           | 6.1⁺            | 5.7b            | 5.6b            | 0.1             |
| C17 iso                         | 4.6             | 4.8             | 4.8             | 0.1             |
| C17 ante                        | 5.3⁺            | 5.0⁺            | 5.0⁺            | 0.1             |
| C17:0                           | 6.6⁺            | 7.5⁺            | 7.5⁺            | 0.1             |
| C17:1                           | 2.4             | 2.8             | 2.4             | 0.1             |
| C18:0                           | 120.1⁺          | 134.7⁺          | 136.0⁺          | 0.5             |
| C18:1 tot                       | 274.7⁺          | 266.5⁺          | 267.0⁺          | 1.2             |
| C18:2 cis112                    | 0.6             | 0.4             | 0.4             | 0.1             |
| C18:2 cis19,12                  | 0.6             | 0.5             | 0.5             | 0.1             |
| C18:3 cis,cis12                 | 12.6            | 12.5            | 12.6            | 0.1             |
| C18:3 cis19,12,115              | 0.2             | 0.2             | 0.3             | 0.1             |
| C18:3 cis19,12,15               | 0.4             | 0.4             | 0.4             | 0.1             |
| C18:3 cis,cis12,cis15           | 6.7             | 7.1             | 7.0             | 0.2             |
| C20:0                           | 2.0⁺            | 2.6⁺            | 2.6⁺            | 0.1             |
| CLA tot                         | 17.1⁺           | 14.3⁺           | 14.3⁺           | 0.1             |
| C18:4                           | 0.3             | 0.5             | 0.5             | 0.1             |
| C20:1                           | 0.4             | 0.4             | 0.4             | 0.1             |
| C20:3                           | 1.1⁺            | 1.0⁺            | 0.05⁺           | 0.1             |
| C20:5                           | 0.7             | 0.7             | 1.1             | 0.2             |

Table 2. C18:1 isomers profile during the ripening of raw milk cheese (mg g⁻¹ of lipids).

| Fatty acid | 0 days n=9 | 30 days n=9 | 60 days n=9 | SEM |
|------------|------------|------------|------------|-----|
| C18:1 cis112-14     | 2.4⁺      | 2.0⁺      | 2.1⁺      | 0.1 |
| C18:1 cis9,11       | 3.2⁺      | 2.7⁺      | 2.5⁺      | 0.1 |
| C18:1 cis10,12      | 9.7⁺      | 3.1⁺      | 3.1⁺      | 0.2 |
| C18:1 cis11,13      | 36.5⁺     | 33.2⁺     | 34.0⁺     | 0.2 |
| C18:1 cis12,13      | 4.9⁺      | 3.6⁺      | 3.8⁺      | 0.1 |
| C18:1 cis9,11       | 210.6⁺    | 215.6⁺    | 214.9⁺    | 0.7 |
| C18:1 cis11,13      | 2.9⁺      | 2.1⁺      | 2.2⁺      | 0.1 |
| C18:1 cis12,13      | 3.6⁺      | 3.2⁺      | 3.3⁺      | 0.1 |
| C18:1 cis14-cis16   | 0.9       | 1.0       | 1.1       | 0.3 |

₃P<0.05.
same starter used in this experiment and considering that in this trial the concentration of this isomer presented a behaviour completely opposite decreasing within 30 days of ripening, it could be hypothesised that this difference is due to cheese making with raw milk because it is well known, that the microflora characterizing it is commonly formed by a great variety of bacteria whose role on fatty acid profile modification is partially known in literature. Hence a decisive contribute to RA decrease could be done by autochthonous microflora of raw milk.

In this study, changes in CLA isomer profile of cheese could be due either to a geometrical conversion between cis and trans double bonds and to a positional isomerisation of CLA. Destaillass et al. (2005) have reported that a pericyclic isomerization, a [1,5] sigmatropic rearrangement, can occur on CLA isomers of butterfat under thermal conditions that are encountered in food processing. A similar mechanism could be happen also in cheese fat, if an enzymatic catalysis, in which microorganisms play a fundamental role, is assumed. In literature, several examples of microorganisms ability to shift double bond have been reported (Teter and Jankins, 2006) A similar behaviour could be assumed also for cheese microflora.

Conclusions

Given the beneficial role of CLA and VA for human health, an improvement of knowledge aimed to better characterize the variables that influence the content of these functional fatty acids during cheese making are needed to define nutritional values of dairy fatty acids obtained from raw milk. Further studies are needed in order to better elucidate the role of native microorganisms in the cheese fatty acid changes during ripening.

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