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Phd thesis

Determination of protein content and fatty acid profile in milk of Girgentana goat breed for evaluation of nutritional characteristics of dairy products

S.S.D. AGR/17

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Contents

Abstract

Goat casein genes showed high polymorphism, which influences not only the quantity of caseins in milk but also the structural and nutritional characteristics and technological properties of milk. One of the aims of this thesis was to separate and quantify the most common allelic variants of caseins in milk of Girgentana goat breed, a Sicilian autochthonous breed, and to evaluate the effect of casein polymorphisms on casein content.

The genotypes and, therefore, the alleles at different casein genes were detected using PCR, PCR-RFLP, AS-PCR protocols and sequencing analysis. Milk samples were prepared following the method proposed by Bobe et al. (1998) and analyzed by RP-HPLC method. A reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, 3.5µm, 300Å, 150×4.6 I.D.) was used and the detection was made at wavelength of 214 nm. The procedure was developed using individual raw milk samples of Girgentana goat breed. For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes, considering that commercial standards for goat allelic variants were not available. In particular, were used animals with AA, BB, FF and NN genotypes at alphas1-casein; CC and C'C' genotypes at beta-casein; AA and FF genotypes at alphas2-casein; and AA and BB genotypes at kappa-casein. Method validation consisted in testing linearity, repeatability, reproducibility and accuracy. A linear relationship between the concentrations of proteins and peak areas was observed over the concentration range, with low detection limits. Repeatability and reproducibility were satisfactory for both retention times and peak areas.

Another aim of this thesis was to investigate the interactions between nutrition and the genotype at αs_1-casein locus (*CSN1S1*) in goats, evaluating the impact of fresh forage-based diets and an energy supplement on the casein and fatty acid (FA) profiles of milk from Girgentana goat breed. Twelve goats were selected for having the same genotype at the αs_2-casein, β-casein, and κ-casein loci and differing in the *CSN1S1* genotype: homozygous for strong alleles (AA) or heterozygous for strong and weak alleles (AF). Goats of each genotype were divided into three groups and, according to a 3×3 Latin square design, fed *ad libitum* with three diets: *Sulla* fresh forage (SFF), SFF plus 800 g/d of barley (SFB), mixed hay plus 800 g/d of barley (MHB).

Riassunto

I geni delle caseine caprine mostrano un elevato polimorfismo, che influenza non solo la quantità di caseine nel latte ma anche le caratteristiche strutturali e nutrizionali e le proprietà tecnologiche del latte. Uno degli obiettivi di questa tesi è stato quello di separare e quantificare le più comuni varianti alleliche caseiniche nel latte di capra di razza Girgentana, una razza autoctona siciliana, e di valutare l'effetto dei polimorfismi caseinici sul contenuto di caseina nel latte.

I genotipi e, quindi, gli alleli ai diversi geni delle caseine sono stati rilevati utilizzando protocolli di PCR, PCR-RFLP, AS-PCR e analisi di sequenziamento. I campioni di latte sono stati preparati seguendo il metodo proposto da Bobé et al. (1998) e analizzati mediante metodo RP-HPLC. È stata utilizzata una colonna analitica in fase inversa C8 (Zorbax 300SB - C8 RP, 3.5μm, 300A, 150 × 4.6 ID) e la rilevazione è stata effettuata ad una lunghezza d'onda di 214 nm. La procedura è stata

sviluppata utilizzando campioni di latte individuale crudo di capre di razza Girgentana. Per gli esperimenti di calibrazione, le varianti genetiche pure sono state estratte da campioni di latte individuale di animali con genotipo noto, in quanto commercialmente non erano disponibili le varianti alleliche estratte da latte caprino. In particolare, sono stati utilizzati animali con genotipo alla alphas1-caseina AA, BB, FF e NN, genotipo alla beta-caseina CC e C'C', genotipo alla alphas2-caseina AA e FF, e genotipo alla kappa-caseina AA e BB. La validazione del metodo prevedeva il test di linearità e le stima di ripetibilità, riproducibilità e precisione. Una relazione lineare è stata osservata tra le concentrazioni delle proteine e le aree sottese dal picco nell'intervallo di concentrazione in analisi, con l'ottenimento di limiti di rilevabilità bassi. La ripetibilità e riproducibilità sono risultate soddisfacenti sia per i tempi di ritenzione e che per le aree sottese ai picchi.

Un altro obiettivo di questa tesi è stato quello di studiare le interazioni tra nutrizione e genotipo al locus dell'αs_1-caseina (CSN1S1) caprina, valutando l'impatto delle diete a base di foraggio fresco e di un supplemento energetico sui profili di caseina e di acidi grassi (FA) nel latte di capra di razza Girgentana. Dodici capre sono state selezionate aventi lo stesso genotipo ai loci di αs_2-caseina, β-caseina e κ-caseina, e differente genotipo alla CSN1S1: omozigote per alleli forti (AA) o eterozigoti per alleli forti e deboli (AF). Le capre di ciascun genotipo sono state divise in tre gruppi e alimentate ad libitum con tre diete, secondo un disegno di quadrato Latino 3×3: foraggio fresco, Sulla, (SFF), SFF più 800 g/d di orzo (SFB), fieno misto più 800 g /d di orzo (MHB).

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General Introduction

Background

The dairy sector worldwide faces challenges including growing consumer demands for animal products increasing need for greater efficiency and growing concerns over ecological sustainability. Therefore, the dairy sector continuously looks for innovative solutions to address the challenges and create added value. Genetic aspects of milk production have been studied extensively to understand the basis of milk yield and composition.

The average protein content in goat milk is about 3.5% however, this percentage can widely vary, also within species, according to several factors such as breed, stage of lactation, genetic polymorphism, feeding etc.

The principal proteins of goat milk are α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulins (Ig), lactoferrin (Lf), αs_1-casein (αs_1-CN), αs_2-casein (αs_2-CN), κ-casein (κ-CN) and β-casein (β-CN) and other minor proteins and enzymes. They can be subdivided in caseins (αs_1-CN, αs_2-CN, κ-CN, β-CN) and whey proteins (α-La, β-Lg, Ig, Lf), so called because they remain in the serum after the precipitation of the caseins.

The four caseins represent ~80% of milk proteins. Among Ca-sensitive caseins (αs_1, β, and αs_2), the αs_1 fraction is the most widely investigated in goat (Martin et al., 2002; Rijnkels, 2002).

The Girgentana goat is a Sicilian autochthonous breed reared for its good dairy production. Due to sanitary policies the size of the Girgentana goat breed decreased of almost 90% in 20 yrs. In 1983, the population

consisted of 30,000 individuals but, nowadays, only 650 heads are reared in Sicily belonging to approximately 30 flocks (ASSONAPA, 2012).

Goats have been widely investigated for polymorphisms 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).

The extensive polymorphism at αs_1-casein locus has been shown to affect not only the quantity of casein in goat milk, but also the structural and nutritional characteristics and technological properties of milk. In fact, polymorphism associated with a quantitative variability in casein synthesis has a significant effect on coagulation properties, micelle size and mineralization, cheese yield, and sensory attributes (Ramunno et al., 2007). With regard to polymorphisms at αs_1-CN locus (*CSN1S1*), 18 alleles have been detected and classified according to their rate of milk casein synthesis: strong (A, B1, B2, B3, B4, B′, C, H, L, M), intermediate (E and I), weak (D, F, and G), and null (01, 02, and N) alleles that synthesize high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and no amounts of αs_1-CN, respectively (Grosclaude et al., 1987; Chianese et al., 1997; Martin et al., 1999; Bevilaqua et al., 2002; Ramunno et al., 2005). For the β-CN fraction (*CSN2*), the A, A1, B, C, C1, D, E variants are associated with a normal content n (5.0 g/L/allele), and the 0 and 01 "null" alleles are associated with the absence of this casein fraction in milk (Chessa et al., 2008).

The αs_2-CN includes the A, B, C, E, F that are "strong" alleles associated with a normal content (about 2.5 g/L per allele) of this protein in milk (Boulanger et al., 1984, Bouniol et al., 1994, Lagonigro et al., 2001,

Ramunno et al., 2001a), an intermediate (D) allele, which expresses a reduced level (about 1.5 g/L per allele) and a null allele (0) which causes no detectable expression (Ramunno et al., 2001a, b). Moreover, Erhardt et al. (2002) reported the G variant associated with a normal content of this protein typed at protein level by isoelectric focusing (IEF), but not characterized at molecular level.

The κ-CN fraction plays an important role in the formation, stabilization and aggregation on casein micelles and thus affects technological and nutritional properties of milk. For this reason, κ-CN locus has been investigated and several genetic variants have been found in different goat breeds. Sixteen allelic variants have been identified so far in goat κ-CN, these have been clustered into two groups by IEF of milk samples. The A^{IEF} group (isoelectric point = 5.53) contains the A, B, B′, B′′, C, C′, F, G, H, I, J, L, N alleles while the B^{IEF} group (isoelectric point = 5.78) contains the D, D′ E, K, M variants (Prinzenberg et al., 2005; Di Gerlando et al., 2013).

Research Interest

Quantification of the different genetic variants were difficult to achieve because commercial standards for goat caseins were not available. In literature, there were not data regarding the quantitative chemical analysis of individual genetic variants of caseins in goat.

Research Objectives

The objectives of the thesis were the quantification of the most common genetic variants of caseins in milk of Girgentana dairy goat breeds, to evaluate the effect of each allele on casein content using a high-

performance liquid chromatographic (HPLC) method and the study of interactions between nutrition and the genotype at αs_1-CN locus (*CSN1S1*) in Girgentana goat breed. It could be interesting to evaluate the possibility of revitalizing interest in the milk produced by Girgenatana goat breed in order to regain an important economic role in the production of "drinking-milk" requested for particular food products, such as milk for infants, using weak and null genotypes, and in the production of niche products, using strong genotypes.

Outline of the Thesis

Chapter 2 assesses the interactions between nutrition and the genotype at αs_1-CN locus (*CSN1S1*) in Girgentana goat breed, evaluating the impact of fresh forage-based diets and an energy supplement on the casein and fatty acid (FA) profiles of milk. The *CSN1S1* genotype also affects the milk fatty acid (FA) composition; specifically, goats that are homozygous for strong alleles (AA) have more short- and medium-chain FA (SMFA) and less delta-9 desaturase activity than goats homozygous for weak alleles (FF) (Bouniol et al., 1994, Chessa et al., 2008). For this study, twelve goats were selected for having the same genotype at the αs_2-CN, β-CN, and κ-CN loci and differing in the αs_1-CN genotype: homozygous for strong alleles (AA) or heterozygous for strong and weak alleles (AF). Goats of each genotype were divided into three groups and, according to a 3×3 Latin square design, fed *ad libitum* with three diets: *Sulla* fresh forage (SFF), SFF plus 800 g/d of barley (SFB), mixed hay plus 800 g/d of barley (MHB).

In Chapter 3 a RP-method was validated in order to separate and quantify the αs_1 genetic variants in goat milk. The analyses were carried out

applying a binary gradient profile to the mobile phase composition using two solvents. Solvent A consisted of 0.1% TFA in water and solvent B of 0.1% TFA in acetonitrile.

Separations were performed with the program proposed by Bonfatti et al. (2008). Pure casein genetic variants were extracted for calibration experiments considering that commercial standards for goat were not available. Method validation consisted in testing linearity, repeatability, reproducibility and accuracy.

Finally, in Chapter 4 the quantification of the most common genetic variants of caseins in milk of Girgentana dairy goat breeds was completed in order to evaluate the effect of each allele on casein content using the high-performance liquid chromatographic (HPLC) method proposed by Bonfatti et al. (2008) and validated for quantification of the αs1 genetic variants (Chapter 3).

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Effects of diet on casein and fatty acid profiles of milk from goats differing in genotype for α_{S1}-casein synthesis

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Abstract

This study investigated the interactions between nutrition and the genotype at αS1-CN loci (CSN1S1) in goats, evaluating the impact of fresh forage-based diets and an energy supplement on the casein and fatty acid (FA) profiles of milk from Girgentana goats. Twelve goats were selected for having the same genotype at the αS2-CN, β-CN, and κ-CN loci and differing in the CSN1S1 genotype: homozygous for strong alleles (AA) or heterozygous for strong and weak alleles (AF). Goats of each genotype were divided into three groups and, according to a 3×3 Latin square design, fed ad libitum three diets: sulla fresh forage (SFF), SFF plus 800 g/d of barley (SFB), mixed hay plus 800 g/d of barley (MHB). The SFB diet led to higher energy intake and milk yield. The energy-supplemented diets (SFB, MHB) reduced milk fat and urea and increased coagulation time. The fresh forage diets (SFF, SFB) increased DM and CP intake and milk β-CN. Diet had a more pronounced effect than CSN1S1 genotype on milk FA profile, which was healthier from goats fed the SFF diet, due to the higher content of rumenic acid, polyunsaturated and omega-3 FA. The AA milk had longer coagulation time and higher curd firmness, higher short- and medium-chain FA (SMFA), and lower oleic acid than AF milk. Significant diet by genotype interactions indicated the higher milk yield of AA goats than AF goats with the higher-energy SFB diet, and the lower synthesis of SMFA in AF than in AA goats with the SFF diet.

Key Words: goat milk, CSN1S1 genotype, nutrition, casein fraction, milk fatty acid

INTRODUCTION

In goats, genetic variants for αS1-casein (αS1-CN) synthesis greatly influence several milk production traits, especially casein content and the cheese making ability of milk [1].

With regard to polymorphisms at αS1-CN loci (CSN1S1), 18 alleles have been detected and classified according to their rate of milk casein synthesis: strong (A, B1, B2, B3, B4, B′, C, H, L, M), intermediate (E and I), weak (D, F, and G), and null (O1, O2, and N) alleles that synthesize high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and no amounts of αS1-CN, respectively [2,3].

Goats with strong alleles have a greater ability to synthesize αS1-CN than goats with weak alleles; they also produce milk higher in casein, fat, calcium, and phosphorus, with smaller casein micelles and higher coagulation time (r) and curd firmness (a30) [1,4].

The CSN1S1 genotype also affects the milk fatty acid (FA) composition; specifically, goats that are homozygous for strong alleles (AA) have more short- and medium-chain FA (SMFA) and less delta-9 desaturase activity than goats homozygous for weak alleles (FF) [5,6].

Because feed also exerts a great influence on the yield and properties of goat milk, there is interest in how nutrition might interact with the genetic polymorphism at αS1-CN. Recent researches showed how AA goats, compared with FF goats, more efficiently utilize dietary protein [7-9] and respond to high-energy diets by utilizing nutrients more efficiently and achieving a higher milk yield [10].

In a more recent research [11], goats homozygous for strong alleles at CSN1S1 loci (AA) and those heterozygous for a weak allele (AF), which

are associated with high and low levels of αS1-CN synthesis, respectively, were compared on the basis of their feeding behavior, metabolic and hormonal responses, and milk production resulting from different nutrient intake. The choice of the AF genotype depended on the high frequency of heterozygous goats at CSN1S1 loci in the farms, but also on the small number of researches focused on the heterozygous CSN1S1 genotype. In that study, the AA goats confirmed, also in comparison with AF goats, the more efficient energy and protein utilization, already evident at the digestive level, and the better productive responses to high-nutrition diets.

Casein and FA play a fundamental role in the nutritional and technological properties of milk. Thus, to further investigate interactions between nutrient intake and the CSN1S1 genotype in goats, this paper reports a successive study, conducted within the same research [11], evaluating the impact of a fresh forage diet and/or an energy supplement on casein fractions and FA profile of milk produced by Girgentana goats with different genetic abilities to synthesize αS1-CN. Goats, that were homozygous (AA) and heterozygous (AF) for CSN1S1 alleles, were fed diets based on fresh sulla (Hedysarum coronarium L.), a legume forage common in Mediterranean areas [12-14], with or without a barley supplement.

MATERIALS AND METHODS

Animals and Experimental Design

The present experiment was carried out on a farm in Sicily (Santa Margherita Belice, Agrigento) for a period of 11 weeks, from March to May. A total of 40 milking goats were genotyped at the CSN1S1,

CSN1S2, CSN2, and CSN3 loci, codifying for αS1-CN, αS2-CN, β-CN, and κ-CN, respectively, using specific PCR protocols at the DNA level [15-18].

Twelve goats in their 3rd or 4th lactation, with 50 or 120 days in milking (DIM) and averaging 37.2 ± 3.5 kg of live weight, were selected for having the same genotype at the CSN1S2 (AA), CSN2 (AA), and CSN3 (AA) loci and a different CSN1S1 genotype: six goats were homozygous for a strong allele (AA) and the other six were heterozygous for strong and weak alleles (AF).

During the entire experiment, the goats were housed in individual large pens placed inside a closed shed. After a 2-week period of adaptation to their changed housing conditions, the six goats of each CSN1S1 genotype (AA and AF) were allocated homogeneously, based on DIM, to three groups and fed three diets in succession, according to a 3×3 Latin square design with three experimental periods of 21 d each (14 d for adaptation to the diets and 7 d for measuring and sampling).

The three experimental diets consisted of sulla (Hedysarum coronarium L.) fresh forage ad libitum (SFF), SFF ad libitum plus 800 g/d of barley meal (SFB), and mixed hay ad libitum plus 800 g/d of barley meal (MHB).

The sulla forage was mowed daily in the morning, cut roughly, and supplied to goats in the feeding trough twice a day, at 10 a.m. and 5 p.m., while the barley meal was divided into two meals.

Sampling and Analysis

At the beginning and the end of each experimental period, all goats were weighed and checked for their body condition score (BCS).

During the last 7 d of each experimental period, the offered and refused forage and barley of each goat were weighed daily and sampled twice to estimate the amount and quality of feed intake. Individual milk yield was recorded daily at morning (7:00 am) and evening (4:00 pm) milking and sampled three times on days 3, 5 and 7 of the sampling week in each period.

The samples of barley and forage were analysed for the determination of dry matter (DM), crude protein (CP) [19], and NDF [20]. Their energy content, expressed in Mcal of net energy for lactation (NEL), was estimated using equations of the National Research Council [21]. In addition, freeze-dried samples of sulla forage were analysed by spectrophotometer for condensed tannins using the butanol-HCl method [22] and delphinidin as the reference standard [23].

Individual milk samples were analysed for fat, protein, casein, and somatic cell count using the infrared method (Combi-foss 6000, Foss Electric, Hillerød, Denmark); pH using a HI 9025 pH-meter (Hanna Instruments, Ann Arbor, MI, USA); titratable acidity using the Soxhlet-Henkel method (°SH/50 mL); and urea by enzymatic method using the difference in pH (CL-10 Plus, Eurochem, Roma, Italy).

Individual milk samples were also evaluated for their clotting ability by measuring coagulation time (r, min), curd firming time (k20, min), and curd firmness after 30 min (a30, mm), according to Zannoni and Annibaldi [24], in 10 ml milk at 35°C with 0.2 mL of a diluted solution (1.6:100) of rennet (1:15,000; Chr. Hansen, Parma, Italy), using the Formagraph (Foss Electric).

Milk Casein Fractions

Milk caseins (αS1-CN, αS2-CN, β-CN, and k-CN) were separated and quantified in individual milk samples collected on day 7 at the end of sampling week in each experimental period. This was done by direct analysis with RP-HPLC (reversed-phase high-performance liquid chromatography), according to Bonizzi et al. [25].

Purified αS-CN (purity 90%), β-CN (purity 98%), and κ-CN (purity 98%) fractions used as standards, HPLC-grade trifluoroacetic acid, water, acetonitrile, and other chemicals were purchased from Sigma-Aldrich (Milano, Italy).

Single-fraction mother solutions were prepared by dissolving 249.4 mg purified αS-CN, 255.2 mg purified β-CN, and 51.7 mg purified κ-CN in 10 mL of a denaturing solution containing 8 M urea, 165 mM Tris, 44 mM sodium citrate, and 0.3% (v/v) β-mercaptoethanol. A mixed standard solution was prepared by mixing 1 mL of each single concentrated solution and adding 2 mL of the denaturing solution, so that the dilution factor at this step was 5 for all casein fractions. Then a set of four mixed concentration standards was obtained from the mixed mother solution by applying the dilution scheme reported by Bonizzi et al. [25]. Because αS1-CN and αS2-CN are not available as single proteins, the corresponding values were calculated from the αS-CN by applying the 4:1 proportion reported in the literature [25]. The resulting standard solutions were analysed to construct the αS1-CN, αS2-CN, β-CN, and κ-CN calibration curves.

Milk samples were lyophilized and preserved frozen at –4°C until analysis. Each milk sample was weighed before and after lyophilization to determine the water percentage content. Before analysis, the lyophilized milk sample was solubilized by adding a corresponding volume of

distilled water, then it was homogenized by Vortex and the fat removed by centrifugation at 1000 × g for 10 min at 4°C. A volume of 400 μL of skimmed milk was diluted with 1.6 mL of the denaturing solution described above. The diluted sample was filtered through a 0.45-μm-pore cellulose membrane (Phenomenex, Torrance, CA, USA) and directly analysed twice.

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of an LC-20AT liquid chromatographer, a DGU-20A 5 degasser, a SIL-20A HT autosampler, a CTO-20A column oven, and a SPD-20A UV/VIS detector, run using LC Solutions software.

Chromatographic separation was performed in reversed-phase mode using a Jupiter C4 column (250 mm × 4.6 mm, 300 Å pores, 5 μm particles; Phenomenex) kept at room temperature. The detection wavelength was 220 nm.

The analyses were carried out by applying a binary gradient profile to the mobile phase composition, according to a modified gradient programme developed recently, as reported by Bonizzi et al. [25]. Eluent A was HPLC-grade water containing 0.1% (v/v) trifluoroacetic acid, and eluent B was HPLC-grade acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The gradient elution programme was run at a constant flow rate of 0.8 mL/min and was set as follows: 0–40 min linear gradient from 30% B to 50% B; 40–42 min linear gradient from 50% B to 100% B; 42–43 min isocratic elution 100% B; 43–46 min linear gradient from 100% B to 30% B, followed by a 5-min isocratic elution at the initial conditions. The total duration of a single run, including column reequilibration, was 51 min.

The quantification of milk casein fractions was performed by comparing the corresponding peak areas in the chromatogram of the sample with

those of the standard solutions used for the construction of the calibration curves.

Milk FA Composition

Milk FA were determined from individual milk samples collected at the end of each experimental period.

FA in lyophilized milk samples (100 mg) were directly methylated with 1 mL hexan and 2 mL 0.5 M NaOCH3 at 50°C for 15 min, followed by 1 mL 5% HCl in methanol at 50°C for 15 min [26].

Fatty acid methyl esters (FAME) were recovered in hexane (1.5 mL). One microlitre of each sample was injected by autosampler into an HP 6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies, Santa Clara, CA, USA). FAME from all samples were separated using a 100 m length, 0.25 mm i.d., 0.25 μm capillary column (CP-Sil 88, Chrompack, Middelburg, The Netherlands).

The injector temperature was kept at 255°C and the detector temperature was kept at 250°C, with an H2 flow of 40 mL/min, an air flow of 400 mL/min, and a constant He flow of 45 mL/min. The initial oven temperature was held at 70°C for 1 min, increased 5°C/min to 100°C, held for 2 min, increased 10°C/min to 175°C, held for 40 min, then finally increased 5°C/min to a final temperature of 225°C and held for 45 min. Helium, with a head pressure of 23 psi and a flow rate of 0.7 mL/min (linear velocity of 14 cm/s), was used as the carrier gas.

A FAME hexane mix solution (Nu-Check-Prep, Elysian, MN, USA) was used to identify each FA. Conjugated linoleic acid (CLA) isomers were identified using a commercial mixture of methyl esters of the C18:2 c9 t11 and C18:2 c10 t12 (Sigma-Aldrich). The Health Promoting Index was

calculated as suggested by Chen et al. [27]: total unsaturated FA/[C12:0 + (4 × C14:0) + C16:0].

Statistical Analysis

Statistical analysis was carried out using the MIXED procedure in SAS 9.1.2 [28]. Experimental phase (1, 2, 3), DIM (50 and 120 d), diet (SFF, SFB, MHB), genotype (AA and AF), and the diet by genotype interaction were fixed factors, and the goat was considered a random factor and used as an error term. Somatic cell count values were transformed in logarithmic form (log10). Means were compared using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Feed Intake and Milk Production

At the end of the experimental period, the live weight and BCS of the goats did not show changes as a function of diet or CSN1S1 genotype, as previously observed [11].

The DM and main nutrients intake was strongly influenced by diet, while it did not reveal a significant effect of CSN1S1 genotype and diet by genotype interaction (Table 1). Similar results were found by Bonanno et al. [29] and Pagano et al. [10]. In particular, Bonanno et al. [29] reported no difference in DM intake between goats with strong (AA) and heterozygous (AF) genotypes, like in the present study, although they observed a lower feed intake in goats with a weak (FF) genotype.

With regard to diet, the sulla fresh forage increased the DM intake compared to hay, regardless of the energy supplementation with barley (Table 1). This confirms the positive effect of sulla forage on voluntary

feed intake [12, 30] attributed to the high protein percentage, the low NDF content and the high ratio of nonstructural to structural carbohydrates of sulla [31]. Intake of protein, as well as condensed tannins, increased with increasing levels of fresh forage ingested. The SFF diet resulted in the maximum NDF intake, followed by the MHB diet, whereas the SFB diet, because of its lower NDF intake, corresponded to the highest energy intake.

Like feed intake, milk production was affected by diet (Table 1). In fact, the daily milk yield increased from the SFF diet to the MHB diet, culminating with the SFB diet.

With regard to the effect of diet on milk composition, the energy supplement with barley reduced the contents of milk fat and urea (Table 1). This reduction in fat was certainly due to the lower forage/concentrate ratio of the supplemented diets and thus to the lower cellulose intake. The reduction of urea was presumably a consequence of the more balanced protein/energy ratio in the diets with barley supplementation which favoured the conversion of dietary nitrogen into microbial protein in the rumen [32].

Moreover, the sulla fresh forage, independent of the barley supplement, resulted in an increase in the percentages of milk protein and casein. This was probably due to the higher intake of condensed tannins (Table 1), secondary metabolites contained in sulla forage in moderate amounts (<6% DM) [33]. These tannins are able to reduce protein degradability in the rumen and consequently enable a greater amount of amino acids to be absorbed in the intestinal tract [34]. This contributes to improving the efficiency of dietary protein utilization for milk casein synthesis in the udder.

Regardless of genotype, diet affected the titratable acidity and coagulation time of milk, which were higher and lower, respectively, when goats received the SFF diet (exclusively sulla fresh forage) than the other diets (Table 1). This result is in line with Todaro et al. [35], who found a negative correlation between titratable acidity and the coagulation time of goat milk. However, generally the relationship between diet and milk coagulation ability is quite complex, even though diet has been shown to affect milk titratable acidity and the coagulation process [36].

For milk yield, there was no influence of genotype, whereas there was a significant interaction between diet and genotype (Table 1). In this regard, the literature has frequently shown the lack of an effect of CSN1S1 genotype on goat milk yield. For example, many researchers have found no significant difference between goats with AA and FF genotypes at CSN1S1 loci [5, 7, 9, 37]; only Avondo et al. [38] reported increased milk production in goats with the strong genotype (AA) compared to the weak genotype (FF). Moreover, the milk yields of goats with the AA and AF genotypes do not differ significantly, and both genotypes result in more milk production than the FF genotype [29]. However, Pagano et al. [10] showed a higher milk yield in AA goats compared to AF and FF goats, which did not differ.

These discrepancies can be attributed to the different milking responses of goats to nutrients in accordance with their CSN1S1 genotype. As evidence of this assertion, in the current study a significant interaction between diet and genotype emerged, because the superior production of AA goats compared to AF goats occurred when the goats were fed with more energy SFB diet (1720 vs. 1606 g/d, $P < 0.05$). Moreover, the milk yield of AA goats fed the SFB diet was 350 g/d more than that of goats

fed the other diets, whereas the differences among diets were markedly lower in AF goats. These results clearly show the existence of relationships between nutrition and αS1-CN polymorphism, as supported by other authors [9, 10], and particularly confirm the better milking response of goats with strong alleles at CSN1S1 loci, compared with FF goats, when fed higher energy diets balanced for energy and protein content [8-10].

The CSN1S1 genotype did not significantly influence milk composition. In this regard, several authors [9, 37-39] have reported that the milk of goats with the strong CSN1S1 genotype (AA) has a higher percentage of casein than that of goats with the weak CSN1S1 genotype (FF); casein levels in the milk of heterozygous goats (AF) are intermediate and statistically different from those of either AA or FF goats [10, 29], contrary to the results of this trial.

Even though the CSN1S1 genotype did not significantly influence the milk casein content, the milk of goats with strong alleles had a longer coagulation time and greater curd firmness (Table 1). Since generally these clotting responses are related to a higher casein level [40], they could be linked to a more favourable partition among the casein fractions compared to in AF goats. Because in this trial the genotypes differed only for the variants of αS1-CN synthesis, this result implicates αS1-CN as key in variations in milk coagulation.

In previous trials [29, 37], milk from goats with the AA genotype at CSN1S1 loci showed greater curd consistency in comparison with milk of FF goats, whereas the coagulation ability of milk from AA goats did not differ from that of milk from AF goats.

Milk Casein Fractions

The analysis of casein components, such as κ-CN, αS2-CN, αS1-CN, and β-CN, showed a higher αS1-CN percentage in the milk of AA goats than AF goats, as expected (Table 2). In Spanish goat breeds, genotypes with strong alleles (BB) also displayed significantly increased levels of milk αS1-CN in comparison with heterozygous genotypes (BF) [41].

Figures 1 and 2 show the chromatograms obtained by RP-HPLC from milk samples of goats with genotypes expressing a high (AA) and low (AF) level of αs1-CN synthesis, respectively.

The levels of k-CN and αS2-CN were not affected by either diet or genotype, whereas the percentage of β-CN, which is the most represented casein fraction, was significantly influenced only by diet. β-CN, in fact, was mostly synthesized with the fresh forage diets, presumably as a consequence of the favourable effects of the higher content in the protein and condensed tannins of the sulla forage [34].

When milk casein profiles were analysed for the daily production of the various fractions, the effect of genotype was again significant for αS1-CN, which was higherin AA than AF milk (Table 2). Moreover, all casein fractions showed an effect of diet, irrespective of genotype; their production, in fact, was favoured by the higher energy and more balanced diet based on sulla forage supplemented with barley.

With regard to the effect of diet on the casein profile of goat milk, researchers have compared animals with strong (AA) and weak (FF) alleles at CSN1S1 loci [9, 39]. In line with the results of the present trial, De la Torre Adarve et al. [9] detected a higher incidence of αS1-CN in the milk of goats with strong than weak alleles regardless of dietary protein intake. However, these same authors also observed an increase in the

percentage of αS2-CN in goats with the strong genotype and an increase in αS1-CN and αS2-CN daily yield in goats with the weak genotype when fed a diet rich in protein.

Valenti et al. [39] observed that goats with a strong genotype for αS1-CN responded to a higher energy diet, increasing both milk casein content and daily casein yield, and that this increase was due to only αS1-CN. Instead, in the present trial, the increase in milk αS1-CN percentage in AA goats was independent of diet, and the daily αS1-CN yield with the higher energy SFB diet increased similarly in goats with the AA and AF genotypes.

Ultimately, with regard to the incidence of casein fractions, the diet affected the level of β-CN similarly in goats of both genotypes, whereas the AA genotype at CSN1S1 loci was linked exclusively to the increase in αS1-CN synthesis, regardless of diet. Therefore, the milk of goats of these genotypes differed only in the level of αS1-CN. Considering the response by genotype in terms of milk coagulation previously described (Table 1), this result shows that in this trial αS1-CN was solely responsible for the coagulation properties of the milk, particularly for curd firmness (a30).

Milk FA Composition

As can be seen in Tables 3 and 4, the milk FA composition was influenced strongly by nutrients intake and only marginally by the polymorphism at CSN1S1 loci and the interaction between diet and genotype.

Both the sulla fresh forage and the hay supplemented with barley induced an increase in the levels of SMFA in milk (from C10:0 to C16:0, Table 3; Σ C4-C14, Table 4).

Moreover, the milk obtained with the SFB diet showed the highest content of linoleic acid (C18:2 n-6, LA) (Table 4), certainly due to the contribution of both feeding sources, sulla forage and barley.

Conversely, the diet based exclusively on green forage (SFF) resulted in an increase in most of the odd and branched chain FA in milk (C14:0 iso, C15:0 iso, C15:0 anteiso, C15:0, C17:0 anteiso, and C17:0, Table 3), grouped under the acronym OBCFA in Table 4. The OBCFA, to which a certain anticancer activity is recognized, derive mainly from the biosynthesis of rumen bacteria; therefore, their presence is considered an indicator of microbial fermentations in the rumen and is favoured by a higher incidence of the forage component in the diet [42].

The SFF diet also resulted in an increase in many FA with 18 carbon atoms (Table 4), such as stearic (C18:0), vaccenic (C18:1 t11, VA), oleic (C18:1 c9), and rumenic (CLA, C18:2 c9 t11, RA) acids. The incidence of sulla forage in the diet also strongly influenced α-linolenic acid content (C18:3 n-3, LNA), which was lowest in the hay-based diet, increased with the SFB, and then further increased with the sulla forage alone (Table 4). This trend was also found for total polyunsaturated and omega-3 FA and then, in reverse, for the omega-6/omega-3 ratio (Table 4).

Like every other green forage, sulla fresh forage is rich in polyunsaturated FA, which can represent more than 70% of the total FA, and consists mainly of LNA and LA acids (about 60% and 10% of the total FA, respectively) [43]. Therefore, sulla fresh forage intake might have favoured the increase in polyunsaturated FA in the milk.

However, the intake of condensed tannins contained in the sulla forage could also have played a determining role in increasing the amount of polyunsaturated FA in the milk; the condensed tannins, in fact, would have been able to inhibit the activity of ruminal microorganisms in biohydrogenating the unsaturated FA, as demonstrated by Cabiddu et al. [44]. In this context, RA represents the first and VA the last of the intermediate products that are formed in the rumen during the saturation of LA and LNA to stearic acid (C18:0) [45, 46], and therefore their levels increase as a consequence of the inhibiting action of the sulla condensed tannins.

Rumenic acid is the most abundant of the CLA isomers; these molecules have beneficial properties for human health and, because of their cytotoxic action against several tumour cell lines, are mainly used to prevent the occurrence of tumours [47, 48]. Rumenic acid originates not only from the biohydrogenation of LA and LNA in the rumen but also from the desaturation of VA in the mammary gland [45]. In this regard, the lower ratio of RA to VA (Table 4) in the SFF and SFB diets compared to the MHB diet would indicate a lower efficiency of the activity of the enzyme delta-9-desaturase in the mammary gland tissue for the conversion of VA to RA, an effect that probably is due to the higher level of VA. However, the ratios between saturated and unsaturated FA of the same chain length (Table 4), used as indicators of FA desaturation in the mammary gland by delta-9 desaturase, were not influenced by diet.

Overall, the exclusive intake of sulla fresh forage by goats improved the FA profile of milk fat, making it more suitable to the health needs of consumers [46, 49, 50]. Indeed, the sulla forage enriched the milk in OBCFA, CLA (RA), and monounsaturated, polyunsaturated, and omega-

3 FA, thereby reducing the ratios of saturated/unsaturated FA and omega-6/omega-3 FA and improving the Health Promoting Index (Table 4), which expresses the health value of dietary fat [27].

Compared to diet, the effect of the genotype at CSN1S1 loci on milk FA composition was weak. However, an effect of genotype was found, at varying levels of significance, for the short- and even-chain FA (from C4:0 to C10:0) (Table 3), which were higher in AA goats, as well as for C17:0 anteiso (Table 3), stearic acid (C18:0), and oleic acid (C18:1 c9) (Table 4), which were higher in AF goats. Therefore, the FA profile of milk fat of goats with a greater ability to synthesize αS1-CN was characterized by more saturated FA, especially for the contribution of SMFA (Σ C4-C14), and less monounsaturated FA, mainly due to the reduced incidence of oleic acid (C18:1 c9) (Table 4). Accordingly, the milk of goats with the strong genotype showed a higher saturated/unsaturated FA ratio, although the Health Promoting Index was not affected by genotype.

Only Todaro et al. [51], studying the effects of genotype at CSN1S1 loci on the FA profile of milk from goats of Maltese breed, also evaluated animals with a heterozygous genotype for a weak allele (AF). They detected differences between the AF goats and goats with a weak genotype (FF) that were mainly due to the high presence of medium-chain FA in the milk of the latter goats. They did not find any differences between the AF and AA goats.

In agreement with Todaro et al. [51], in this trial, the level of RA did not differ by CSN1S1 genotype, although it was slightly higher in heterozygous goats than in goats with the strong genotype, as was VA. Also, FA desaturation occurred in the mammary gland by the enzyme

delta-9 desaturase, as indicated by ratios of saturated and unsaturated FA of the same chain length (Table 4), did not appear to be affected by genotype. However, Chilliard et al. [5] found an increasing content of RA in the milk of goats with the weak genotype (FF), and, in line with Valenti et al. [52], also found higher ratios of FA desaturation in comparison with milk of the strong genotype (AA).

When the goats carrying strong alleles at CSN1S1 loci were compared with those homozygous for the weak alleles (FF), the effect of genotype for αS1-CN was more pronounced than that detected in this trial, and differences emerged mainly for SMFA (∑ C4-C14), which was higher with the genotype with strong alleles [5-7, 9, 51]. This shows that the proportion of SMFA is normally higher in animals with a high capacity for αS1-CN synthesis, in line with the findings of this study.

With regard to the OBCFA, only Valenti et al. [6] found a higher content of C15:0 anteiso in the milk of goats with the strong genotype than the weak genotype, while no study in literature reports an increase in C17:0 anteiso with the weak genotype as emerged in this current trial.

Furthermore, as in this study, Chilliard et al. [5] and De la Torre Adarve et al. [9] found a lower oleic acid (C18:1 c9) content in the milk of goats with the strong genotype. Since a negative energy balance increase in milk the amount of long chain FA mobilized from adipose tissue, especially oleic acid (C18:1 c9) [5], this results would indicate that AA goats, compared to those with the heterozygous and weak genotypes, had less of a need to mobilize their body fat reserves. In this regard, Valenti et al. [52] observed that goats with the strong genotype for αS1-CN did not show the increase in oleic acid (C18:1 c9) content that occurred in goats

with the FF genotype when fed the lower energy diet, which further supports the greater efficiency of energy utilization in these animals.

In the present experiment, an interaction between diet and genotype emerged, at a tendency level, only for the sum of SMFA (Σ C4-C14, Table 4). These FA increased when the goats with the low genetic capacity for αS1-CN synthesis received the SFB and MHB diets with the energy supplement. Similarly, Valenti et al. [52] found a greater synthesis of SMFA in the milk of goats with the weak genotype when these animals were fed a higher energy diet.

Finally, this study, as well as the other investigations discussed, points to the weak link between goat polymorphism at CSN1S1 loci and milk FA composition. According to Leroux [53], the absence of a more pronounced effect of genotype may be justified by the fact that milk fat content does not seem to depend on a different expression of enzymes involved in lipogenesis. However, other enzymes seem to be involved in the de novo synthesis of SMFA in the udder tissue. In this regard, Ollier et al. [54] hypothesized that weak variants at CSN1S1 loci may interfere negatively with the expression of genes coding for the enzyme that catalyzes the de novo synthesis of SMFA in the mammary gland.

CONCLUSIONS

In this study, Girgentana goats with genotypes associated with a high (AA) or low (AF) level of αS1-CN synthesis were compared on the basis of milk casein and FA profiles deriving from different nutritional treatments.

The diet highest in energy, a combination of sulla fresh forage and barley (SFB), maximized the goats' energy intake and milk yield; however, milk

production with SFB diet was more efficient in AA goats than in AF goats.

Regardless of CSN1S1 genotype and the presence of a barley supplement, the fresh forage diets (SFF and SFB) increased DM and protein intake and milk β-CN content. The diet based exclusively on sulla fresh forage (SFF) improved the health properties of milk fat which was richer in CLA (RA), OBCFA, monounsaturated, polyunsaturated, and omega-3 FA, and had lower saturated/unsaturated FA and omega-6/omega-3 FA ratios and a more favourable Health Promoting Index. These improvements were presumably the result of condensed tannins of sulla in the inhibiting the biohydrogenation of unsaturated FA in the rumen.

With regard to genotype, AA goats differed from AF goats in terms of their superior ability to synthesize αS1-CN, regardless of diet. Therefore, the higher αS1-CN content in the AA milk was responsible for the improved milk clotting properties, as a result of the longer coagulation time and higher curd firmness, in comparison with the AF milk.

Compared to the AA goats, the heterozygous AF goats showed less of an ability to biosynthesize SMFA (Σ C4-C14) in the mammary gland tissue, but this effect disappeared when they received the energy supplement. Whereas the lesser exigency to mobilize body fat depots of AA goats, and thus their more efficient energy utilization, was confirmed by the lower content of oleic acid (C18:1 c9) in the milk.

Ultimately, this study confirms the better nutritional and productive efficiency and the higher capacity for αS1-CN synthesis of goats with the strong genotype at CSN1S1 loci in comparison with heterozygous AF goats. In addition, this study demonstrates that the milk production potential of AA goats, besides being higher than that of the FF goats

which have the least ability to synthesize αS1-CN, as reported in the literature, is also superior to that of heterozygous AF goats.

Moreover, the results provide evidence of the pronounced effect of diet on milk FA composition (i.e., the improved health properties of the milk of goats fed exclusively sulla fresh forage) and, in contrast, the weak influence of goat polymorphism at CSN1S1 loci on milk FA composition.

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Table 1 Effects of diet and *CSN1S1* genotype of goats on nutrient intake and milk yield, composition, and clotting ability

Genotype (G)[a]				AA	AF		AA			AF			SEM	Significance[b]		
Diet (D)[c]	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
Intake																
DM, g/d	1820^{a}	1807^{a}	1655^{b}	1746	1776	1769	1776	1692	1872	1837	1618	86.8	**	ns	ns
CP, g/d	321^{a}	290^{b}	203^{c}	272	270	322	286	209	320	293	197	15.3	***	ns	ns
NDF, g/d	632^{a}	483^{c}	539^{b}	535	568	592	463	550	673	503	527	37.4	***	ns	ns
Condensed tannins, g/d	47.2^{a}	35.6^{b}	3.50^{c}	29.1	28.5	47.7	35.7	3.70	46.6	35.4	3.31	1.64	***	ns	ns
NE_L, Mcal/d	2.40^{b}	3.03^{a}	2.34^{b}	2.60	2.58	2.36	3.05	2.37	2.44	3.01	2.31	0.088	***	ns	ns
Milk traits																
Milk yield, g/d	1353^{c}	1664^{a}	1423^{b}	1487	1473	1356^{cd}	1720^{a}	1384^{cd}	1348^{d}	1606^{b}	1465^{c}	44.3	***	ns	**
Fat, %	3.59^{a}	3.17^{b}	2.95^{c}	3.17	3.31	3.52	3.06	2.92	3.66	3.28	2.99	0.21	***	ns	ns
Protein, %	3.34^{a}	3.28^{a}	3.21^{b}	3.29	3.26	3.35	3.26	3.24	3.33	3.29	3.17	0.10	**	ns	ns
Urea, mg/dL	35.4^{a}	32.1^{b}	30.9^{b}	33.8	31.8	35.8	33.4	32.3	35.0	30.9	29.5	2.19	***	ns	ns
SCC, $\log_{10}$ n/mL	5.27	5.28	5.27	5.13	5.42	5.15	5.09	5.14	5.39	5.46	5.41	0.15	ns	ns	ns
pH	6.63	6.65	6.65	6.66	6.64	6.64	6.67	6.65	6.63	6.63	6.65	0.018	ns	ns	ns
Titratable acidity, °SH/50 mL	2.81^{a}	2.67^{b}	2.62^{b}	2.59	2.82	2.81	2.56	2.66	2.81	2.79	2.58	0.11	**	ns	ns
Coagulation time (r), min	13.8^{b}	15.0^{a}	14.9^{a}	15.2^{a}	13.9^{b}	14.6	15.8	15.3	13.1	14.2	14.5	0.55	*	*	ns
Curd firming time (k_{20}), min	2.67	2.82	2.65	2.79	2.63	2.82	2.94	2.62	2.53	2.70	2.67	0.33	ns	ns	ns
Curd firmness (a_{30}), mm	32.2	34.2	30.9	35.9^{a}	29.0^{b}	37.3	37.5	32.8	27.0	31.0	29.1	2.09	ns	***	ns

[a] Genotypes are as follows: AA = homozygous for strong alleles, AF = heterozygous for a weak allele.
[b] * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = not significant. [a, b, c, d]Means within a row with different superscripts differ ($P \leq 0.05$).
[c] Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/d barley meal, MHB = mixed hay plus 800 g/d barley meal.

Table 2 Effects of diet and *CSN1S1* genotype of goats on percentage in milk and daily yield of casein fractions

Genotype (G)[a]				AA	AF		AA			AF			SEM	Significance[b]		
Diet (D)[c]	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
κ-CN, %	0.34	0.36	0.37	0.36	0.35	0.36	0.36	0.36	0.31	0.36	0.38	0.022	ns	ns	ns
α_{S2}-CN, %	0.68	0.68	0.73	0.69	0.70	0.70	0.67	0.71	0.66	0.69	0.76	0.043	ns	ns	ns
α_{S1}-CN, %	0.58	0.53	0.53	0.67^{a}	0.42^{b}	0.74	0.63	0.64	0.42	0.42	0.41	0.062	ns	**	ns
β-CN, %	1.33^{a}	1.28^{a}	1.21^{b}	1.21	1.33	1.31	1.18	1.15	1.35	1.37	1.27	0.086	**	ns	ns
κ-CN, g/d	4.56^{b}	5.98^{a}	4.89^{b}	5.26	5.03	5.00	6.13	4.64	4.12	5.83	5.14	0.58	**	ns	ns
α_{S2}-CN, g/d	9.22^{b}	11.2^{a}	9.62^{b}	9.96	10.1	9.76	11.2	8.91	8.68	11.3	10.3	1.24	*	ns	ns
α_{S1}-CN, g/d	7.80^{ab}	8.90^{a}	7.01^{b}	9.77^{a}	6.03^{b}	10.10	10.90	8.31	5.50	6.89	5.71	1.35	*	*	ns
β-CN, g/d	18.0^{b}	20.9^{a}	15.6^{b}	17.4	18.9	18.5	19.3	14.5	17.5	22.5	16.8	2.20	**	ns	ns

[a] Genotypes are as follows: AA = homozygous for strong alleles, AF = heterozygous for a weak allele.
[b]* $P \leq 0.05$; ** $P \leq 0.01$; ns = not significant. [a, b]Means within a row with different superscripts differ ($P \leq 0.05$).
[c] Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/d barley meal, MHB = mixed hay plus 800 g/d barley meal.

Table 3 Effects of diet and *CSN1S1* genotype of goats on short- and medium-chain fatty acid composition (g/100 g FAME) of milk

Genotype (G)[a]				AA	AF		AA			AF			SEM	Significance[b]		
Diet (D)[c]	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
C4:0	0.97	0.84	0.95	1.01	0.83	1.15	0.93	0.96	0.80	0.75	0.94	0.13	ns	+	ns
C6:0	2.01	2.05	1.89	2.22	1.74	2.48	2.34	1.85	1.54	1.75	1.94	0.32	ns	+	ns
C8:0	2.52	2.89	2.28	3.02^{a}	2.10^{b}	3.31	3.51	2.24	1.73	2.26	2.32	0.44	ns	*	ns
C9:0	0.36	0.32	0.25	0.28	0.33	0.39	0.22	0.24	0.32	0.42	0.25	0.073	ns	ns	ns
C10:0	9.77^{b}	12.5^{a}	11.1^{ab}	12.0^{a}	10.2^{b}	11.3	13.7	11.0	8.25	11.3	11.1	0.83	**	*	ns
C11:0	0.37	0.48	0.49	0.47	0.42	0.42	0.49	0.50	0.33	0.46	0.48	0.052	+	ns	ns
C12:0	4.45^{b}	6.78^{a}	6.24^{a}	6.09	5.55	4.98	6.85	6.45	3.92	6.71	6.02	0.69	**	ns	ns
C13:0	0.18^{b}	0.26^{a}	0.30^{a}	0.23	0.26	0.18	0.23	0.27	0.17	0.30	0.32	0.033	**	ns	ns
C14:0 *iso*	0.18^{a}	0.13^{b}	0.13^{b}	0.14	0.15	0.20	0.10	0.12	0.17	0.16	0.14	0.026	*	ns	ns
C14:0	9.14^{b}	12.3^{a}	12.9^{a}	11.3	11.6	9.25	12.0	12.5	9.02	12.5	13.3	0.63	***	ns	ns
C15:0 *iso*	0.27^{a}	0.16^{c}	0.21^{b}	0.21	0.21	0.26	0.17	0.20	0.27	0.15	0.22	0.022	***	ns	ns
C15:0 *anteiso*	0.46^{a}	0.32^{b}	0.36^{b}	0.36	0.40	0.44	0.28	0.34	0.48	0.35	0.38	0.043	**	ns	ns
C14:1 *c9*	0.10^{b}	0.18^{a}	0.21^{a}	0.14	0.19	0.09	0.15	0.20	0.12	0.22	0.23	0.033	*	ns	ns
C15:0	1.59^{a}	0.86^{b}	1.00^{b}	1.08	1.22	1.42	0.80	1.03	1.77	0.92	0.97	0.13	***	ns	ns
C16:0 *iso*	0.28	0.28	0.25	0.26	0.28	0.28	0.29	0.23	0.29	0.27	0.28	0.032	ns	ns	ns
C16:0	23.3^{b}	28.4^{a}	31.1^{a}	27.7	27.5	23.1	28.0	32.0	23.6	28.8	30.2	1.46	***	ns	ns
C17:0 *iso*	0.36^{a}	0.26^{b}	0.38^{a}	0.31	0.35	0.33	0.26	0.35	0.38	0.26	0.41	0.033	**	ns	ns
C17:0 *anteiso*	0.37^{a}	0.21^{b}	0.20^{b}	0.24^{b}	0.28^{a}	0.33	0.20	0.19	0.41	0.23	0.21	0.027	***	*	ns
C16:1 *c9*	0.47	0.50	0.62	0.49	0.57	0.45	0.45	0.58	0.48	0.56	0.66	0.065	+	ns	ns
C17:0	1.17^{a}	0.78^{b}	0.77^{b}	0.89	0.92	1.08	0.75	0.85	1.27	0.82	0.68	0.073	***	ns	ns
C17:1	0.30^{a}	0.18^{b}	0.22^{b}	0.22	0.25	0.27	0.17	0.22	0.32	0.19	0.23	0.022	***	ns	ns

[a] Genotypes are as follows: AA = homozygous for strong alleles, AF = heterozygous for a weak allele.
[b] + $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = not significant. [a, b, c]Means within a row with different superscripts differ ($P \leq 0.05$).
[c] Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/d barley meal, MHB = mixed hay plus 800 g/d barley meal.

Table 4 Effects of diet and *CSN1S1* genotype of goats on long-chain and grouped fatty acid composition (g/100 g FAME) of milk

Genotype (G)[a]				AA	AF		AA			AF			SEM	Significance[b]		
Diet (D)[c]	SFF	SFB	MHB			SFF	SFB	MHB	SFF	SFB	MHB		D	G	D × G
C18:0	11.2[a]	8.18[b]	7.23[b]	8.39	9.36	10.2	7.96	7.00	12.2	8.39	7.46	0.64	***	+	ns
C18:1 *t11* , VA[d]	1.44[a]	0.64[b]	0.43[b]	0.77	0.91	1.23	0.62	0.45	1.65	0.66	0.42	0.15	***	ns	ns
C18:1 *c9*	15.6[a]	11.6[b]	12.2[b]	12.5[b]	13.9[a]	14.6	11.2	11.6	16.7	12.0	12.9	0.86	***	*	ns
C18:2 n-6 *c9 c12*, LA[e]	1.67[b]	2.32[a]	1.89[b]	1.87	2.05	1.76	2.19	1.65	1.58	2.44	2.14	0.18	**	ns	ns
C18:3 n-3 *c9 c12 c15*, LNA[f]	1.94[a]	0.97[b]	0.41[c]	1.11	1.10	1.88	0.88	0.56	2.00	1.05	0.26	0.17	***	ns	ns
CLA C18:2 *c9 t11*, RA[g]	0.56[a]	0.29[b]	0.27[b]	0.35	0.41	0.48	0.28	0.28	0.65	0.30	0.27	0.066	***	ns	ns
CLA isomers	0.27	0.24	0.18	0.23	0.23	0.29	0.25	0.16	0.25	0.23	0.20	0.1014	ns	ns	ns
C20:5 n-3, EPA[h]	0.20	0.16	0.17	0.19	0.16	0.22	0.15	0.19	0.18	0.17	0.14	0.034	ns	ns	ns
C22:6 n-3, DHA[i]	0.13	0.11	0.11	0.11	0.12	0.09	0.13	0.12	0.17	0.09	0.10	0.075	ns	ns	ns
C22:5 n-3, DPA[l]	0.25	0.18	0.29	0.23	0.25	0.20	0.17	0.33	0.29	0.20	0.26	0.10	ns	ns	ns
Saturated FA	70.8[b]	78.6[a]	78.8[a]	77.2[a]	74.9[b]	72.5	79.7	79.4	69.1	77.5	78.1	1.42	***	*	ns
Monounsaturated FA	21.8[a]	15.8[b]	16.5[b]	17.1[b]	18.9[a]	20.3	15.2	15.9	23.3	16.5	17.0	1.08	***	*	ns
Polyunsaturated FA	6.35[a]	4.78[b]	3.91[c]	4.85	5.17	6.25	4.44	3.88	6.46	5.12	3.94	0.37	***	ns	ns
Unsaturated FA	28.1[a]	20.6[b]	20.4[b]	22.0[b]	24.1[a]	26.5	19.6	19.8	29.8	21.6	21.0	1.36	***	*	ns
Saturated/Unsaturated	2.56[b]	3.92[a]	4.04[a]	3.74[a]	3.28[b]	2.78	4.17	4.25	2.33	3.67	3.83	0.29	***	*	ns
Σ omega-6	2.53	2.88	2.50	2.55	2.72	2.69	2.70	2.27	2.37	3.06	2.72	0.23	ns	ns	ns
Σ omega-3	2.56[a]	1.30[b]	0.91[b]	1.56	1.61	2.39	1.20	1.10	2.72	1.40	0.71	0.21	***	ns	ns
omega-6/omega-3	1.14[c]	2.29[b]	3.96[a]	2.53	2.40	1.40	2.28	3.91	0.88	2.31	4.01	0.58	***	ns	ns
OBCFA[m]	6.58[a]	4.71[b]	5.37[b]	5.32	5.79	6.25	4.30	5.42	6.92	5.12	5.32	0.39	***	ns	ns
Σ C4-C14	30.0[b]	38.7[a]	36.7[a]	36.9[a]	33.4[b]	33.7[b]	40.5[a]	36.4[ab]	26.3[c]	36.8[ab]	37.0[ab]	1.73	***	*	+
C14:1/C14:0	0.009	0.014	0.016	0.012	0.013	0.008	0.013	0.016	0.010	0.014	0.015	0.003	+	ns	ns
C16:1/C16:0	0.020	0.018	0.020	0.018	0.021	0.020	0.016	0.019	0.021	0.020	0.022	0.003	ns	ns	ns
C17:1/C17:0	0.26	0.23	0.35	0.25	0.31	0.26	0.22	0.27	0.26	0.23	0.43	0.060	ns	ns	ns
C18:1/C18:0	1.45	1.52	1.75	1.55	1.59	1.51	1.43	1.70	1.39	1.59	1.79	0.15	ns	ns	ns
RA/VA[n]	0.40[b]	0.47[b]	0.65[a]	0.49	0.53	0.39	0.44	0.63	0.42	0.50	0.67	0.065	**	ns	ns
HPI[o]	0.44[a]	0.25[b]	0.24[b]	0.30	0.32	0.41	0.24	0.24	0.47	0.26	0.24	0.026	***	ns	ns

[a] Genotypes are as follows: AA = homozygous for strong alleles, AF = heterozygous for a weak allele.

[b] + $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = not significant. [a, b, c] Means within a row with different superscripts differ ($P \leq 0.05$).

[c] Diets are as follows: SFF = sulla (*Hedysarum coronarium* L.) fresh forage, SFB = sulla fresh forage plus 800 g/d barley meal, MHB = mixed hay plus 800 g/d barley meal.

[d] VA = vaccenic acid. [e] LA = linoleic acid. [f] LNA = α-linolenic acid. [g] RA = rumenic acid.

[h] EPA = eicosapentaenoic acid. [i] DHA = docosahexaenoic acid. [l] DPA = docosapentaenoic acid.

[m] OBCFA = odd and branched chain fatty acids. [n] RA/VA = rumenic acid/vaccenic acid.

[o] HPI = Health Promoting Index [27] = unsaturated fatty acids/[C12:0 + (4 × C14:0) + C16:0].

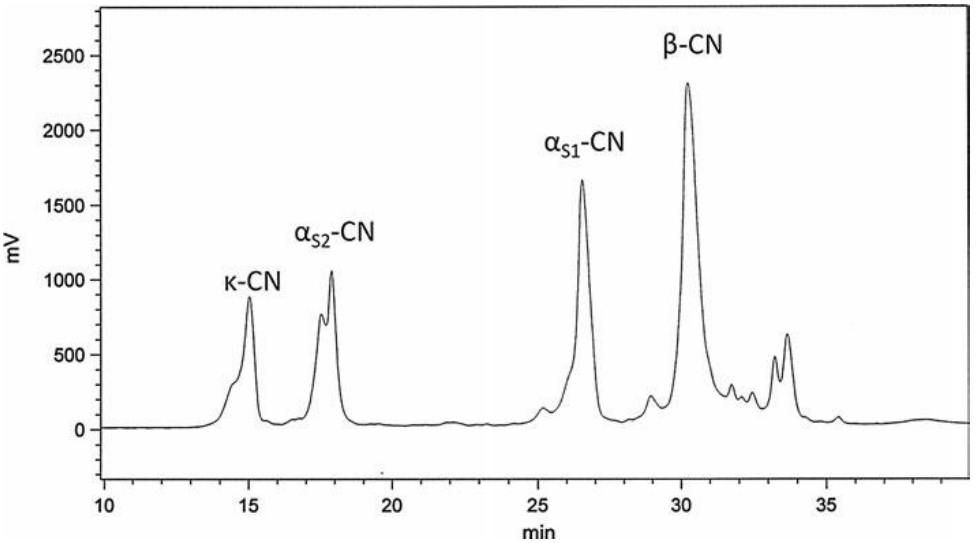

Figure 1 Chromatogram obtained by RP-HPLC from a milk sample of a goat with AA genotype at *CSN1S1* loci, showing a high expression of α_{S1}-CN synthesis.

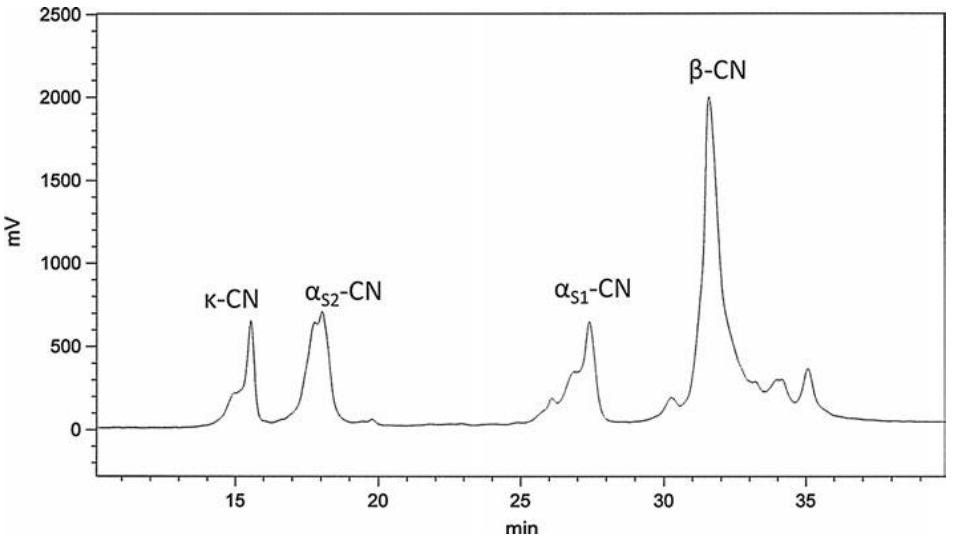

Figure 2 Chromatogram obtained by RP-HPLC from a milk sample of a goat with AF genotype at *CSN1S1* loci, showing a low expression of α_{S1}-CN synthesis.

3

Development and validation of RP-HPLC method for the quantitative estimation of αs_1-genetic variants in goat milk

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed and validated for separation and quantification of the most common genetic variants of α_{s1}-casein in goat's milk, to evaluate the effect of α_{s1}-casein polymorphisms on casein content. Chromatography was carried out by binary gradient technique on a reversed-phase C8 Zorbax column and the detection was made at a wavelength of 214 nm. The procedure was developed using individual raw milk samples of Girgentana goats. For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes, considering that commercial standards for goat genetic variants were not available. The data obtained for Girgentana goat breed showed that A, B, F variants were alleles associated with a content of αs_1-casein in milk of 3.2 ± 0.4, 5.4 ± 0.5, 0.7 ± 0.1 g/L, respectively, whereas N variant was a 'null' allele associated with the absence of αs_1-casein in milk.

Key Words: Genetic variants; αs_1-casein; HPLC; Goat milk.

INTRODUCTION

In the milk of ruminants, more than 95% of proteins are synthesized by six structural genes, four caseins (αs_1-, β-, αs_2- and κ-caseins) and two whey proteins (α-lactalbumin and β-lactoglobulin). Among Ca-sensitive caseins (αs_1, β, and αs_2), the αs_1-casein fraction is the most extensively investigated in goat species (Martin, Szymanowska, Zwierzchowski & Leroux, 2002; Rijnkels, 2002). The extensive polymorphism at αs_1-casein locus has been shown to affect not only the quantity of casein in goat milk, but also the structural and nutritional characteristics and technological properties of milk. In fact, polymorphism associated with a quantitative variability in casein synthesis has a significant effect on coagulation properties, micelle size and mineralization, cheese yield, and sensory attributes (Ramunno, Pauciullo, Mancusi, Cosenza, Mariani & Malacarne, 2007). So far, at least 17 codominant alleles have been identified at DNA level, which are associated with different expression levels of αs_1-casein in milk. A first group of alleles (A, B1, B2, B3, B4, C, H, L, M) are associated with a high content of αs_1-casein (about 3.5 g/L), alleles I and E are associated with an intermediate content (about 1.1 g/L), and alleles D, F, and G with a low level (about 0.45 g/L) of this protein in milk. Alleles αs_1-casein N, 01 and 02 are 'null' alleles and have been associated with the absence of αs_1-casein in milk (Grosclaude, Mahé, Brignon, Di Stasio & Jeunet, 1987; Chianese, Ferranti, Garro, Mauriello & Addeo, 1997; Martin, Ollivier-Bousquet & Grosclaude, 1999; Bevilaqua et al., 2002; Ramunno et al., 2005). The presence of alleles associated with "low" and "null" content of αs_1-casein in goat milk, may be interesting considering that very low levels of αs_1-casein

were found to be less allergenic than milk characterized by high level of αs_1-casein (Haenlein, 2004). Hence, the quantification of different genetic variants at αs_1-casein locus became very important for the quality of milk and also for the possible valorization of the products that are linked to a specific breed (i.e. mono-breed labeled cheeses). Nowadays, a great variety of methods have been developed to analyze milk protein fractions: alkaline urea polyacrylamide gel electrophoresis (urea-PAGE) and RP-HPLC (Reversed Phase-High Performance Liquid Chromatography) for whole caseins analysis and Cation-Exchange Chromatography (CEC) of whole casein for the fractionation of the lyophilized casein (Moatsou, Samolada, Panagiotou & Anifantakis, 2004); Capillary Zone Electrophoresis (CZE) (Brambilla, Felibini & Enne, 2003; Valenti, Pagano & Avondo, 2012), RP-HPLC (Clark & Sherbon, 2000; Bonfatti, Grigoletto, Cecchinato, Gallo & Carnier, 2008), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to identify allelic polymorphisms and Rocket Immunoelectrophoresis to estimate the contents of individual caseins (Grosclaude et al., 1987); Isoelectric focusing (IEF) and RP-HPLC/Electrospray Ionization Mass Spectrometry (ESI-MS) to analyze the protein fractions and polymorphism of caseins of goat milk (Moatsou, Vamvakaki, Mollé, Anifantakis & Léonil, 2006; Moatsou, Moschopoulou, Mollé, Kandarakis & Léonil, 2008) RP-HPLC/ESI-MS and Matrix-Assisted Laser Desorption/ionization Mass Spectrometry (MALDI-MS) to identify and characterize caseins (Cunsulo et al., 2005; Cunsulo, Muccilli, Saletti, Marletta & Foti, 2006); Hydrophobic Interaction Chromatography (HIC) to separate and determine caseins (Bramanti, Sortino, Onor, Beni & Raspi, 2003); Capillary Electrophoresis (CE) for quantitative determination of caseins (Gómez-

Ruiz, Miralles, Agüera & Amigo, 2004). Separation and quantification of the different αs_1- genetic variants were difficult to achieve. In literature, the main cited values on the different levels of allelic casein content were obtained by Rocket Immunoelectrophoresis (Grosclaude et al., 1987). Capillary Electrophoresis (CE) was used for αs_1- quantitative determination confirming the results of Grosclaude et al. (1987) for the analyzed genotypes. However, till now, there were not data in literature regarding the quantitative chemical analysis of individual genetic variants of αs_1-casein in goat milk. The Girgentana goat is a Sicilian autochthonous breed reared for its good dairy production. Due to sanitary policies the size of the Girgentana goat breed decreased of almost 90% in 20 yrs. In 1983, the population consisted of 30,000 individuals but, nowadays, only 522 lactating goats in 25 farms are present in Sicily (AIA, 2012). The aims of this work were to separate and quantify the most common genetic variants of αs_1-casein in milk of Girgentana goat breeds, to compare our results with the quantitative data proposed by Grosclaude et al. (1987) and to evaluate the effect of each allele on αs_1-casein content. Moreover, it could be interesting to evaluate the possibility of revitalizing interest in the milk produced by Girgenatana goat breed in order to regain an important economic role in the production of "drinking-milk" requested for particular food products, such as milk for infants, using weak and null genotypes, and in the production of niche products, using strong genotypes.

EXPERIMENTAL

Reagents, standards and samples

Acetonitrile and Water ultra Plus (Carlo Erba Reagents, Italy) were of HPLC grade, Trifluoroacetic acid (TFA) was from Romil Pure Chemistry (Cambridge, United Kingdom). All other chemicals were of analytical grade. BisTris buffer, Dithiothreiol (DDT), Guanidine hydrochloride (GdnHCl), Sodium citrate were from Sigma-Aldrich (Milano, Italy). Purified αs-casein standard from bovine milk was purchased from Sigma-Aldrich (Milano, Italy). A total of 200 individual milk and blood samples of lactating goats of Girgentana breed were randomly collected in 15 different flocks located in different areas of Sicily. Samples were collected from 10-15 unrelated individuals per herd. A subset of 40 samples were used for validation and quantification procedure and a total of 100 individual goat milk samples, previously genotyped, was analyzed by RP-HPLC method. Goat αs_1-casein genetic variants, used as standards for calibration, were obtained by extraction and lyophilization from individual milk samples with homozygous genotypes. The samples belonged to different αs_1-casein genotypes: four samples corresponding to genotype AA, four samples to genotype BB, five samples to genotype AB, five samples to genotype FF, six samples to genotype AF, five samples to genotype BF, one sample to genotype NN, five samples to genotype AN, two samples to genotype BN, and finally three samples to genotype FN.

All goat milk collected samples were lyophilized and frozen at −20°C until analysis. Before analysis, the lyophilized milk sample was solubilized by adding a corresponding volume of ultrapure water. Milk samples were prepared following the method proposed by Bobe, Beitz, Freeman & Lindberg (1998). The diluted samples were analyzed by direct chromatographic.

HPLC equipment

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of a model LC-20AT liquid chromatographer, a model DGU-20A 5 degasser, a model CTO-20A column oven, a model SPD-20A UV/VIS detector and a model FRC-10A fraction collector. It was operated by means of the LC Solutions software which sets solvent gradient, data acquisition and data processing.

Separations were performed on a reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5µm, 300Å, 150×4.6 I.D.). A security Guard Cartridge System (product No. 820999-901, Agilent Technologies) was used as pre-column (Zorbax 300SB-C8, Agilent Technologies).

The sample vial was injected via an auto-sampler (Shimadzu SIL-20A HT series). An injection loop of a 100 µl was used.

Chromatographic conditions

The analyses were carried out applying a binary gradient profile to the mobile phase composition using two solvents. Solvent A consisted of 0.1% TFA in water and solvent B of 0.1% TFA in acetonitrile.

Separations were performed with the program proposed by Bonfatti et al. (2008) except for duration of the final re-equilibration condition under the starting conditions that was 13 min. Therefore, the total analysis time per sample was 50 min. This ensured the maintenance of chromatographic performance in sample run.

The flow rate was 0.5 ml/min, the column temperature was kept at 45°C and the detection was made at a wavelength of 214 nm. The injection volume consisted of 5 µl.

Purified proteins

Pure αs_1-casein genetic variants were extracted for calibration experiments considering that commercial standards for goat were not available. Each variant was purified by RP-HPLC, starting from individual milk samples of DNA-genotyped animals, and then lyophilized and weighted.

For this purpose, the same elution conditions were used in semi-preparative experiments by collecting the correspondent peaks. A semi-preparative Zorbax 300SB-C8 (5µm, 300Å, 250 mm x 9.4 mm, Agilent Technologies) column and a fraction collector were used. The flow rate was 2 ml/min. After lyophilization, in order to obtain a standard solution, purified proteins were solubilized in a solution containing 4.5 M GndHCl and solvent A, and stored at -20°C.

DNA genotyping

For our study, 200 blood samples of Girgentana goat breed were randomly collected and genomic DNA was extracted from buffy coats of nucleated cells using a salting out method (Miller, Dykes & Polesky, 1988). The αs_1-caseinA*/01, B*/E, F and N alleles were simultaneously investigated by PCR-RFLP using *Xmn*I restriction enzyme (Ramunno et al., 2000). This protocol allowed the identification of F and N alleles, but not distinguish allele A* from 01, and allele B* from E. Allele Specific-PCR was used for the detection of the αs_1-casein E (Dettori, Vacca,

Carcangiu, Pazzola, Mura & Rocchigiani, 2009) and αs_1-casein 01 alleles (Cosenza et al., 2001: Cosenza, Illario, Rando, Di Gregorio, Masina & Ramunno, 2003). The A* indicated A, G, I, and H alleles while B* indicated B1, B2, B3, B4, and C alleles.

Validation

In validation tests, ten individual milk samples from Girgentana goats were used. Linearity was tested by running the same sample at increasing injecting volume 5-80 µl in triplicate. To estimate the precision of method, the repeatability and the reproducibility were evaluated. Repeatability was established by consecutive injections of samples while reproducibility by analyzing each sample on four different days. The accuracy was determined by quantifying each genetic variant in two samples and by repeating the quantification on different mixtures of them (at 75, 50 and 25%). Each mixture was analyzed in duplicate.

For each genetic variant of αs_1-casein, calibration curves were computed injecting increasing volume (5, 10, 20, 40 and 80 µl) of corresponding purified standard solution.

RESULTS AND DISCUSSION

Separation

The identification of αs_1-casein peak in milk samples was confirmed by comparison with commercial standard that consisted of purified genetic variants from bovine milk. Since in commercial standards αs_1 and αs_2 are not available as single proteins, assignment was made on the basis of the 4:1 proportion known for cow milk (Alais, 1984). The identification of αs_1-casein genetic variants of Girgentana goat breed was confirmed by

comparison with chromatograms of individual milk samples of animals with homozygous genotypes. For homozygous animals, genetic variants gave rise to a single peak. The assignment of peaks of αs_1-casein genetic variants was made by comparing the chromatograms of individual milk samples of homozygous animals with those of heterozygous animals (Fig. 1, Fig. 2).

Fig. 1 Chromatograms relative to individual milk samples with different αs_1-casein (αs_1-CN) genotypes obtained using the optimized condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml/min at 45°C, UV detection at 214 nm.

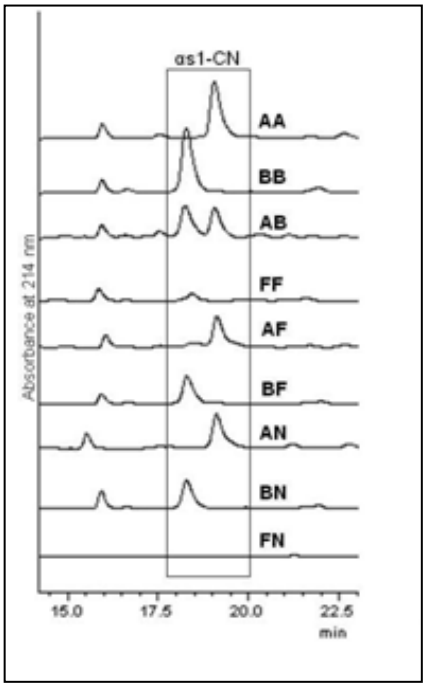
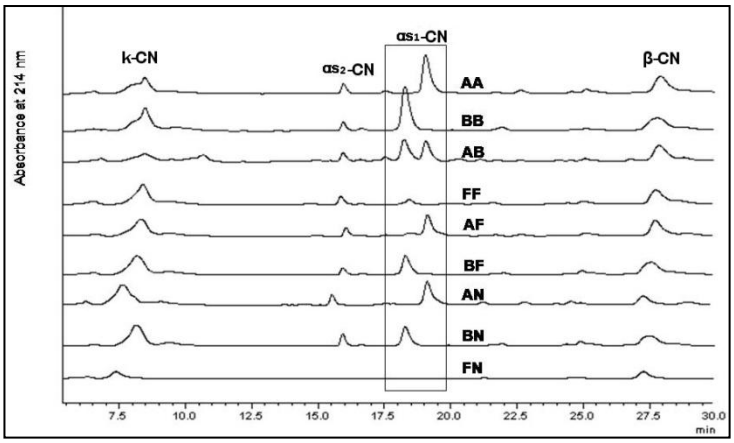


Fig. 2 Chromatograms relative to individual milk samples with different casein (CN) genotypes obtained using the optimized condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml/min at 45°C, UV detection at 214 nm.



In this study, A and B genetic variants of αs_1-casein were perfectly resolved with the current method; in fact, the resolution between these two peaks exceeded 1.05 which is considered satisfactory value in chromatographic separation. Despite the gradient optimization, B and F genetic variants co-eluted and this made impossible to quantify the genetic variants in the heterozygous condition (BF), whereas, A and F variants were perfectly resolved. Nevertheless, the validity of this method was confirmed by the fact that the most frequent genotype at this locus in Girgentana breed was AF (0.365) followed by AA (0.340) and FF (0.090), whereas the frequencies for BF genotype was very low (0.015) (Mastrangelo, Sardina, Tolone & Portolano, in press). Chromatographic analysis confirmed that N was a 'null' allele associate with the absence of αs_1-casein in milk (Ramunno et al., 2005).

In the group of sampled animals, no individuals carrying E and 0′ were found therefore, assessment for these two variants was not feasible.

To analyze genetic polymorphism of caseins several electrophoretic techniques have been used but none of them appears to be fully satisfactory for resolution of αs_1- and αs_2 caseins and the identification of the relevant variants (Grosclaude et al., 1987; Boulanger, Grosclaude, & Mahè, 1984; Russo, D'Avoli, Dall'Olio & Tedeschi, 1986). As alternative, chromatographic techniques such as RP-HPLC, have been shown to achieve genetic variants of casein fraction of bovine milk (Bonfatti et al., 2008). Consequently, the comparison of our results with other reported in literature was impossible. However, it was possible to compare our results with those reported by Bonfatti et al. (2008) on bovine milk proteins. In fact, while their data for αs_1-casein genetic variants showed a co-eluting of the two found variants B and C, our data on a wider number of genotypes showed a separation of αs_1-casein genetic variants with very high resolution.

Quantitative analysis

Quantification by RP-HPLC was performed for individual milk samples. The external standard method was used to calibrate the chromatographic system for αs_1-casein genetic variants quantifications. Five points calibration curves were generated for each genetic variant by estimating parameters of the linear regression of the peak area on the amount injected, with increasing injection volume of each standard solution (5, 10, 20, 40 and 80 µl). Each solution was analyzed in triplicate.

The data obtained for Girgentana goat breed showed that A and B variants were strong alleles associated with a high content of αs_1-casein

with some quantitative differences respect to Grosclaude et al. (1987), and that F variant was a weak allele associated with a low level of αs_1-casein in milk (Tab. 1). In our study, quantification data of B genetic variant compared to A showed that the expression of this allele determines a higher content of αs_1-casein in milk.

Table 1 Content (g/l) in αs_1-casein (αs_1-CN) for allele

Genetic Variant	g/L for allele	Samples (n)[a]
αs_1-CN $_A$	3.2 ± 0.4	8
αs_1-CN $_B$	5.4 ± 0.5	7
αs_1-CN $_F$	0.7 ± 0.1	9

[a]Homozygous and heterozygous analyzed samples

3.1. Linearity, Repeatability, Reproducibility and Recovery

The linearity of method was evaluated by the least square regression method using unweighted calibration data. The linear relation was estimated between peak area and injected amount of genetic variants of αs_1-casein (R^2>0.999; data not shown). Parameters of calibration curves are reported in Table 2.

Table 2 Parameters of regression equations for calibration curves, response factors, and limit of detection (LOD) for single αs_1-casein (αs_1-CN) genetic variants[a]

Allelic Variant	Intercept ± SE[b]	Slope ± SE[b]	R^2	Response ± SD (μg/area)·10^5	LOD[c] (μg)	Injected amount (μg)	Theorical plates [e](N·10^3)
αs_1-CN_A	187031 ± 26856	187536 ± 964	0.9997	0.49 ± 0.03	0.5	3.4-54.0	20.00
αs_1-CN_B	66967 ± 20990	120428 ± 756	0.9995	0.80 ± 0.02	0.6	3.2-52.0	11.57
αs_1-CN_F	-44579 ± 4370	165551 ± 705	0.9998	0.66 ± 0.04	0.1	0.75-12.00	20.00

[a]Separated solutions of purified αs_1-CN allelic variants injected at volume of 5, 10, 20, 40 and 80 μl in triplicate.
[b]Standard error.
[c]Calculated on the basis of calibration curve slope.
[d]Different injected amounts were used in respect to the average proportions of the proteins in milk.
[e]For computation of the number of theoretical plates, peak width at the baseline was obtained by tangential lines drawn at half-height.

The precision studies were composed of repeatability and reproducibility and, in Table 3, were shown the values of relative standard deviation (RSD) for retention times and peaks areas. All RSD values were similar to those reported in literature for within- and between-days variation for genetic variants in bovine milk (Martin et al., 1999; Moatsou et al., 2004). Results indicate that the precision of the method was acceptable. The RSD values for retention times were below 0.22% within analytical day (repeatability) and below 0.60% across analytical days (reproducibility). Values of RSD for peak areas were below 0.77% within day and below 5.00% among days.

Table 3 Relative standard deviation of retention times and peak areas for milk proteins fractions or genetic variants obtained in the analysis of repeatability and reproducibility

Allelic Variant	Repeatability[a]		Reproducibility[b]		Samples (n)
	Retention time RSD (%)	Area RSD (%)	Retention time RSD (%)	Area RSD (%)	
αs_1-CN $_A$	0.13	0.47	0.57	5.00	10
αs_1-CN $_B$	0.22	0.77	0.57	4.72	10
αs_1-CN $_F$	0.07	0.67	0.60	4.50	7

[a]Separated solutions of purified α_{s1}-CN allelic variants injected at volume of 5, 10, 20, 40 and 80µl in triplicate.
[b]Standard error.

Pre-column conditions might have also affected the reproducibility of quantification of whey

proteins. Thus, a frequent guard-cartridge turnover was advisable. In addition, a blank injection might be used after each sample run.

Recovery studies were carried out to determine the accuracy of the method (Table 4). Recoveries ranged from 99.33% to 103.13% and results of Student's t-test indicated that recovery rates were not significantly different from 100% at $P<0.05$.

Table 4 Results of the analysis of accuracy[a]

Allelic Variant	Recovery rate (%)	RSD%
αs_1-CN $_A$	99.33	4.13
αs_1-CN $_B$	103.13	4.35
αs_1-CN $_F$	101.97	3.54

[a]Mixtures of two raw milk samples were obtained following relative proportions of 75, 50 and 25%. Mixtures and whole samples were analyzed in duplicate and recovery rates were calculated using expected areas provided by calibration curves and observed areas

CONCLUSIONS

In this study, RP-HPLC method for separation and quantification of αs_1-casein genetic variants in goat milk was developed and validated. The proposed method was simple and selectively providing satisfactory accuracy with low limits of detection. It ensures a precise quantification of the αs_1-casein variants and could be a useful tool for studies on composition of goat milk proteins. The data obtained for genetic variants were in agreement with the only available data published by Grosclaude et al. (1987) but, in addition showed significant differences in the protein contents per allele. Finally, this chromatographic method appears to be

particularly interesting, because it provides fractionation and resolution of several genetic variants of αs_1-casein goat milk.

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4

Quantitative determination of casein genetic variants in milk of Girgentana dairy goat breed

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Abstract

Forty five milk samples of Girgentana lactating goats (seven with AA genotype and six with FF genotype at αs_2-casein; nine with CC genotype and seven with C1C1 genotype at β-casein; eight with AA genotype and eight with BB genotype at κ-casein) were used to quantify genetic variants of caseins by a high-performance liquid chromatographic (HPLC) method.

Chromatography was carried out by binary gradient technique on a reversed-phase C8 Zorbax column and the detection was made at a wavelength of 214 nm. The procedure was developed using individual raw milk samples of Girgentana goat breed.

For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes, considering that commercial standards for goat allelic variants were not available. Several analytical parameters were evaluated showing the reliability of RP-HPLC method. The data obtained for Girgentana goat breed show following levels of caseins for allele: αs_2-casein A=2.9 ± 0.8 g/L and F=1.8 ± 0.4 g/L; β -casein C=3.0 ± 0.8 g/L and C1=2.0 ± 0.7 g/L and κ-casein A=1.6 ± 0.3 g/L and B=1.1 ± 0.2 g/L.

Key Words: Genetic variants; caseins; HPLC; Goat milk.

INTRODUCTION

The production and consumption of goat's milk and its dairy products are increasing worldwide. Goat milk is a valuable source of protein in many countries, including a large number of African, Asian and European countries such as Norway, France, and Italy. The most abundant proteins in goat milk, as in other milks, are the caseins (Hayes et al., 2006).

Goat caseins show high quantitative variability, caused by difference in protein expression, qualitative variability due to structural polymorphism of casein genes, differential splicing patterns and post-translation modifications (Marletta et al., 2007).

Another important aspect to be considered is the study of nutritional and metabolic properties of goat's milk, especially for its potential use in infants and patients with cow's milk protein intolerance, although the exact role of these protein components in milk allergies remains still uncertain (Lamblin et al., 2001).

Goat breeds have been widely investigated for polymorphisms of milk proteins, which have been related to milk chemical composition, cheese-making 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).

A great variety of methods have been developed to analyze milk casein fractions: Capillary Zone Electrophoresis (CZE) (Brambilla, Felibini & Enne, 2003; Valenti, Pagano & Avondo, 2012), RP-HPLC (Clark & Sherbon, 2000; Bonfatti, Grigoletto, Cecchinato, Gallo & Carnier, 2008), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to identify allelic polymorphisms and Rocket Immunoelectrophoresis to estimate the contents of individual caseins (Grosclaude et al., 1987); Isoelectric

focusing (IEF) and RP-HPLC/Electrospray Ionization Mass Spectrometry (ESI-MS) to analyze the protein fractions and polymorphism of caseins of goat milk (Moatsou, Vamvakaki, Mollé, Anifantakis & Léonil, 2006; Moatsou, Moschopoulou, Mollé, Kandarakis & Léonil, 2008) RP-HPLC/ESI-MS and Matrix-Assisted Laser Desorption/ionization Mass Spectrometry (MALDI-MS) to identify and characterize caseins (Cunsulo et al., 2005; Cunsulo, Muccilli, Saletti, Marletta & Foti, 2006); Hydrophobic Interaction Chromatography (HIC) to separate and determine caseins (Bramanti, Sortino, Onor, Beni & Raspi, 2003); Capillary Electrophoresis (CE) for quantitative determination of caseins (Gómez-Ruiz, Miralles, Agüera & Amigo, 2004).

In literature, there were not data regarding the quantitative chemical analysis of single genetic variants of caseins in goat milk probably because commercial standards for goat casein were not available, therefore, the quantification is difficult to achieve. Only recently, some data for quantification of allelic variants of αs_1-casein in Girgentana goat milk was proposed by Montalbano et al. (2012).

As it is well known, a first group of alleles (A, B1, B2, B3, B4, C, H, L, M) are associated with a high content of αs_1-casein (about 3.5 g/L), alleles I and E are associated with an intermediate content (about 1.1 g/L), and alleles D, F, and G with a low level (about 0.45 g/L) of this protein in milk. Alleles N, 01 and 02 are 'null' alleles and have been associated with the absence of αs_1-casein in milk (Grosclaude et al., 1987; Bevilacqua et al., 2002).

For the β-casein fraction, the A, A1, B, C, C1, D, E alleles are associated with a normal content of this protein in milk (5.0 g/L/allele), and the

CSN2 0 and 01 “null” alleles are associated with the absence of this casein fraction in milk (Chessa et al., 2008a).

The αs_2-casein comprises the A, B, C, E, F variants that are “strong” alleles associated with a normal content (about 2.5 g/L per allele) of this protein in milk (Boulanger et al., 1984, Bouniol et al., 1994, Lagonigro et al., 2001, Ramunno et al., 2001a), an intermediate D allele, which expresses a reduced level of αs2-casein in milk (about 1.5 g/L per allele) and a null 0 allele which causes no detectable amount (Ramunno et al., 2001a, b). Moreover, Erhardt et al. (2002) reported the G variant associated with a normal content of αs2-casein typed at protein level by isoelectric focusing (IEF), but not characterized at DNA molecular level.

Sixteen allelic variants have been identified so far in goat κ-casein, these have been clustered into two groups by IEF of milk samples. The A^{IEF} group (isoelectric point = 5.53) contains the A, B, B′, B″, C, C′, F, G, H, I, J, L alleles while the B^{IEF} group (isoelectric point = 5.78) contains the D, E, K, M alleles (Prinzenberg et al., 2005). Moreover, two new alleles (D′ and N) have been identified in Girgentana goat breed and characterized at molecular level (Di Gerlando et al., 2013).

The Girgentana goat is a Sicilian autochthonous breed reared for its good dairy production. According to morphology, this breed probably came from Afghanistan and Himalaya regions (Portolano, 1987). Due to sanitary policies the size of the Girgentana goat breed decreased of almost 90% in 20 yrs. In 1983, the population consisted of 30,000 individuals but, nowadays, only 650 heads are reared in Sicily belonging to approximately 30 flocks (ASSONAPA 2012). Over the last years this breed has become almost extinct, in part as a consequence of the marked decrease in fresh goat milk consumption.

The aims of this work was the quantification of the most common genetic variants of caseins in milk of Girgentana dairy goat breeds, to evaluate the effect of each allele on casein content using a high-performance liquid chromatographic (HPLC) method proposed by Bonfatti et al. (2008) was and validated for separation and quantification of bovine milk protein genetic variants. It could be interesting to evaluate the possibility of revitalizing interest in the milk produced by Girgenatana goat breed in order to regain an important economic role in the production of "drinking-milk" requested for particular food products, such as milk for infants, using weak and null genotypes, and in the production of niche products, using strong genotypes.

MATERIALS AND METHODS

Reagents, standards and samples

Acetonitrile and Water ultra Plus (Carlo Erba Reagents, Italy) were of HPLC grade, Trifluoroacetic acid (TFA) was from Romil Pure Chemistry (Cambridge, United Kingdom). All other chemicals were of analytical grade. BisTris buffer, Dithiothreiol (DDT), Guanidine hydrochloride (GdnHCl), Sodium citrate were from Sigma-Aldrich (Milano, Italy). Purified αs-, κ-, β-casein standard from bovine milk was purchased from Sigma-Aldrich (Milano, Italy). A total of 200 individual milk and blood samples of lactating goats of Girgentana breed were randomly collected in 15 different flocks located in different areas of Sicily. Samples were collected from 10-15 unrelated individuals for herd. A subset of 45 samples were used for validation and quantification procedures and all samples, previously genotyped, was analyzed by RP-HPLC method. Goat casein genetic variants, used as standards for calibration, were obtained

by extraction and lyophilization from individual milk samples with homozygous genotypes.

In particular, seven samples with AA genotype and six samples with FF genotype at αs_2-casein; nine samples with CC genotype and seven samples with C1C1 genotype at β-casein, and eight samples with AA genotype and eight samples with BB genotype at κ-casein were used.

All goat milk samples were lyophilized and frozen at −20°C until analysis. Before analysis, the lyophilized milk samples were solubilized by adding a corresponding volume of ultrapure water. Milk samples were prepared following the method proposed by Bonfatti et al. (2008). The diluted samples were analyzed by direct chromatography.

Chemical analysis

The chromatographic system (Shimadzu, Kyoto, Japan) used to perform the analyses consisted of LC-20AT liquid chromatographer, DGU-20A 5 degasser, CTO-20A column oven, SPD-20A UV/VIS detector, and FRC-10A fraction collector.

It was operated by means of the LC Solutions software which sets solvent gradient, data acquisition and data processing.

Separations and calibration experiments were performed with the program proposed by proposed by Bonfatti et al. (2008).

Sampling, DNA extraction and genotyping of caseins in Girgentana goat

All blood samples were used to extract genomic DNA from buffy coats of nucleated cells using a salting out method (Miller et al., 1988). The *CSN2* A, A1, C, C1, E, and 0' alleles were identified using PCR protocols of Chessa et al. (2005; 2008a) followed by sequencing of amplified

fragments with ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems). At *CSN1S2* locus alleles B and C/E were detected by multiplex AS-PCR (Vacca et al., 2009). The allele E was identified by PCR-RFLP protocol of Lagonigro et al. (2001) using primers by Chessa et al. (2008b). The alleles D, 0, and F were genotyped by PCR-RFLP (Ramunno et al. 2001a). The allele A at this locus has been assigned when all the other alleles were not present. The several alleles at *CSN3* locus were identified by PCR protocol described by Prinzenberg et al. (2005) with primers by Di Gerlando et al. (2013), followed by sequencing of amplified fragments with ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems).

Statistical analysis

Individual data for each casein were analyzed to test the linearity, repeatability, reproducibility and accuracy of the method for each casein genetic variant. Linearity was tested by running the same sample at increasing injecting volume 5-80 µl in triplicate. To estimate the precision of method, the repeatability and the reproducibility were evaluated. Repeatability was established by consecutive injections of samples while reproducibility by analyzing each sample on four different days. The accuracy was determined by quantifying each genetic variant in two samples and by repeating the quantification on different mixtures of them (at 75, 50 and 25%). Each mixture was analyzed in duplicate.

For each genetic variant of investigated caseins, calibration curves were computed injecting increasing volume (5, 10, 20, 40 and 80 µl) of corresponding purified standard solution.

RESULTS AND DISCUSSION

Separation of casein

The identification of casein peak in goat milk samples was confirmed by comparison with commercial standard that consisted of purified caseins from bovine milk. Therefore, it was ascertained that goat proteins eluted in the same order of bovine ones: κ-CN, αs_2-CN, αs_1-CN and β-CN (Fig.1). As expected, the retention times of eluted caseins were not the same probably due to different protein chemical structure.

The validated RP-HPLC method, that allows the quantification of content for allele (g/L) for αs_2-, β- and κ-casein was carried out by analyzing only milk samples of homozygous animals.

Comparing our data with those reported by Bonanno et al. (2013), which used a different chromatographic method , it can be possible to state that our applied chromatographic conditions allowed in less time running a greater separation and resolution of caseins.

Quantitative analysis

Quantification by RP-HPLC was performed for individual milk samples. The external standard method was used to calibrate the chromatographic system for casein genetic variants quantifications as proposed by Bonfatti et al. (2008). The comparison of our data (Tab.1) with other studies on αs_2-casein (Boulanger et al., 1984, Bouniol et al., 1994, Lagonigro et al., 2001, Ramunno et al., 2001a) showed a similar content of A and F alleles that were defined “strong” alleles associated with a normal content of this protein in milk.

The β-casein data compared with those published by Chessa et al. (2008a), which report the CSN2 A, A1, B, C, C1, D, E variants are

associated with a normal content (5.0 g/l/allele) showed a lower content of this protein associated to C and C1 alleles (3.0±0.8 and 2.0±0.7, respectively).

There are no data reported the quantification of single allelic variants for κ-casein. The only data available confirmed that B^{IEF} group represents the more favorable variants group in terms of milk κ-casein content (Chiatti et al., 2007). The application of our analytical method allowed the separation of A and B alleles (Tab. 1).

Linearity, Repeatability, Reproducibility and Recovery

The method linearity was evaluated by the least square regression method using unweighted calibration data. The linear relation was estimated between peak area and injected amount of genetic variants of casein (R^2>0.987). Parameters of calibration curves are reported in Table 2. The precision studies were composed of repeatability and reproducibility and, in Table 3, were shown the values of relative standard deviation (RSD) for retention times and peaks areas. All RSD values were similar to those reported in literature for within- and between-days variation for genetic variants in bovine milk (Martin et al., 1999; Moatsou et al., 2004). Results indicate that the precision of the method was acceptable. The RSD values for retention times were below 0.23% within analytical day (repeatability) and below 0.43% across analytical days (reproducibility). Values of RSD for peak areas were below 1.04% within day and below 5.08% among days.

Recovery studies were carried out to determine the accuracy of the method and results of Student's t-test indicated that recovery rates were not significantly different from 100% at P<0.05 (data not show).

CONCLUSIONS

In conclusion a RP-HPLC method was successfully applied for quantitative determination of αs_2-casein genetic variants A and F, β-casein C and C1, κ-casein A and B. These data, specific for the Girgentana goat breed, were obtained by methods and techniques with high precision and accuracy. The studied analytical parameters for HPLC method (linearity, repeatability, reproducibility and recovery) are suitable for caseins quantification in milk. A good correlation was found between the quantities of αs_2-casein genetic variants A and F, and β-casein C and C1 with other method previously described. The main important obtained result was for κ-casein because, till now, no data were available for quantification of single genetic variants of this protein.

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Table 1 Content (g/L) in casein (CN) for allele

Genetic Variant	g/L for allele	Samples (n)[a]
αs_2-CAN	2.9 ± 0.8	7
αs_2-CN_F	1.8 ± 0.4	6
β-CN_C	3.0 ±0.8	9
β –CN_{C1}	2.0 ± 0.7	7
K-CAN	1.6 ± 0.3	8
K-CN_B	1.1 ± 0.2	8

Table 2 Parameters of regression equations for calibration curves, response factors, and limit of detection LOD) for single casein (CN) genetic variants[a]

Allelic Variant	Intercept ± SE[b]	Slope ± SE[b]	R^2	Response ± SD (μg/area)· 10^5	LOD[c] (μg)	Injected amount (μg)	Theorical plates e($N \cdot 10^3$)
αs_2-CN_A	-12825±6750	19870±251	0.9979	5.4±0.4	1.3	3.3-52.0	36.14
αs_2-CN_F	-46528±9093	102320±801	0.9992	1.1±0.1	0.3	1.4-22.0	34.26
β-CN_C	12516±11977	36365±748	0.9949	2.7±0.3	1.2	1.9-30.0	12.12
β –CN_{C1}	54276±10352	17642±236	0.9979	4.8±0.7	2.2	5.1-82.0	16.47
K-CN_A	-433648±218151	210486±6870	0.9874	0.54±0.06	3.9	3.7-60.0	-
K-CN_B	-162912±106761	222446±2946	0.9977	0.47±0.03	1.9	4.4-70.2	-

[a] Separated solutions of purified CN allelic variants injected at volume of 5, 10, 20, 40 and 80μl in triplicate.
[b] Standard error.
[c] Computed as LOD= 10×(3×SD) where SD is the standard deviation of the background noise.
[d] Different injected amounts were used in respect to the average proportions of the proteins in milk.
[e] For computation of the number of theoretical plates, peak width at the baseline was obtained by tangential lines drawn at half-height.

Table 3 Relative standard deviation of retention times and peak areas for milk proteins fractions or genetic variants obtained in the analysis of repeatability and reproducibility

Allelic Variant	**Repeatability**[a]		**Reproducibility**[b]		**Samples (n)**
	Retention time RSD (%)	Area RSD (%)	Retention time RSD (%)	Area RSD (%)	
αs_2-CN_A	0.12	0.90	0.17	4.43	8
αs_2-CN_F	0.06	1.04	0.15	5.08	7
β-CN_C	0.21	0.52	0.22	4.11	10
β –CN_{C1}	0.05	0.41	0.24	4.12	9
K-CN_A	0.23	0.43	0.40	1.71	8
K-CN_B	0.20	0.40	0.43	2.96	9

[a] Ten aliquots of the same individual goat milk sample were injected consecutively.
[b] A sequence of 10 individual goat milk samples was injected over 4 days.

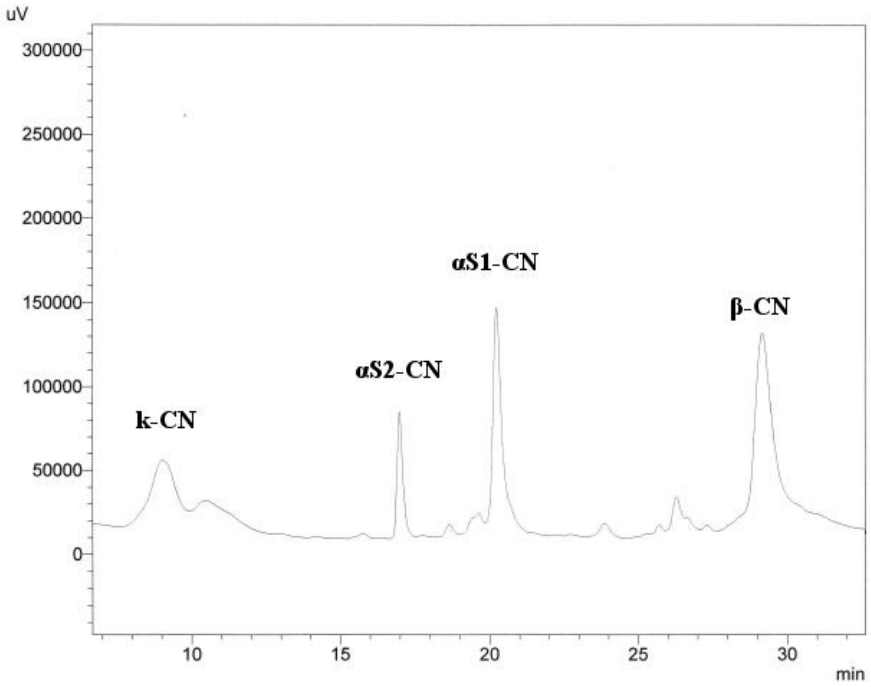

Fig. 1 Chromatograms relative to individual milk sample obtained using the optimized condition: Zorbax 300SB-C8 RP (Agilent Technologies), binary gradient, flow rate 0.5 ml/min at 45°C, UV detection at 214 nm

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5

General Conclusion

The overall objectives of this thesis were the quantification of the most common genetic variants of caseins in milk of Girgentana dairy goat breeds and the interaction studies between nutrition and genotypes at αs_1-CN locus (*CSN1S1*) in Girgentana goat breed.

The research was motivated by the fact that little knowledge exists on the implication of analytical method that have been proposed recently to give quantitative information of common genetic variants of caseins in goat milk.

Knowing the protein composition is important because of its effect on manufacturing properties of milk. For example, cheese manufacturing properties (cheese yield, milk coagulation time and curd firmness) are influenced by protein composition (Ramunno et al., 2007). Therefore, improving milk protein composition for dairy processes by selecting breeds on genetic basis can increase the economic outcome of dairy industry.

Moreover, it could be interesting to evaluate the possibility of revitalizing interest in the milk produced by Girgenatana goat breed in order to regain an important economic role in the production of "drinking-milk" requested for particular food products, such as milk for infants, using weak and null genotypes, and in the production of niche products, using strong genotypes.

An important scientific implication of the thesis is that it presents results that can be regarded as a general framework to assess the technical and economic implications of innovative strategies on dairy productions. Specifically, the thesis presented a two-step approach, where the first step assessed the interactions between nutrition and the genotype at αs_1-CN

locus (*CSN1S1*), whereas the second one assessed the quantitative determination of common caseins genetic variants in Girgentana goat breed.

In the first step, it was investigated the interactions between nutrition and the genotype at αs_1-CN locus (*CSN1S1*), evaluating the impact of fresh forage-based diets and an energy supplement on the casein and fatty acid (FA) profiles of milk from Girgentana goat breed. The results provide evidence of the pronounced effect of diet on milk FA composition (i.e., the improved health properties of the milk of goats fed exclusively *Sulla* fresh forage) and, in contrast, the weak influence of goat polymorphism at CSN1S1 locus on milk FA composition.

In the second step, a RP-HPLC method, proposed by Bonfatti et al. (2008), was successfully applied for quantitative determination of αs_1-CN genetic variants A, B and F, αs_2-CN genetic variants A and F, β-CN genetic variants C and C1, κ-CN genetic variants A and B.

The main importance of this study was that our data specific to the Girgentana goat breed were obtained by such methods and techniques that have a high precision and accuracy.

The main important results are for κ-CN because there aren't data in literature for quantification of single allelic variant. The only data available confirmed that B^{IEF} group represents the more favorable variants group in terms of milk κ-CN content (Chiatti et al., 2007).

In conclusion, it was possible to use these double results of the pronounced effect of diet on milk FA composition and of influence of goat polymorphisms at caseins loci on casein content on milk, to realize dairy processing by selecting breeding on genetic basis and/or on nutritional treatments. It was clear as genetic selection of strong casein

genetics variants, which produce a milk with high protein level, influence the cheese manufacturing properties, as genetic selection of weak and null genotypes produce a “drinking-milk” request for particular food products, such as milk for infants and finally as a diet based exclusively on fresh forage improved the health properties of milk fat which is richer in CLA (RA), OBCFA, monounsaturated, polyunsaturated, and omega-3 FA, and have lower saturated/unsaturated FA and omega-6/omega-3 FA ratios and a more favorable Health Promoting Index.

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