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STUDY OF FATTY ACIDS PROFILE IN OVINE AND CAPRINE SICILIAN DAIRY BREED AND ASSOCIATION WITH POSSIBLE CANDIDATE GENES

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Abstract

The Sicilian cattle, sheep and goat breeds and their dairy products are an important source for the economy of the livestock sector.

In particular, the economic importance of sheep and goat milk lies for the first one in the processing into dairy products and for the second one in the drinking milk production. For both these species, the role of fat and protein content is as important as the amount of milk yield.

The identification of genes responsible for quantitative and qualitative characteristics of small ruminant milk could increase the efficiency of genetic improvement and consider new objectives such as the production of milk with good nutritional properties.

The aims of this work were:

i) To provide new data to better understand the influence of polymorphism genetic of casein on fatty acid profile of milk in Girgentana goat breed;

ii) To determine the fatty acid profile in Valle del Belice sheep breed;

iii) To verify a possible association, statistical significant, between FA composition and polymorphisms in ovine ACACA gene, encoding an enzyme directly involved in fatty acid metabolism.

In Chapter 2, the association between genetic polymorphism of casein loci and fatty acid profile of milk of Girgentana goat was investigated. One hundred lactating Girgentana goats, homogeneous for milk production and days of lactation were used. The procedure was developed using individual raw milk samples, collected in three different stages of lactation, October, February and June, from individuals with known genotypes at casein loci. The determination of the fatty acid profile was performed by gas chromatography with flame ionization detector (GC-FID). The statistical analysis showed an interesting association between the polymorphisms at κ-casein and the concentration of long-chain fatty acids, in particular C18:0 (stearic acid), C18:01 (oleic acid), C18:03 (linoleic acid), and C20:0 (arachic acid) which are potentially involved as positive factors in human health.

In Chapter 3, the fatty acid composition of Valle del Belice sheep milk was determined providing, for the first time, data on fatty acid profile for this Sicilian breed. In this study, the milk fat separation was obtained with a rapid method reliable and simple which allowed us to

analyze a large number of samples. The fatty acid methyl esters (FAMEs) were determined by gas chromatography coupled with mass spectrometer detector (GC-MS) to achieve more accurate peak identification and a novel fused capillary gas chromatography, the ionic liquid SLB-IL111 column, was used. More than 400 samples of milk fat were processed. Saturated, *cis*-monounsaturated, and polyunsaturated fatty acids were present at 4.20%, 1.05%, and 0.42% of total fat, respectively. Good resolution of all fatty acids commonly found in milk sample was achieved.

In Chapter 4, a study of association between fatty acid profile of Valle del Belice sheep milk and polymorphisms within Acetyl-CoA carboxylase (ACACA) gene was carried out. In order to reduce the costs of the typical sequencing of a large number of DNA samples, the selective genotyping approach was used. Only those individuals with extreme phenotypic values were genotyped. In this way, two extreme groups of 16 animals were chosen considering the mean level of each fatty acid ± 2 standard deviations. Seven exons of the ACACA gene were sequenced and 19 polymorphic sites were identified. The association analysis with fat content performed for all SNPs showed the correlation between two different SNPs and the concentration of three fatty acids: caproic acid (C6:0), palmitoleic acid (C16:1), and linoleic acid (C18:2). The obtained results showed an increased concentration of C6:0 and C16:1 associated with T allele of SNP1. As regards the SNP3, allele T determined an increased concentration of C6:0, whereas allele C an increased concentration of C16:1. The concentration of C18:2 decreases when homozygote genotype is present and increased with heterozygote genotypes.

These results confirm the influence of ACACA gene, an ovine candidate gene potentially controlling milk fat trait, on fatty acid synthesis in the mammary gland.

Riassunto

In Sicilia, le razze bovine, ovine e caprine e le produzioni lattiero-casearie rappresentano una risorsa importante per l'economia del settore zootecnico. L'importanza economica del latte ovino e caprino è soprattutto legata per il primo alla sua trasformazione in prodotti lattiero-caseari, per il secondo al possibile utilisso per il consumo fresco. Per entrambe queste specie, il contenuto di grasso e proteine è importante tanto quanto la produzione di latte. L'individuazione dei geni responsabili delle caratteristiche quanti-qualitative del latte dei piccoli ruminanti permetterebbe quindi di aumentare l'efficienza del miglioramento genetico e di considerare nuovi obiettivi di selezione come le caratteristiche nutrizionali del latte. Gli scopi di questo lavoro sono stati:

i) Fornire nuovi dati per meglio comprendere l'influenza dei polimorfismi genetici delle caseine sul profilo acidico del latte di capra di razza Girgentana.
ii) Determinare la composizione in acidi grassi del latte di pecora di razza Valle del Belice;
iii) Verificare possibili associazioni statisticamente significative tra il profilo acidico del latte di pecora Valle del Belice e il gene ACACA, che codifica un enzima direttamente coinvolto nel metabolismo lipidico.

Nel Capitolo 2, è stato condotto uno studio di associazione tra i polimorfismi genetici delle caseine ed il profilo acidico del latte di capra Girgentana. Cento capre in lattazione, omogenee per produzione di latte e giorno di lattazione sono state oggetto di studio. Campioni di latte individuale crudo sono stati prelevati in tre diversi periodi di lattazione (Ottobre, Febbraio e Giugno) da animali con genotipo noto ai loci caseinici. La determinazione del profilo acidico è stata effettuata in gas-cromatografia con rivelatore di fiamma (GC-FID). L'analisi statistica ha mostrato un'interessante associazione tra i polimorfismi della κ-caseina e la concentrazione di quattro acidi a lunga catena, C18:0 (acido stearico), C18:01 (acido oleico), C18:03 (acido linoleico) e C20:0 (acido arachico), i quali sono considerati potenziali fattori positivi per la salute umana.

Nel Capitolo 3, è stata determinata la composizione in acidi grassi del latte di pecora Valle del Belice, fornendo, per la prima volta, i dati sul profilo acidico del latte di questa razza siciliana. In questo studio, la separazione del grasso del latte è stata ottenuta con un metodo rapido, semplice e affidabile, che ha permesso di analizzare un gran numero di campioni. Gli esteri metilici degli acidi grassi (FAMEs) sono stati determinati con un gas-cromatografo (GC) dotato di rivelatore di massa (MS) per ottenere una migliore identificazione dei picchi ed è stata usata una colonna capillare, la SLB-IL111, dotata di fase stazionaria liquida ionica. Sono stati analizzati più di 400 campioni di latte. I grassi saturi, *cis*-monoinsaturi, e polinsaturi sono all'incirca il 4.20%, l' 1.05% e lo 0.42% del grasso totale, rispettivamente. Una buona risoluzione di tutti gli acidi grassi che si trovano comunemente nei campioni di latte è stata cosi ottenuta.

Nel Capitolo 4, è stata effettuato uno studio di associazione tra il profilo degli acidi grassi del latte di pecora Valle del Belice ed i polimorfismi presenti all'interno del gene Acetil-CoA carbossilasi (ACACA). Al fine di ridurre i costi tipici del sequenziamento di un gran numero di campioni di DNA, è stata eseguita una genotipizzazione selettiva. Sono stati genotipizzati, cioè, solo gli animali che presentavano valori fenotipici estremi per il carattere quantitativo considerato. Nel nostro caso sono stati scelti due gruppi estremi di 16 animali tenendo conto del livello medio di ciascun acido grasso ± 2 deviazioni standard. Sette esoni del gene ACACA sono stati sequenziati e 19 siti polimorfici sono stati identificati. L'analisi di associazione del carattere quantitativo eseguita per tutti gli SNPs ha mostrato la correlazione tra due SNPs differenti e la concentrazione di tre acidi grassi: l'acido caproico (C6:0), l'acido palmitoleico (C16:1) e l'acido linoleico (C18:2). I risultati ottenuti hanno mostrato un'incrementata concentrazione degli acidi grassi C6:0 e C16:1 in corrispondenza dell'allele T dello SNP1. Per quanto riguarda lo SNP3, l'allele T ha determinato un aumento della concentrazione del C6:0, mentre l'allele C un incremento della concentrazione del C16:1. La concentrazione del C18:2 diminuisce quando è presente il genotipo omozigote e aumenta con i genotipi eterozigoti.

Questi risultati confermano l’influenza del gene ACACA, un gene ovino potenzialmente candidato per il controllo del grasso nel latte, sul processo di sintesi degli acidi grassi che avviene nella ghiandola mammaria

Chapter 1

General introduction

Introduction

Milk is a complex biological fluid produced by the mammary glands of female mammals after they have given birth and it contains all nutritional elements required by newborn. Milk is a very important product for human consumption due to its complex and heterogeneous composition which allows different uses and, at the same time, makes it a complete food (Brunelli, 2008). It is basically composed of water, which represents 87% of the total weight, and of components of different nature both in solution (salts, water soluble vitamins, non-protein nitrogenous substances, sugars), either colloidal (part of the proteins and calcium phosphates and citrates) and emulsion (lipids and fat-soluble vitamins) state. The presence of macromolecules and micronutrients (carbohydrates, lipids and proteins) is one of the main features that make milk a so important food. Milk proteins have a high biological value and contribute to the nutritional value of milk as source of nitrogen and amino acids (Brunelli, 2008). As it is well known, among various functional foods, the milk has gained a new role as source of bioactive molecules able to influence some aspects of human health, such as some proteins and peptides originated from their hydrolysis which have been associated with physiological properties (Michalski, 2007). An important component of milk is represented by lipid fraction which has been the subject of several studies focusing on nutritional properties of Omega-3 and Omega-6 fatty acids, of CLA (Conjugated Linoleic Acids), and of the possible beneficial effects of the latter on human health identified through the efforts of scientific researches in the field of nutrition and foods (Glanz *et al.*, 2005; Hartmann *et al.*, 2007). Furthermore, milk contains other elements really important for human nutrition such as vitamins, enzymes, hormones, minerals and trace elements. All these milk constituents are closely linked among them and their concentration is different depending on species, breed, diet, lactation period, etc. (Table 1) (Salvadori del Prato, 1998; 2005). Depending on the species (goat, sheep, cattle), milk presents different physico-chemical characteristics. Goat milk differs from cow or human milk in having better digestibility, alkalinity, buffering capacity, and certain therapeutic values in medicine and human nutrition (Haenlein and Caccese, 1984; Park, 1989; 1994). Sheep milk has higher specific gravity, viscosity, refractive index, titratable acidity, and lower freezing point than average cow milk (Haenlein *et al.*,

2006). Lipids in sheep and goat milk have higher physical characteristics than in cow milk, but there are variations between different reports (Anifantakis, 1986; Park, 2006a). The composition of milk can be influenced by several factors, which may affect the characteristics of its structural elements and, consequently, the physico-chemical properties of milk itself. Depending on their origin, the factors of variation can be divided into endogenous and exogenous. The endogenous factors are divided into genetic (breed and individual characteristics) and physiological (health status and stage of lactation). Exogenous factors are mostly livestock and include feeding, climate, type of farming, relaying, technical and milking intervals. As for genetic causes, there is a hereditary individual variability in production and milk composition, typical of each animal of the same breed and in the same rearing conditions. Therefore, the milk composition varies depending on the breed considered and in particular, the fat content varies greatly between breeds and between individuals within the same breed (Mariani *et al*., 1987; Alais, 2000). In fact, fat and protein percentage in milk is determined for 40% by genetic (heritability) and 60% by environmental factors, while milk, fat, and protein yields (kg of fat and protein) are almost determined for 25% by genetics and 75%by environmental factors (Salvadori del Prato, 1998). Lactose and mineral salts have less variability and are indices used for the detection of normal milks, also because lactose is a limiting factor of the breast synthesis capacity and then of milk production. The most significant changes in milk composition are those associated with physiological state of the animal, in fact, during lactation changes occur in the content of various components (Alais, 2000; Strzalkowska *et al.*, 2002). Seasonal variations lead to an increase of the fat, dry matter, proteins and minerals during the winter while with temperatures between 0°C and 29°C, the percentage composition of constituents does not show significant changes. Nevertheless, temperatures above 30°C decrease level of production, protein content, non-fat solids and lactose. Furthermore, heat stress for temperatures above 30°C can lead to ipoacidic milk. Although changes in milk composition can be influenced by breeding system and management, feeding can have a fundamental role because it is able to induce changes more rapidly than other factors (Fredeen, 1996). Of particular interest is the influence that energy balance (Table 2) of feed ration may exercises on lipid composition, as well as on the amount of produced fat.

The lipid fraction is a crucial component from human nutrition point of view and it is a key element in the structure and aroma of many dairy products (Michalski, 2007). The fatty acid composition has different effects on quality (including physical properties: melting, crystallization and fractionation of the lipid fraction), organoleptic, as well as on nutritional properties of milk. Among the short chain fatty acids, the most representative is butyric acid which has protective role of the colon mucus membrane (Ballarini, 1998; Parodi, 1999; Russo *et al.*, 1999). The medium chain fatty acids have mainly metabolic and energetic functions, although in some studies it was demonstrated the hypercholesterolemic effect of lauric, myristic and palmitic acids due to the increase of LDL (Low-Density Lipoprotein) cholesterol (Temme *et al.*, 1996; Mensink *et al.*, 1998). The stearic acid seems to be neutral and not have an atherogenic effect although it is a saturated fatty acid (Mensink *et al.*, 1998). In particular, lauric and palmitic acids present medium harmfulness to human health, unlike myristic acid which is considered the most harmful (Ulbricht *et al.*, 1991).

Secchiari *et al.* (2002) focused their study on the determination of monounsaturated fatty acids (MUFA) effects on human health and some authors demonstrated that MUFA are able to reduce the level serum cholesterol as well as PUFA (polyunsaturated fatty acids). On the contrary of the ω-6 PUFA, the MUFA do not decrease the level of HDL (High-Density Lipoprotein) cholesterol (Mensink *et al.*, 1998). This last aspect is very important because the HDL has a protective role against coronary heart diseases. The protective role of unsaturated fatty acids in general and in particular of the oleic acid against various diseases is due to the maintenance of the functional integrity of cell membranes. The greater unsaturation of cell membranes leads to an increase of their fluidity with a consequent increase in cellular metabolism and, also, to an increase in the rate of cell division. The polyunsaturated fatty acids in milk, therefore, in addition to having energetic, metabolic and structural functions, have health-relevant activity, in particular extra-nutritional type. Nowadays, it is known that linoleic acid and in particular conjugated linoleic acid, also known for its anti-cancer and antioxidant activity, plays metabolic protective actions in case of infections, immunizations and stimulation of the immune system (Ballarini, 2000).

In recent years, the research on the relationship between the fatty acid composition and other milk components, mainly their interaction and their influence on technological and sensory

properties have increased (Chilliard *et al.*, 2004; Zarzynska *et al.*, 2014). The fact that milk constituents are influenced by genetic factors was demonstrated through the traditional quantitative approach. As reported by Moioli *et al.* (2007), this effect was simply treated as breed effect, so that statistically significant differences between breeds for fat and protein contents have been considered as significant genetic effects on these traits. However, genetic components have been calculated through analysis of milk records including genealogy registration. Estimates of the heritability are highly variable, depending on the breed and the used sample. Barillet *et al.* (1998) reviewed the genetic variation of dairy traits for small dairy ruminants, and concluded that differences in the estimates of heritability depend on the sampled animals and also on the employed statistical procedure, either lactation records or test-day records. However, Moioli *et al*. (2007) agree that heritability values on total lactation basis followed the same patterns as in cattle, and are between 0.5 and 0.6 for fat and protein contents. A realistic strategy to identify genes responsible for a trait is to verify the associations between polymorphisms of candidate genes (presumably involved in the trait according to their function or to analogies with other species) and different phenotypic expression levels. One of the research topics has been the study on the relationship between fatty acid composition and different genetic polymorphism of milk proteins (MacGibbon *et al.*, 1997; Bobe *et al.*, 1999, 2004; Chilliard *et al.*, 2006). In particular, Bobe *et al.* (2004) studied the possible association between different phenotypes at κ-casein and β-lactoglobulin and fatty acid composition of cow milk with special attention to the presence of certain fatty acids synthesized in the mammary gland. Moioli *et al.* (2007) reported a list of the so far identified variants in protein encoding genes for sheep and goats that have effects on milk constituents: caseins, β-lactoglobulin, α-lactalbumin; and encoding genes for enzymes directly involved in fatty acid metabolism: delta 9 desaturase (SCD), acetyl-CoA carboxylase (ACACA), diacylglycerol acyltransferase1 (DGAT1), lipoprotein lipase (LPL) and fatty acid-binding protein type 3 (FABP3).

This thesis is focused on these research topics and had the following objectives:

1. To verify the association between fatty acid composition and the genetic polymorphisms at casein loci in Girgentana goat milk.
2. To determine the fatty acid profile in Valle del Belice sheep milk.

3. To verify the association between fatty acid composition and the polymorphisms of Acetyl-CoA carboxylase (ACACA) gene in Valle del Belice sheep milk.

Table 1: Average composition of milk of different species. All components are expressed in percentage.

Species	**Water**	**Dry matter**	**Fat**	**Lactose**	**Sub. nitrogenous**	**Casein**
cow	87-89	11-13	3.4-3.6	4.6-4.7	3.4-3.6	2.5
buffalo	78-84	16-22	6-9	4.7-4.9	4.4-4.8	3.9
goat	83-89	11-17	4.3-4.4	4.3-4.7	4.0-4.2	3.0
sheep	79-82	18-21	5-7	4.5-5.0	5.6-6.0	4.5
she-ass	89-90	10-11	1.5	6.7	1.65	0.95
whale	52-55	45-48	35.0	0.7	10.0	-
bitch	81-82	18-19	4.0	4.8	9.0	4.5
mare	90-91	9-10	1.1	5.6	2.0	1.25
doe rabbit	70-71	29-30	12.0	1.8	13.0	9.0
woman	87-88	12-13	3.3	6.6	1.4	0.85
cat	81-83	17-19	4.0	4.9	9.1	2.8
reindeer	66-68	32-34	17.5	2.8	9.9	7.9
sow	82-84	16-18	5.0	3.0	7.2	3.7
zebu	81-82	18-19	5.2	5.1	4.2	3.3

Table 2: Effect of energy balance of feed ration on fatty acid composition (%) of milk fat (Corradini, 1995)

Fatty acid level energy	**100%**	**75%**	**50%**
Butyric acid	6.9	7.1	6.5
Caproic	3.4	3.1	2.3
Caprylic	1.8	1.4	1.0
Caprico	4.1	1.9	1.8
Sum 4:0-10:0	**16.2**	**14.5**	**11.6**
Lauric	4.9	3.1	1.9
Myristic	13.3	11.5	8.5
Sum 12:0-16:0	**57.3**	**48.0**	**38.7**
Palmitic	39.1	33.4	28.3
Stearic	7.2	7.8	7.9
Oleic	16.6	26.5	38.3
Linoleic	1.9	1.9	2.2
Linolenic Acid	0.8	1.3	1.3
Sum 18:0-18:3	**26.5**	**37.5**	**49.7**

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Chapter 2

Association between the polymorphism at casein loci and milk fatty acid composition in Girgentana goats

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Abstract

Goat polymorphism at casein genes can affect casein, fat and milk fatty acid (FA) composition. FA composition is an important trait for the goat dairy industry because of its influence on cheese yield and the organoleptic properties of dairy products. The aim of this work was to provide new data to better understand the influence of polymorphism at casein loci on fatty acid profile in Girgentana goat milk. One hundred milk samples from lactating Girgentana goats were collected. The procedure was developed using individual raw milk samples, collected in three different stages of lactation: October, February and June, from individuals with known genotypes at casein loci. The determination of the FA profile was performed by gas chromatography with flame ionization detector (GC-FID). Data set was analyzed using GLM procedures for repeated measure of SAS System v9.2. Our results showed that FA composition appears to be influenced by κ-casein genotypes especially for long-chain monounsaturated and polyunsaturated FAs which have positive effects on human health.

Keywords: Genetic polymorphism; caseins; fatty acid composition; GC-FID; Girgentana goat milk.

1. Introduction

Lipid composition is one of the most important components of the technological and nutritional quality of goat milk (Chilliard *et al.*, 2003). Lipids are involved in cheese yield (per kilogram of milk) and firmness, as well as in color and flavor of goat dairy products (Delacroix-Buchet *et al.*, 2000). Besides their quantitative contribution to the amount of dietary energy, the different fatty acids (FA) (short- and medium-chain, saturated, branched, mono- and polyunsaturated, cis and trans, conjugated) are potentially involved as positive or negative predisposing factors for the health of human consumers (Parodi, 1999; Sébédio *et al.*, 1999; Williams, 2000). Furthermore, the peculiarities of goat milk lipolytic system (Chilliard, 1982) and medium-chain FA (Ha *et al.*, 1993) could greatly change the content in free FA playing a major role in the occurrence of the characteristic goat flavor. The fat of goat's milk normally presents a 35% medium-chain FA (C6-C14) compared to cow's milk that contains 17%, whereas monounsaturated and polyunsaturated FA (MUFA and PUFA, respectively) are less abundant (Fontecha *et al.*, 2000). Among saturated fatty acids, caproic, caprylic and capric constitute 15% of the total fat in goat milk compared with 5% in bovine milk. Goats have been widely investigated for polymorphism of milk proteins which has been related to milk chemical composition, processing properties (coagulation properties, micelle size and mineralization, cheese yield, and sensory attributes), structural, biological and nutritional characteristics (Martin *et al.*, 2002; Ramunno *et al.*, 2007).

Goat polymorphism at casein genes can affect casein, fat and milk fatty acid (FA) composition (Pagano *et al.*, 2010). The aim of this work was to provide new data to better understand the influence of polymorphism at casein loci on fatty acid profile in Girgentana goat milk, a Sicilian autochthonous endangered breed reared for its good dairy production.

2. Materials and methods

2.1. Milk samples

Individual milk samples were collected from 100 lactating Girgentana goats during morning milking. Sampling was performed in three different stages of lactation October, February and

June from individuals with known genotypes at casein loci from one dairy farm located in Sicily. In particular, animals with A*A*, B*B*, A*B*, FF, A*F, B*F, A*E, EF, FN, NN and A*N genotypes (where A* indicates A, G, I, and H alleles, while B* indicates B1, B2, B3, B4 and C alleles) at αs_1-casein; CC, AC, A0', AC', C0', CC', and C'C' genotypes at β-casein; AA, AC, AF, CF, EF and FF genotypes at αs_2-casein; and AA, AB, AD, AN, BB, BD, BN, DD and D'G genotypes at κ-casein were analyzed. Goat raw milk samples were lyophilized and stored at -20 °C until analysis.

2.2. Fatty acids extraction

Milk fatty acids (FA) extraction was performed according to Rose-Gottlieb's method based on extraction with solvents. After reconstitution of lyophilized milk, 10 g of sample were weighed into 100 ml cylinder with cap. A volume of 1.5 ml of 25% ammonia solution and subsequently 10 ml of ethyl alcohol were added. The sample was stirred and after 25 ml of ethyl ether were added. The cylinder was then closed and stirred for 1 min. After that 25 ml of petroleum ether were added and the sample was mixed vigorously for 30 seconds. The obtained suspension was left to rest until the top layer became clear and separated from the lower one. The top layer is transferred to a vacuum flask. The extraction was repeated two more times in the same way only reducing the volumes of the two ethers to 15 ml and the extracts were collected in the same volumetric flask. The solvents evaporation was carried out with a Buchi Rotavapor R-215 and sample was incubated at 40°C in oven for 1 hour. For lipids transesterification, 5 ml of pentane was added to the obtained sample. Fatty acid methyl esters (FAMEs) were obtained by adding 0.5 ml of 2 N KOH in methanol. Finally, samples were analyzed by gas chromatography (Sağdiç *et al.*, 2004).

2.3. Gas chromatographic equipment

The FA profile determination was performed by gas chromatography using SHIMADZU GC-2010 System (Shimadzu, Kyoto, Japan) equipped with autosampler AOC-20i+s and flame ionization detector (GC-FID). A capillary column (Zebron ZB-WAX Plus 30m x 0:32 mm id, 0.2 mM film) (Phenomenex, Torrance, CA, USA) was used for FAMEs analyses. Helium (He) was used as carrier gas with a flow of 3 mL/min. The split ratio was 5:1. A sample aliquot was injected under the following GC conditions: oven temperature was set at 60°C for

2 min then increased to 150°C with a rate of 13°C/min, finally to 240°C at 2°C/min until the end of the run (Total Program Time: 53.92 min). The injector temperature was set at 240°C whereas the detector temperature at 250°C. The external standard method was used to calibrate the chromatographic system for FAMEs analysis.

2.4. Analysis of FAMEs

FAMEs were identified by comparison of their retention times with those of authentic standards (AccuStandard, Inc., New Haven, CT, USA). All FA were analytical reagent grade with minimum 99% purity. In particular, standards of 13 methyl esters were first analyzed as individual standard for peaks identification according to retention time, and then in mixture with known concentration. For each standard, four mixtures were prepared at different concentrations (Table 1) for the construction of curves to four points which relate peak areas with concentration of corresponding standard. Each FA was quantified using a four-point calibration of mixed standard solution. All the results concerning the milk FA composition were expressed as w/w (%) total FA (Donmez, 1998; Tatar *et al.*, 2001; Arici *et al.*, 2002).

2.5. Method validation

2.5.1 Linearity

Linearity of the method was estimated by calculating the regression line with the method of least squares and the corresponding correlation coefficient plotting the value of peak areas as a function of the concentrations of the standard solutions at different concentrations. For each concentration three measurements were performed.

2.5.2 Precision

Precision of the method was checked through the repeatability and reproducibility experiment. Repeatability of the method was evaluated by four preparations of FAME samples and each preparation was analyzed in duplicate according to the method.

The obtained analytical data for each FA were used to estimate the average value of concentration, the standard deviation and the coefficient of variability (CV) (Table 2).

Reproducibility was evaluated comparing the results of repeatability with the ones obtained on the same sample prepared by different analysts during two different days. The difference between two analyses was determined using *t*-test.

2.6. Statistical analysis

Data were analyzed according to the linear model (GLM procedures) for repeated measure of SAS System v9.2. Student *t*test was performed to compare mean concentrations of each FA relative to the sampling season and evaluations were based on a significance level of 5% ($p<0.05$). The model included the genotype and the interaction genotype-sampling season: y= genotype+genotype *x* sampling season+*e*

where y is the mean concentration of individual FA in milk fat and *e* is random error.

3. Results and discussion

The average values of FA composition of analyzed milk samples belonging to individuals with different genotype for the four casein loci were estimated for the three main stages of lactation (Table 3).

Statistical analysis of our data showed statistically significant associations between FAs concentration and genetic polymorphism at κ-casein gene.

In particular, it is possible to note that fatty acid composition appears to be influenced by κ-casein genotypes especially for long-chain monounsaturated and polyunsaturated FAs which have positive effects on human health. Considering the same sampling season, the fat of individuals with AA genotype showed higher concentration of stearic acid (C18:0) and oleic acid (C18:1) than other genotypes. At the same time, a reduced concentration of linoleic acid (C18:2) and arachidonic acid (C20:0) was found in heterozygous individuals AD respect to the others.

Genetic polymorphisms of the other three casein genes did not produce statistically significant changes in the fatty acid profile.

The κ-casein protein is really important in milk production and processing as it significantly affects cheese yield and milk technological properties (Ikonen *et al.*, 1999). In fact, goat A allele at κ-casein gene seems to confer greater stability to dispersed casein micelles and

consequently milk with this variant is more suitable for cheese production (Russo *et al.*, 1978).

Our results on FA composition in Girgentana goat milk were in agreement with those reported by Bonanno *et al.*, (2013). Although κ-casein polymorphism could affect fatty acid profile the greatest difference in fatty acid composition was due to several factors such as species, breed (Malacarne *et al.*, 2001.; Carroll *et al.*, 2006), feeding, farming system and environmental conditions (Palmquist *et al.*, 1993.; Ferlay *et al.*, 2006.; Ferlay *et al.*, 2008).

4. Conclusions

Our results showed that genetic polymorphisms at κ-casein could have effects on FA composition especially for MUFA and PUFA potentially involved as positive factors in human health. The association between κ-casein AA and AD genotypes and fatty acids in Girgentana goat milk could be useful for re-evaluating the possible use of fresh drinking milk from this breed. Furthermore, preservation of breeds in danger of extinction could be achieved by establishing economic reasons for their survival.

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FAMEs	Standard 1 mg/ml	Standard 2 mg/ml	Standard 3 mg/ml	Standard 4 mg/ml
Methyl linolenate	0.1	0.2	0.4	0.8
Methyltrans9octadecenoate	0.1	0.2	0.4	0.8
Methyl cis-9-octadecenoate	0.2	0.4	0.8	1.6
Methyl cis-9hexadecenoate	0.1	0.2	0.4	0.8
Methyl octadecanoate	0.2	0.4	0.8	1.6
Methyl heptadecanoate	0.1	0.2	0.4	0.8
Methyl hexadecanoate	0.1	0.2	0.4	0.8
Methyl tetradecanoate	0.2	0.4	0.8	1.6
Methyl dodecanoate	0.2	0.4	0.8	1.6
Methyl decanoate	0.2	0.4	0.8	1.6
Methyl octanoate	0.2	0.4	0.8	1.6
Arachidic acid methyl ester	0.2	0.4	0.8	1.6
Methyl tetranoate	0.2	0.4	0.8	1.6
Methyl hexanoate	0.2	0.4	0.8	1.6

Table 1: Dilute solutions for fatty acids calibration

Table 2: Estimated values for each fatty acid within goat milk analyzed samples.

Fatty acid	Number of analyzed sample	Mean g/100g	Standard deviation	Variability coefficient %	Repeatability limit
C4	8	0.51	0.10	20.11	0.34
C6	8	2.05	0.46	22.27	1.53
C8	8	0.77	0.18	23.90	0.61
C10	8	9.13	2.31	25.37	7.74
C12	8	4.11	1.06	25.95	3.56
C14	8	2.31	0.60	26.08	2.02
C16	8	6.27	1.59	25.44	5.33
C16:1	7	0.24	0.11	44.70	0.38
C17	7	0.22	0.06	25.96	0.20
C18	5	8.29	2.10	25.37	7.03
C18:1	8	8.52	1.98	23.28	6.63
C18:2	8	0.19	0.08	42.93	0.28
C20	8	0.08	0.02	23.68	0.06

Table 3: Mean concentration (g/100 g total fat) of fatty acids in relation to genotypes at κ-casein and to the stages of lactation. Values with different letters within same rows are significantly different (a,b=P<0.05; A,B=P<0.01). Season 1=October, Seanon 2=February, Season 3=June.

Fatty acid	κ-casein genotypes								
	AA			AB			AD		
	Season			Season			Season		
	1	2	3	1	2	3	1	2	3
C4:0	0.37±0.025	0.45±0.027	0.42±0.03	0.34±0.02	0.44±0.02	0.37±0.02	0.37±0.03	0.41±0.03	0.38±003
C6:0	1.48±0.10	1.73±0.11	1.54±0.11	1.42±0.08	1.64±0.07	1.41±0.07	1.60±0.12	1.53±0.12	1.41±0.11
C8:0	0.57±0.04	0.62±0.04	0.54±0.05	0.55±0.03	0.58±0.03	0.50±0.03	0.63±0.05	0.54±0.05	0.50±0.05
C10:0	7.69±0.56	8.55±0.60	6.77±0.62	7.55±0.42	8.01±0.41	6.67±0.37	9.02±0.63	7.21±0.67	5.97±0.63
C12:0	4.40±0.34	4.36±0.36	3.06±0.37	4.29±0.26	4.07±0.25	3.24±0.23	5.28±0.38	3.45±0.41	2.63±0.38
C14:0	2.53±0.18	2.82±0.19	2.08±0.20	2.38±0.14	2.79±0.13	1.99±0.12	2.49±0.20	2.45±0.22	1.64±0.20
C16:0	4.47±0.38	6.27±0.40	3.90±0.42	4.95±0.29	6.06±0.28	4.01±0.25	4.60±0.43	5.62±0.46	3.38±0.43
C16:1	0.19±0.02	0.21±0.02	0.14±0.02	0.20±0.01	0.22±0.01	0.14±0.01	0.19±0.02	0.23±0.02	0.11±0.02
C17:0	0.14±0.02	0.23±0.02	0.15±0.02	0.14±0.01	0.24±0.01	0.13±0.01	0.15±0.02	0.27±0.02	0.14±0.02
C18:0	3.90 ± 0.34^{a}	3.88 ± 0.37^{ab}	4.67 ± 0.38^{ab}	3.26 ± 0.26^{ab}	3.93 ± 0.25^{ab}	4.23 ± 0.23^{ab}	2.70 ± 0.39^{b}	3.95 ± 0.42^{ab}	4.77 ± 0.39^{ab}
C18:1	5.83 ± 0.47^{a}	6.32 ± 0.50^{ab}	5.47 ± 0.52^{ab}	5.40 ± 0.36^{ab}	7.17 ± 0.34^{ab}	5.04 ± 0.31^{ab}	4.46 ± 0.53^{b}	7.20 ± 0.57^{ab}	4.79 ± 0.53^{ab}
C18:3	0.15 ± 0.02^{A}	0.16 ± 0.02^{AB}	0.14 ± 0.02^{AB}	0.17 ± 0.01^{AB}	0.19 ± 0.01^{AB}	0.13 ± 0.01^{AB}	0.22 ± 0.02^{B}	0.20 ± 0.02^{AB}	0.14 ± 0.02^{AB}
C20:0	0.044 ± 0.006^{AB}	0.077 ± 0.006^{A}	0.036 ± 0.006^{AB}	0.041 ± 0.004^{AB}	0.088 ± 0.004^{AB}	0.037 ± 0.004^{AB}	0.040 ± 0.006^{AB}	0.103 ± 0.007^{B}	0.037 ± 0.007^{AB}

Chapter 3

GC-MS quantification of fatty acid profile in sheepmilk of Valle del Belice breed

Abstract

Aim of this study was to evaluate the fatty acid profile in milk of Valle del Belice breed. The fatty acid methyl esters (FAMEs) were determined by GC coupled with MS to achieve more accurate peak identification. A novel fused capillary gas chromatography, the ionic liquid SLB-IL111 column, available from Supelco Inc., was used. It is GC column capable of providing enhanced separations of FAMEs compared to the highly polar cyanopropyl siloxane columns currently recommended for analysis of *cis* and *trans* isomers of fatty acids. Good resolution of all fatty acids commonly found in milk sample was achieved. More than 400 samples of fat extracted from whole milk were processed. Saturated, *cis*-monounsaturated, and polyunsaturated fatty acids were present at 4.20%, 1.05% and 0.42% of total fat, respectively.

Keywords: fatty acids profile, GC-MS, sheep milk, Valle del Belice breed

1. Introduction

In recent years, consumer's attention has grown in importance due to nutritional needs and consumption of healthy foods that could have positive effects on health or prevent the onset of certain diseases. Several studies have shown that products of animal origin contributed significantly in providing beneficial effects to human health (Williams, 2000; Parodi, 2009). In particular, lipid fraction of milk has been the subject of numerous studies focusing on its nutritional characteristics and the possible beneficial effects on human health identified through the efforts of scientific researches in the field of nutrition and feeding (Glanz *et al.*, 2005; Parodi, 2009). The quality of dietary lipids could be an important modulator in terms of morbidity and mortality of a lot of diseases like hyperlipidemia, arteriosclerosis, obesity, diabetes mellitus and hypertension; it is also assumed that one-third of human cancers is associated with dietary habits and lifestyle (Williams, 2000). Because of the rapid increase in the number of elderly people these diseases are medically and socio-economically important. In this context, the fat cheese content and in particular the milk fatty acid composition can play an important role. The fatty acid (FA) composition and in particular the presence of some monounsatured acids such as oleic acid (C18:1n9c) and polyunsatured acids such as α-linolenic acid (C18: 3n-3) (Parodi, 2009) was of primary importance. FA composition influenced milk fat quality, contributing to its physical (crystallization and fractionation of fat, hardness and melting point of the butter) and sensory (free short chain FA, oxidation products) properties (Chilliard *et al.*, 2000).

However, the consumption of milk fat has been subject to many criticisms due to in the presence of some saturated fatty acids which would be responsible of raising the level of plasma cholesterol associated with lipoproteins of low density. The milk of ruminants is, in fact, characterized by a higher content in short-chain fatty acids than human milk, mainly saturated fatty acids and a ratio unsaturated/saturated lower than human one. However, the milk of ruminants showed content in PUFA-ω3 which has been shown to exert potential benefits to human health including protection against carcinogenesis, atherosclerosis, diabetes, inflammation, cardiovascular and autoimmune diseases (Parodi, 2009).

The FA composition of milk fat determined by gas chromatography with capillary columns has been studied for many decades and is still highly relevant for dairy research and authentication studies (Hartig, 2008; Kandhro *et al.*, 2008). Milk FA analysis presented some complexities due to the wide range of the molecular size and the presence of relatively large quantities of short-chain FAs. The problem of converting milk FAs to methyl esters before analysis by gas chromatography has been solved with the rapid reference methylation procedure (ISO-IDF, 2002). The most widely used techniques, such as the Rose-Gottlieb procedure (Rapporti ISTISAN) for milk fat separation, are primarily based on extraction with solvents. In the reference procedure (ISO-IDF, 2002), lipids are extracted using a mixture of diethyl ether and n-pentane after first adding an ammonium hydroxide solution. However, these extraction methods are time consuming requiring a lot of time to analyze just few samples. In this study, the milk fat separation was obtained with a rapid method reliable and simple which allowed us to analyze a large number of samples. The aim of this work was to evaluate the fatty acid profile in Valle del Belice sheep milk, providing, for the first time, data on milk fatty acid composition of this Sicilian breed.

2. Materials and methods

2.1. Chemicals and reagents

N-heptane (EMPLURA), sodium hydrogen sulphate monohydrate and potassium hydroxide (2N) in methyl alcohol solution were obtained from VWR (Milano, Italy). A standard mixture of common FAs (Supelco 37 component FAME Mix) was obtained from Supelco (Supelco, Sigma-Aldrich, Milano, Italy).

2.2. Animals and experimental design

The experiment was carried out in one farm of Valle del Belice dairy ewes located in Agrigento province (Sicily). The Valle del Belice is a Sicilian autochthonous dairy sheep breed obtained through repeated crosses between Pinzirita, Comisana and Sarda sheep breed and subsequent selective crossbreeding and inbreeding. The Valle del Belice sheep is reared because of its high productivity and adaptability and represents the most important dairy breed in Sicily, and an important element of the local dairy sector. Sheep milk is processed

into high quality cheese mainly POD cheeses. From Valle del Belice sheep milk it's possible to obtain a particular cheese that in 2010 became a POD cheese: *Vastedda of Valle del Belice.* For our aim individual milk samples from 113 lactating ewes were collected every two weeks for a two-month period, from April to May 2014. Two aliquots of each milk samples were collected during morning milking and were immediately stored at 4°C in a portable refrigerator. One aliquot (50 mL) was analyzed to determine fat, protein, lactose, casein, non-fat solids and somatic cell count (MilkoScan FT and Fossomatic connected in series, Foss Elecrtic, Hillerod, Denmark) content. The other aliquot (50 mL) was frozen at -20°C and successively analyzed for the FA composition.

2.3. Milk fat extraction

Milk fat extraction was obtained by centrifugation of individual milk samples at 7300 rpm for 30 min at -4°C (Renna *et al*., 2012). Subsequently, the top fat layer was removed, washed once with distilled water and centrifuged at the same speed and temperature for 15 min. A sample of 100 mg of pure fat was dissolved in 5 ml of N-heptane. Fatty acid methyl esters (FAMEs) were obtained by trans-esterification of glycerides, adding 0.2 ml of KOH 2N in methanol as described by International Standard method ISO-IDF (ISO, 2002). Each tube containing the sample was vortexed for 1 min. The solution was neutralized by addition of 0.5 g of sodium hydrogen sulphate monohydrate. Finally, after centrifugation at 350 *g* for 5 min at room temperature, 2 mL of upper phase were transferred to a vial for gas-chromatographic analysis. FAs were determined by GC coupled with MS.

2.4. Determination and quantification of FAMEs

GC/MS analyses were performed at programmed temperature on an Agilent-6890 gas chromatograph (GC) equipped with an Agilent-7683 automated liquid sampler, split/splitless injector, and an Agilent-5973N quadrupole mass selective detector (Agilent Technologies, Santa Clara, CA, USA). A novel fused capillary column SLB-IL 111 with dimension of 100 m x 0.25 mm, 0.20 μm film thickness (Sigma-Aldrich, Bellefonte, Pennsylvania, USA) was used for FAMEs separation. The SLB™-IL111 column exhibits the highest polarity of any GC phase allowing resolving key *cis/trans* FAME isomers that cannot be resolved on other columns. To optimize the analysis condition, the following temperature programming were

used: oven temperature was set at 140°C for 5 min, then increased to 180°C at a rate of 8°C/min, to 260°C at 5°C/min for 5 min until the end of the run (Total Program Time: 31 min). The split ratio was set to 1:50 and the typical injection volume was 2 µl. The carrier gas was helium at a column flow rate of 1 ml/min. The injector and detector temperatures were 230°C and 250°C, respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z. Peak identification of each FAs was carried out comparing retention times from milk samples with retention times and mass spectra of known standards used for confirmation of GC-MS libraries result (NIST 2005) [10]. FAMEs were identified using a commercial mixture of methyl esters, the 37 Component FAME Mix, available from Supelco, (Supelco, Sigma-Aldrich, Milano, Italy). Chromatogram peak areas were acquired and calculated by Chemstation software MSD G1701 DA (Agilent Technologies, Santa Clara, CA, USA) and expressed in percentage of the total identified FAMEs.

2.5. Method's validation

Linearity of the method was estimated by calculating the regression line with the method of least squares and the corresponding correlation coefficient, plotted the value of the peak areas as a function of the concentrations of the standard solutions at different concentrations (R^2>0.987). For each concentration three measurements were performed.

3. Results and discussion

The method was validated was and then used for analysis of FA profile in different milk samples. The chromatographic separations presented in this study were all obtained using the 100 m Supelco SLB-IL111 capillary column and were reproducible. Highly polar stationary phase was favourably used to obtain a good separation of positional and geometrical isomers. As described by Delmonte *et al*. (2011), for each FA chain length, the saturated FA eluted first followed by MUFAs with *trans* double bonds and then those with *cis* double bonds. The increased polarity of the ionic column generally resulted in the elution of saturated FAs in the transition area between the major *trans* and *cis* clusters of FAs with one carbon less. Regardless of chain length and geometric configuration, the positional isomers of MUFAs

eluted in the order of increasing Δ values. Coupling gas chromatography and mass spectrometry (GC/MS) provides multidimensional information about an analyte *per sè*. The mass spectrum of FAMEs was carried out in Scan Mode. In GC/MS, saturated, *iso*- and *anteiso*-FAMEs as well as MUFA were determined with m/z 74 and m/z 55. The molecular ions appear at the corresponding molecular weight. PUFA were determined with m/z 79 (three and more double bonds) and m/z 81 (dienoic FAs) (Thurnhofer and Vetter, 2005). The obtained resolution of FAMEs was better in comparison with data shown in references for *cis* and *trans* isomers especially for elaidic and oleic acid (Delmonte *et al.*, 2011). Mean results for milk yield, percentages of fat and protein, concentrations of each FA present in milk for primiparae and multiparous Valle del Belice sheep are presented in Table 1. Table 2 shows FAs profile of milk by different breeds: Altamurana, Gentile di Puglia and Sarda (Signorelli *et al*., 2008). Our results showed differences between breeds for many FAs and in particular for those that contribute to more than 50% of the FAs composition (C14:0; C16:0; C18:1). There were no differences in C16:1 belonging to MUFA and in C18:2 belonging to PUFA). Compared to Altamurana, Gentile di Puglia and Sarda breeds, the Valle del Belice sheep milk showed a higher content of caproic acid (C6:0), caprylic acid (C:8), lauric acid (C12:0), myristic acid (C14:0), α-linolenic acid (C18:3n3) and a smaller content in palmitic acid (C16:0) and oleic acid (C18:1 cis-9).

The milk of the Valle del Belice is similar to Sarda in the content of C4:0 and C14:0, but has a higher content than Sarda of short and medium FAs. MUFA are lowest in Valle del Belice breed, but no differences between breeds were evident for PUFA, except for C18:3n3. Our obtained values for n-3 FA content of milk, and that of ALA (C18:3 n-3) in particular, are higher than those reported previously by several authors in different breeds (Dimitrov *et al*., (2001); Mihaylova *et al*., (2004), Mele *et al*., (2006), Federica *et al*., (2008), de Gerchev *et al.*, (2009), De La Fuente *et al*., (2009), Sanchez *et al*., (2010)).

4. Conclusions

The method for the determination of fatty acids profile of ovine milk samples has been optimized on SLB-IL111column and results showed that it has acceptable selectivity, linearity

and precision and it is suitable for routine analysis of samples of animal origin with a very distinct FA profile. No previous data on FA composition of milk fat of the Valle del Belice breed were found in literature. The obtained results confirm that differences in content and properties of some FAs among and within breed are an important factor explaining the differences in sensory characteristics and quality of milk. The FAs profile of Valle del Belice milk showed a total FA, in general, and in particular of n3-FA content able to improve nutritional value of fat for human consumption, thanks to their role in the prevention of coronary heart disease and to their anti-carcinogenic and anti-lipogenic properties (Williams, 2000).

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Table 1: Mean values and standard deviation (S.D.) of milk yield, percentages of fat and protein and concentration (g/100g total fat) of fatty acid in Valle del Belice sheep breed

Variable	Mean g/100 g total fat	S.D.
Milk yield, g/d	1337.45	534.28
Fat, %	7.13	1.071
Protein, %	5.85	0.63
Saturated		
C4:0	5.07	3.15
C6:0	3.10	1.43
C8:0	3.47	1.23
C10:0	7.02	3.81
C11:0	1.06	0.05
C12:0	4.82	2.15
C13:0	0.97	0.05
C14:0	9.22	5.47
C15:0	1.09	0.40
C16:0	17.48	10.69
C17:0	0.81	0.12
C18:0	6.82	3.71
C22:0	0.97	0.08
MUFA		
C14:1	1.18	0.19
C16:1	1.35	0.78
C18:1n9t	2.95	1.75
C18:1n9c	11.24	6.49
PUFA		
C18:2n6t	1.08	0.38
C18:2n6c	1.76	0.83
C18:3α	2.55	1.44
C20:2	1.29	0.47

Table 2: Mean values and standard deviation (S.D.) of milk yield, percentages of fat and protein and concentration (g/100g total fat) of fatty acids in different Italian sheep breeds (Signorelli *et al.*, 2008)

Variables	Altamurana		Gentile di Puglia		Sarda	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Milk yield (cl)	320.08	173.33	195.00	99.60	519.54	198.23
Fat, %	9.53	1.90	9.65	1.667	7.46	1.288
Protein, %	6.73	0.72	6.93	0.84	5.45	0.39
C4:0	4.58	0.79	4.18	0.67	5.13	0.79
C6:0	2.87	0.47	2.83	0.55	2.75	0.57
C8:0	2.39	0.48	2.52	0.65	2.20	0.64
C10:0	6.32	1.53	6.94	2.08	5.56	1.78
C10:1	0.24	0.07	0.26	0.09	0.20	0.07
C12:0	3.96	0.93	4.07	1.16	3.51	0.88
C14:0	10.48	1.29	10.03	1.19	9.25	1.09
C14:1	0.27	0.08	0.29	0.10	0.21	0.06
C16:0	25.26	2.82	23.51	1.90	23.24	2.08
C16:1	1.27	0.25	1.28	0.32	1.11	0.25
C18:0	9.27	2.30	9.91	2.23	9.43	2.37
C18:1	23.66	3.48	24.84	3.09	26.00	3.19
C18:2	3.14	0.75	2.94	0.17	4.18	1.31
C18:3	0.60	0.17	0.55	0.20	0.83	0.31
Saturated FA	66.700	4.17	65.62	3.83	62.85	4.70
PUFA	5.31	1.56	5.03	1.56	7.00	2.34
MUFA	25.74	3.33	26.99	2.98	27.80	3.14

Chapter 4

Association between ovine Acetyl-CoA carboxylase gene polymorphisms and fatty acids profile in Valle del Belice sheep milk

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Abstract

The present study gives evidence for association between polymorphisms of gene coding for the ACAC-α (acetyl-CoA carboxylase alpha) and the fatty acid (FA) profile of ovine milk. Sheep milk was chosen because it contains higher total fat percentage than milk of other ruminants. In this study, a selective genotyping approach was applied to select the animals to be genotyped. On the basis of the mean level of each FA ± 2 times the standard deviation, we obtained two extreme groups of 8 animals each. Seven exons of the ACACA gene were sequenced and 19 SNPs were identified. The association analyses performed in the present study revealed 5 significant associations at the 5% nominal level between two different SNPs and the concentration of three fatty acids: caproic acid (C6:0), palmitoleic acid (C16:1) and linoleic acid (C18:2). These results confirm the influence of ACAC-α gene, an ovine candidate gene potentially controlling milk fat trait, on fatty acid synthesis in the mammary gland.

Keywords: Milk fatty acids; Acetyl-CoA carboxylase gene; Single nucleotide polymorphisms.

1. Introduction

Early studies on ruminants have been defined and quantified major metabolic aspects of mammary lipid metabolism including *de novo* synthesis and fatty acid (FA) uptake from blood (Akers, 2002; Neville *et al.*, 2002; Ferreira *et al.*, 2013). Moioli *et al.* (2007), through candidate gene approach, identified in sheep the molecular mechanisms of the genes encoding enzymes directly involved in fatty acid metabolism: Acetyl-CoA carboxylase (ACACA), Stearoyl-CoA desaturase (SCD), and diacyl-glycerol acyl-transferase (DGAT1). A candidate gene is a gene supposed to be responsible for a considerable amount of the genetic variation of a trait. The candidate gene approach is based on the search for DNA polymorphism in genes that are expected, from knowledge of their physiological role (also for their position along the genome or for their level of expression) to have an influence on target traits. Therefore, rather than randomly looking for genes throughout the genome, it is desirable to focus on genes, which may already be suspected to have a role in the expression of the investigated trait. The FA composition is a function of the action of several enzymes and accurate knowledge of lipid metabolism may help identify candidate genes that significantly affect the content of particular FAs. A gene that encodes one of the key enzymes of FA synthesis in the mammary gland is ACACA which is the rate-limiting step in FA synthesis. ACACA is a complex multifunctional enzyme, biotin-containing, expressed ubiquitously with higher levels of expression in lipogenic tissues like liver, adipose tissue and mammary gland during lactation. This enzyme catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA which is the substrate for palmitic acid (C16:0) synthesis and very long-chain FAs (acyl-CoA>C22:0) by the fatty acid synthase (FAS) enzyme (Smith *et al.*, 2003; Leonard *et al.*, 2004). Multiple alternatively spliced transcript variants, divergent in the 5' sequences and encoding distinct isoforms, have been found for this gene. Milk quality is an important economic trait for dairy ewes because it is totally used for dairy products. Milk fat composition can be changed by selective breeding systems which offer to consumer the opportunities to know healthy and technological aspects. In fact, the presence of direct association between markers and traits of interest can be used to select animals with different genotypes for those markers. Different expression level of ACACA gene in lactating

mammary glands of two Italian sheep breeds (Gentile di Puglia and Sarda) suggested a direct involvement of this gene in FA synthesis during lactation due to different milk fat content of the two breeds (Veltri, 2000). Single nucleotide polymorphisms (SNPs) were detected both in Promoter I (Moioli *et al*., 2005a) and Promoter II (Moioli *et al*., 2005b) of ACACA gene with frequencies significantly different between the Sarda breed and three other Italian sheep breeds (Gentile di Puglia, Comisana, and Sopravissana). García-Fernández *et al*. (2010) sequenced approximately 6.6 kb of the coding sequence of the ACACA ovine gene and identified a total of 22 synonymous SNPs. Polymorphisms at ACACA gene are also detected in goat species (Badaoui *et al*., 2007a).. In fact, Badaoui *et al*. (2007a) identified a silent mutation in exon 45 (C5493T) that was associated with fat yield, lactose content and somatic cells count.

In this work we aimed i) to sequence part of the encoding region of ACACA gene in Valle del Belice dairy ewes in order to identify polymorphic sites; ii) to analyse the FA profile of Valle del Belice dairy ewes during different lactation periods; iii) to estimate possible associations among polymorphisms and FA profile.

2. Materials and methods

2.1. Sampling and fatty acid methyl esters (FAMEs) analysis

The study was conducted on 113 Valle del Belice dairy ewes belonging to the same farm. From April to May 2014, individual milk samples were collected every two weeks. More than 400 samples of extracted fat were processed and analysed with gas chromatograph (GC) equipped with quadrupole mass selective (MS) detector (Agilent-6890, Agilent Technologies, Santa Clara, CA, USA).

The milk FA composition was determined as explained by Renna *et al*. (2012). Briefly, after extraction of total fat milk, the FA content (grams per 100 grams of total FA) was determined by GC-MS after methylation of free FAs with KOH 2N in methanol ISO-IDF (ISO, 2002).

After FAMEs analyses on all samples, in order to identify the animals to be genotyped, a selective genotyping approach was used (Darvasi *et al*., 1992). This approach aim to genotype only individuals at the phenotypic extreme of the population and to reduce the genotyping

costs in marker-QTL linkage determination. As criteria, the mean values of each FA ± 2 standard deviations were used to identify the highest and lowest phenotypic values. In this study, two groups of 8 animals each were genotyped.

2.2. Genomic DNA extraction and genotyping of ACACA gene

A total of 113 blood samples from Valle del Belice sheep were collecting from a local farm located in Agrigento. Genomic DNA was extracted using salting out method (Miller *et al*., 1988). After checking quantity and quality of extracted DNA with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), all samples were diluted and stored at 4°C until use. A total of 16 DNA samples were genotyped for 11, 13, 17, 19, 37, 42, 53 exons of ACACA gene. Amplifications were done in a final volume of 25 µl containing: 0.5 µM of each primers (Table 1), 0.6 mM of dNTD Mix, 1 U of Taq DNA Polymerase (Fermentas, Hanover, MD, USA), 1x PCR buffer with KCl, 2.5-3.5 mM $MgCl_2$, and approximately 100 ng of genomic DNA. Thermal cycling conditions were an initial denaturation at 95°C for 3 min, 30 cycles at 95°C, 60°-68°C and 72°C for 3 min each and a final extension at 72°C for 5 min. PCR fragments were checked by electrophoresis on agarose gel at 2% stained with ethidium bromide and then purified using 10 U of Exonuclease I and 1 U of Shrimp Alkaline Phosphatase (Fermentas, Hanover, MD, USA). DNA sequencing reaction was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) with 5 µM of the same primers used in the PCR reaction. Cycle sequencing reaction was performed according to manufacturer's instruction following Ethanol/EDTA/Sodium Acetate precipitation. Sequencing analyses were performed in an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The obtained nucleotide sequences were analysed by Sequencing Analysis v5.3.1 software and SeqScape v2.5 software (Applied Biosystem, Carlsbad, CA, USA) in order to detect polymorphic sites.

2.3. Statistical analysis

A preliminary analysis of data was performed with SAS System v9.2 using the PROC ALLELE procedures to estimate genetic diversity (allelic and genotypic frequencies, heterozygosity, etc.) and Hardy Weinberg Equilibrium (HWE) of the identified polymorphic sites. Only polymorphic sites (SNPs) were used for further statistical analysis. The association

study between each SNPs and FA consisted in a series of single locus statistic test, examining each SNP independently for association with phenotype. In particular, all FAs were analyzed using a generalized least square method implemented in ASReml 3 software. Analysis of variance (ANOVA) was performed to detect significant factors within the following model:

$y_{ij} = \mu_i + SNP_j$

where y_{ij} is the FA concentration, μ is the mean level of each FA and SNP_j represents the number of copies of a particular allele that animal is carrying at the studied locus (e.g 0, TT; 1, TC; 2, CC). The test was considered significant for a $P \leq 0.001$.

3. Results and discussion

Sequencing analysis performed on 16 animals revealed the presence of 19 polymorphic sites in seven exons (11, 13, 17, 19, 37, 42, and 53) of ACACA gene. Of these 19 polymorphic sites, 5 were monomorphic and were discarded from the further analysis. These 5 polymorphic sites were considered in the total number of found polymorphisms (19 in total) because they were different comparing with GenBank sequence (Acc. No. ENSOARG00000000829). In Table 2, Polymorphism Information Content (PIC), Observed Heterozygosity and Allelic diversity are reported. Results of Hardy–Weinberg equilibrium test on considered population are in agreement with for all SNPs (data not shown).

Analysis of the SNPs frequencies (Table 3) showed that the major allele was always at frequency >50% in all cases. The statistical analysis showed that of 14 SNPs, only two had a statistical significant association with the concentration of several FAs. In particular, SNP1 (in exon 11) was associated with caproic acid (C6:0) and palmitoleic acid (C16:1). The second association involved SNP3 (in exon 13) and C6:0, C16:1 and linoleic acid (C18:2) content ($P \leq 0.001$). The percentage effects of SNP1 and SNP3 genotypes on FA concentration are reported, respectively, in Table 4 and 5.

The obtained results showed an increased concentration of C6:0 and C16:1 associated with T allele of SNP1. For SNP3, allele T determined an increased concentration of C6:0, whereas allele C an increased concentration of C16:1. The concentration of C18:2 decreased when

homozygote genotypes (TT and CC) are present and increased with heterozygote genotype (CT).

Our results agree with those reported in a previous study published by Garcìa-Fernandez *et al.* (2010) which showed significant associations between ACACA polymorphisms and several FAs in Churra sheep breed. Moioli *et al.* (2013) carried out an association study among one SNP in Promoter III of ACACA gene and fat and protein content and milk yield in two different breeds, Altamurana and Gentile di Puglia, and concluded that fat content was the only trait to be significantly influenced by SNP effect.

The caproic and linoleic acids, commonly found in milk of small ruminants, have an important role from the therapeutic point of view (Babayan, 1981). Capric acid was used as specific treatment for patients suffering from pancreatic insufficiency, deficiency or absence of bile salts. This acid is also used in diets of undernourished patients, premature babies and those who suffer of childhood epilepsy, thanks to the large capacity of "Energy giving" which derives from the structure of this compound (Babayan, 1981; Haenlein, 1992; Garcia Unciti, 1996). The linoleic acid is known for its anti-cancer and antioxidant activity, plays metabolic protective actions in case of infections, immunizations and stimulation of the immune system (Ballarini, 2000).

4.Conclusions

Within ACACA gene of Valle del Belice sheep breed, only two SNPs presented a statistical significant association with three FAs content: caproic, palmitoleic and linoleic acid. Our results suggest that the ACACA gene, which is the major regulatory enzyme of FA biosynthesis, is certainly a candidate gene affecting milk quality and controlling FA synthesis in the mammary gland. In addition, polymorphic information regarding this gene could be used within selection scheme of Valle del Belice sheep breeds in order to obtain milk with specific FA profile and, therefore, specific nutritional values.

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Table 1: Amplified ACACA exons, with respectively primer sets and length of amplification fragments.

ACACA exon	Primers	Length of fragment
11	FW:ACATGGTCCCTGACACTTCC RV: ACTCCACTATTCTCAGTTGGGC	410 bp
13	FW:AGGTGGTGGCTACTGAAGTG RV:ACAAGACTCCCTTTCCCATGC	884 bp
17	FW:AGCCAATCACCTTAGAGAGTCC RV:AGACCTTGAAAACCCCAAGAGT	1033 bp
19	FW:TGGCATTGTAAACCAAGATGC RV:CCTAGTCCATCCCAGCCAAC	380 bp
37	FW:CCCCAAAGAGTCGGTCGTAT RV:ACATACTGCAAGCCGAGTGG	1441 bp
42	FW:TCAAGGAGCCTGGAACAAAA RV:AAACCTCTACTTCTCTCCCACA	437 bp
53	FW:CCAGTTATCAGCAGAGGCGG RV:GTGGGACTCAGTTTCCCGTC	528 bp

Table 2: Polymorphism Information Content (PIC), Observed heterozygosity and Allelic diversity for each SNP identified in ACACA gene of Valle del Belice sheep breed.

Locus	N° of individual sampled	PIC	Observed Heterozygosity	Allelic diversity
SNP1	16	0.2834	0.3125	0.3418
SNP2	16	0.3494	0.1875	0.4512
SNP3	15	0.3739	0.2667	0.4978
SNP4	5	0.3648	0.0000	0.4800
SNP5	16	0.2583	0.2500	0.3047
SNP6	16	0.3494	0.4375	0.4512
SNP7	16	0.1555	0.1875	0.1699
SNP8	16	0.2834	0.3125	0.3418
SNP9	14	0.3750	0.4286	0.5000
SNP10	16	0.3711	0.3750	0.4922
SNP11	16	0.3711	0.3750	0.4922
SNP12	16	0.3374	0.3750	0.4297
SNP13	16	0.3711	0.3750	0.4922
SNP14	16	0.3711	0.3750	0.4922

Table 3: Genotypic frequencies of 14 SNPs and number of individuals for each genotype.

Locus	N° individuals	Genotype	Frequency
SNP1	10	C/C	0.63
	5	C/T	0.31
	1	T/T	0.06
SNP2	4	G/G	0.25
	3	G/T	0.19
	9	T/T	0.56
SNP3	6	C/C	0.40
	4	C/T	0.27
	5	T/T	0.34
SNP4	3	C/C	0.60
	2	T/T	0.40
SNP5	11	A/A	0.69
	4	A/G	0.25
	1	G/G	0.06
SNP6	7	C/T	0.44
	7	C/T	0.44
	2	T/T	0.13
SNP7	3	A/G	0.19
	13	G/G	0.81
SNP8	10	C/C	0.63
	5	C/T	0.31
	1	T/T	0.06
SNP9	4	C/C	0.29
	6	C/T	0.43
	4	T/T	0.29
SNP10	4	C/C	0.25
	6	C/T	0.38
	6	T/T	0.38
SNP11	6	C/C	0.38
	6	C/T	0.38
	4	T/T	0.25
SNP12	2	A/A	0.12
	6	A/G	0.38
	8	G/G	0.50
SNP13	6	C/C	0.38
	6	C/G	0.38
	4	G/G	0.25
SNP14	4	C/C	0.25
	6	C/T	0.38
	6	T/T	0.38

Table 4: Percentage effects of SNP1 on fatty acid concentration

	SNP1		
Fatty acid	TT	TC	CC
C6:0	12.110	5.804	0.000
C16:1	0.2327	7.218	0.000

Table 5: Percentage effects of SNP3 on fatty acid concentration

	SNP3		
Fatty acid	CC	CT	TT
C6:0	-3.224	1.367	4.021
C16:1	0.2486	-6.982	0.000
C18:2	-2.370	-0.713	-1.710

Chapter 5

General Conclusion

The overall objective of this thesis was the study of fatty acids profile in sheep and goat Sicilian breeds and the possible association with polymorphisms in candidate genes affecting milk quality.

In Chapter 2, we determined the fatty acid profile in Girgentana goat milk, according to Rose-Gottlieb's method, based on extraction with solvents, for milk fatty acids (FAs) extraction. The FA analysis was performed with GC-FID. The results reported showed that genetic polymorphisms at κ-casein could have effects on FA composition especially for MUFA and PUFA potentially involved as positive factors in human health. The association between κ-casein AA and AD genotypes and FAs in Girgentana goat milk could be useful for re-evaluating the possible use of fresh drinking milk from this breed. Furthermore, preservation of breeds in danger of extinction could be achieved by establishing economic reasons for their survival. The fatty acids composition is function of action of several enzymes. The knowledge of lipid metabolism may help identify possible candidate genes which can significantly affect the content of particular fatty acids. In Valle del Belice sheep breed, one possible candidate gene is the gene encoding an enzyme directly involved in fatty acid metabolism: ACACA (acetyl-CoA carboxylase). In Chapter 3, the fatty acid profile of Valle del Belice sheep milk was determined by gas chromatography coupled with mass spectrometer (GC-MS). In this way, the milk fat separation was obtained with a rapid method reliable and simple which allowed us to analyze a large number of samples, based on centrifugation at 7300 rpm at -4°C. After determining the FA profile, a study of association between FA profile of Valle del Belice sheep milk and polymorphisms within Acetyl-CoA carboxylase (ACACA) gene was carried out. The results reported in Chapter 4 showed the correlation between two different SNPs of ACACA gene and the concentration of three fatty acids: caproic acid (C6:0), palmitoleic acid (C16:1) and linoleic acid (C18:2). The caproic acid, a medium-chain fatty acid, has mainly metabolic and energetic functions, whereas linoleic acid are important for human health due to its anti-cancer and antioxidant activity and it plays metabolic protective actions in case of infections, immunizations and stimulation of the immune system.

In conclusion, our results confirm the influence of polymorphisms of caseins in goat and ACACA gene in sheep on FA composition of milk. Our studies showed that it could be possible to select individuals on genetic basis which produce milk with a higher proportion of

monounsatured, polyunsatured and omega-3 FAs that play a positive role in relation to human health.

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