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Doctoral thesis

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Genetic polymorphisms at lipogenic loci in Modicana cows: effects on milk production traits in different feeding systems

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ABSTRACT

Sicily has one of the richest heritages of animal biodiversity of Italy. The dairy sector is characterized by many typical products, often related to breeds and indigenous cows, to feeding techniques that make extensive use of grazing and traditional cheese-making techniques. Dairy cattle breeds have been subjected to an intensive selection towards improvement of milk production traits. Milk composition is very variable, being conditioned by the complex metabolic activity linked to individual variability of animals, to nutritional and environmental factors.

In the present study, it was investigated for the first time in Modicana breed, novel information about polymorphisms at key genes involved in lipid metabolism and interaction between these polymorphisms and the feeding systems. It was studied how the presence of pasture in the diet can interfere with the effects of polymorphisms at the studied loci on milk yield, gross composition and fatty acid profile.

Modicana cows reared in two farms of the Ragusa province with an extensive and semi-intensive system were characterized under the genetic profile for the lipogenic polymorphisms (ABCG2-Y581S, DGAT1-K232A, STAT5A-V686A, SCD1-A293V). Subsequently, on the basis of the genotypes detected, 36 and 38 cows were selected from the semi-intensive and extensive systems, respectively. From these animals, individual phenotypic data were collected relative to productivity, milk composition, fatty acid profile and mineral composition, in order to evaluate the effect of the genetic polymorphisms and interaction with feeding systems on the quantitative and qualitative characteristics of milk.

The entire population resulted monomorphic at ABCG2 Y581S polymorphism while for the other loci the allele with higher frequency were: 91% for GC (232A) at DGAT1, 68% for T (686V) at STAT5A and 73% for T (293A) at SCD1.

Feeding system influenced milk yield and protein content that resulted higher in animals fed with natural pasture while, when feeding systems were considered in interaction with the polymorphisms, results do not allow to find significant differences within the different phenotypes in milk in both systems, extensive and semi-intensive.

Feeding system also affected milk FAs composition and animals reared in an extensive system, showed a lower content of SFA and a higher fraction of MUFA, PUFA and trans FAs. Then, considering the interaction between the feeding systems and the polymorphisms on milk FAs composition, results showed that some FAs were significant affected by this interaction. The DGAT1-K232A polymorphism in interaction with feeding system, affected the content of C4, CLA and C22:4. Milk of animals with DGAT1 GC/GC genotype (232A) and fed with pasture showed a higher content of CLA. The STAT5A-V686A polymorphism affected OBCFA composition, the content of which resulted higher in animals with TT genotype (686V). Animals with TT genotype showed also a higher content of C18:1*t11*, C18:1*c9*, C20:4 and C22:5*n3* and a lower C20:5*n3* content. No interaction has been detected between the identified genotypes and the feeding systems. The SCD1-A293V polymorphism in interaction with feeding systems affected the contents of C4, C6, C8, C18:1*t11* and C20:3 that gradually decreased in animals reared in an extensive system from CC to heterozygous to TT genotype.

Finally, regarding mineral composition, the only significant effect was reported for the K content that was associated with STAT5A-V686A polymorphism and resulted higher in heterozygous animals.

This study described that relationship between the polymorphisms at lipogenic loci and feeding systems in Modicana cows affected some milk production traits but further research are required to describe and better understand the variations in milk traits, within and between the breeds, not only through a genetic or nutritional point of view but as a dynamic interaction between these two aspects.

RIASSUNTO

La Sicilia presenta uno dei piu' ricchi patrimoni di biodiversita' animale d'Italia. Il settore lattiero-caseario e' caratterizzato da molti prodotti tipici, spesso legati a razze e popolazioni autoctone, ai sistemi di allevamento e alle tecniche di caseificazione. I bovini da latte sono stati sottoposti ad una intensa selezione verso il miglioramento della produzione lattifera.

Nel presente studio sono state indagate, per la prima volta nella razza Modicana, nuove informazioni riguardo i polimorfismi in alcuni geni coinvolti nel metabolismo dei lipidi ed a' stata presa in considerazione l'interazione tra questi polimorfismi e i sistemi di allevamento e l'influenza di questa sulla composizione del latte. Nello specifico, come il pascolo influenza gli effetti dei polimorfismi ai loci analizzati, sulla produzione, composizione del latte e profilo degli acidi grassi.

I bovini di razza Modicana considerati, sono allevati in due aziende della provincia di Ragusa secondo un sistema estensivo e semi-intensivo. Gli animali sono stati caratterizzati sotto il profilo genetico per i polimorfismi ai seguenti loci lipogenici: ABCG2-Y581S, DGAT1-K232A, STAT5A-V686A e SCD1-A293V. In base ai genotipi individuati, sono stati selezionati 36 e 38 animali allevati rispettivamente secondo il sistema semi-intensivo ed estensivo. Sugli animali selezionati, sono stati raccolti i dati fenotipici relativi alla produttivita', composizione del latte, minerali e profilo degli acidi grassi, al fine di valutare l'effetto dei polimorfismi e l'interazione con i sistemi di allevamento sulle caratteristiche quanti-qualitative del latte.

Tutta la popolazione e' risultata monomorfa per il polimorfismo Y581S al locus ABCG2 mentre per gli altri loci, gli alleli con piu' alta frequenza sono: 91% per GC al locus DGAT1 (232A), 68% per T al locus STAT5A (686V) e 73% per T al locus SCD1 (293A).

Il sistema di allevamento ha influenzato significativamente la produzione di latte e il contenuto di proteine che sono risultati piu' elevati negli animali alimentati al pascolo. Quando il sistema di allevamento e' stato considerato in relazione ai polimorfismi, i risultati non hanno mostrato differenze significative all'interno dei diversi fenotipi in entrambi i sistemi.

Il sistema di allevamento ha influenzato anche la composizione degli acidi grassi. Gli animali alimentati al pascolo hanno mostrato un minor contenuto di SFA e una maggiore frazione di MUFA, PUFA e acidi grassi trans. Il polimorfismo K232A al locus DGAT1 in interazione con il sistema di allevamento ha influenzato il contenuto di C4:0, CLA e C22:4. Il latte di animali con genotipo GC/GC al locus DGAT1 e alimentati al pascolo, hanno mostrato un piu' alto contenuto di CLA. Il polimorfismo V686A al locus STAT5A ha influenzato la composizione degli OBCFA il cui contenuto e' risultato piu' elevato negli animali con genotipo TT che hanno anche mostrato un maggior contenuto di C18:1t11, C18:1c9, C20:4 e un piu' basso contenuto di C20:5n3. Nessuna interazione e' stata rilevata quando il polimorfismo e' stato considerato in relazione al sistema di allevamento. Il polimorfismo A293V al locus SCD1 in interazione con il sistema di allevamento ha influenzato il contenuto di C4:0, C6:0, C8:0, C18:1t11 e C20:3 negli animali allevati con il sistema estensivo che e' diminuito passando dal genotipo CC a quello TT.

Riguardo alla composizione minerale, l'unico effetto significativo e' stato riportato per il contenuto di K associato al polimorfismo V686A al locus STAT5A il cui contenuto e' risultato maggiore negli animali eterozigoti.

Questo studio ha dimostrato l'esistenza di una relazione tra i polimorfismi ad alcuni loci lipogenici e i sistemi di allevamento che ha influenzato alcuni tratti della produzione del latte in bovine di razza Modicana. Ulteriori ricerche sono necessarie per descrivere e comprendere al meglio queste relazioni, non solo da un punto di vista genetico o nutrizionale, ma come interazione dinamica tra questi.

INTRODUCTION

The Sicilian dairy industry is characterized by production methods linked to breeds and/or indigenous cattle population, to feeding techniques that make extensive use of grazing, and to traditional cheese-making procedures that employ raw milk, rennet and wooden tools.

One of the strengths of the Sicilian dairy sector is the extraordinary range and variety of products derived from bovine milk. Among them, the traditional products are a pivotal component for the economy of the livestock system. Each product, having been developed and become established in relation to specific areas, environmental and socio-economic contexts, bears important historical and cultural value.

In the Ibleo area, the development of innovative and objective systems for the characterisation, authentication, traceability and promotion of local products (i.e. PDO and PGI trademarks) is crucial, alongside to the preservation and valorisation of bovine breeds and autochthonous populations linked to such products. The certification of the dairy products has become increasingly important in the last decade and has led to the necessity to develop genotyping systems, with improved nutrition and health. Furthermore, the commercial globalization and the industrialization of production processes have lead the need of the consumer for a direct and immediate objective control of the products. Moreover, the characterization of feeding system and the certification of animal welfare could provide incentives for the development of marginal areas for the conservation and maintenance of particular breeds at risk of extinction, such as Modicana.

The valorisation of dairy goes through a characterization process under the aspects concerning nutrition, sanitation, health and welfare of animals through the integrated use of chemical and molecular biotechnology considering the different feeding systems.

The use of biotechnology allows to overcome the limitations of conventional systems for authentication and traceability, which are based on the use of trademarks, documents and records that, for the high number of steps involved, the type of information and media being used, increase the possibility of errors and the risk of counterfeiting. Dairy products are characterised and tracked using the species/breed, the region of origin, the rearing system and the type of feed used as parameter. They are based, on the one hand, on the differences present in regions of the genome (which being variable from one species or one breed to another represent an indelible, unmistakable and unique trait) and, second, on the differences inherent in farming systems and feeding that affect the biochemical and nutritional characteristics of the final product.

1. Sicilian dairy cattle breeding

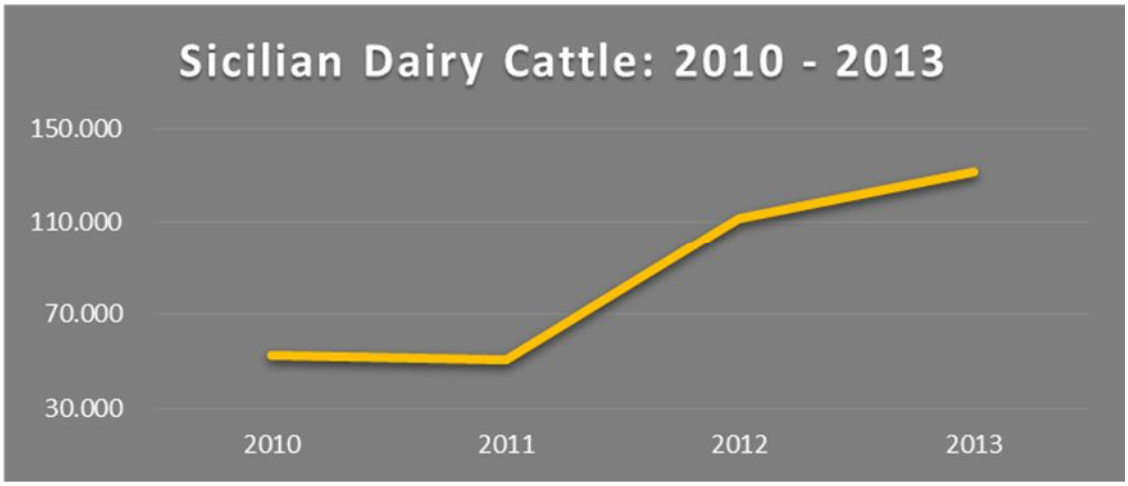
Sicilian livestock originates from the earliest anthropogenic settlements and today constitutes an important component of the island's agriculture. Although it has gradually established intensive livestock farming with modern facilities, a good portion is still carried out in traditional farms using extensive and semi-extensive systems in which the indigenous breeds are still widely used. This strong link with traditions and territory gives to dairy products an important historical and cultural value to be enhanced and preserve.

Sicily has one of the richest heritages of animal biodiversity of Italy. The dairy sector is characterized by many typical products, often related to races and/or indigenous cows and traditional cheese-making techniques. Technological innovations based on animal breeding have resulted in a strong increase in productivity of new breed selections, widening the gap of income between farms set on advanced selections and those that continue with indigenous and traditional breeds.

Since the middle of last century, a few high-performance breeds have spread around the world, outnumbering traditional breeds. Over the years, a steady increase in consistency and in economic importance of many population and local breeds has been witnessed, which over time were diversified and characterized by their genetic characteristics.

According to CLAL[1], the number of dairy cattle raised in Sicily from 2010 to 2013 has more than doubled (increase of 148.29%) from a little over 52 thousand heads in 2010 to over 130,000 in 2013 (Figure 1).

Figure 1: Sicilian milk cattle reared from 2010 to 2013



The dairy sector, made up of 130,000 heads is characterized by intensive farming with modern facilities and well organized both in lowland areas and in the hill, but still a good part of livestock farming takes place in disadvantaged inland areas and mountain farms with traditional rearing system that uses the classic cow-calf scheme.

Regarding the structure of bovine herds, in recent years there has been a reduction in numbers, both farms and animals bred, and a growing, albeit limited, of the average size

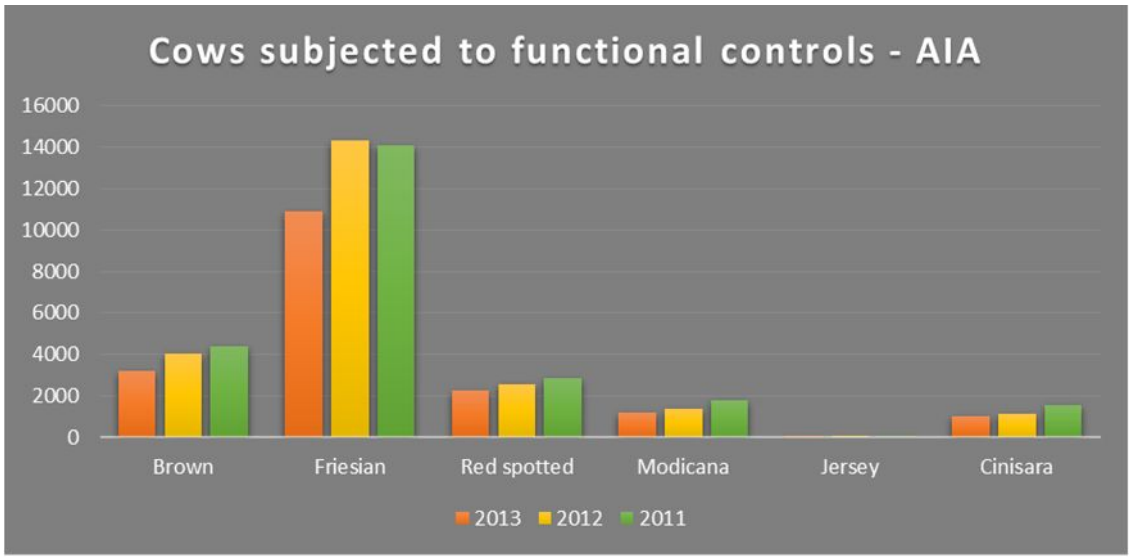
[1] CLAL - http://www.CLAL.it/index.php

of farms. The number of dairy farms in the period between 2008 and 2013 in Sicily passed from 2,254 to 1,989 with an 11.8% decline. The most represented category, in 2013, had a number of head between 10 and 19, which includes 677 farms, accounting for 34% of the regional total. Following, there are farms with a number of heads between 20 and 49 (564, 28% of the total) and farms with 1-9 heads (541, 27% of the total). The geographical location of cattle farms, despite a widespread presence throughout the Sicilian territory, it is greater in the provinces of Ragusa, Messina, Enna and Palermo, where fall 77% of companies and 80% of dairy cows[2].

In lowland areas intensive livestock systems prevail with high levels of technology, capital intensive and little land use. In hilly and mountain areas, conversely, semi-extensive and extensive systems methods prevail and are characterized by a low level of productivity. In these systems capital use is somewhat limited, while very high is the use of land. Here, it is very important the role plays by the peasant family.

Specialized farms in milk production are concentrated in the plains and low hills of the provinces of Siracusa, Messina, Palermo and Catania and is particularly flourishing in the northwestern part of the province of Ragusa. The most represented breeds are Friesian (average production: 7,551 kg per lactation) and Brown (average production: 5,961 kg per lactation), which due to their higher production performance have supplanted breeding of Modicana, race native of dual purpose which, for its hardiness, is less productive (average production: 3,500 kg per lactation)[3]. According to AIA functional controls (2010-2013), main dairy breeds in the island are reported in the Figure 2.

Figure 2: Cows subjected to functional controls by AIA from 2010 to 2010



Percentage distribution of dairy breed, in 2013, amounted to 59% for Friesian, 17% for Brown and 6% for Modicana. It is interesting note that, according to AIA, in 2001, the

[2] Source ISTAT, 2014
[3] Source AIA 2013

percentage was significantly different: 47% Friesian, 27% Brown and 10% Modicana[4]. This shows that the process of substitution of native breeds is still in progress.

Regarding the distribution of the controlled dairy breeds AIA in 2013 in the provinces (Figure 3), data indicate that Friesian is reared in all Sicilian provinces and that 79% is concentrated in Ragusa province. Brown breed is also reared in all provinces, and in large numbers in the provinces of Ragusa, Siracusa and Palermo (43%, 30% and 16% respectively). Finally, the Modicana breed is mainly bred in the provinces of Messina, Ragusa and Palermo (35%, 30% and 16% respectively)[1].

Figure 3: Distribution of the controlled dairy breeds in Sicily in 2013 (Source AIA 2013)

Intensive animal husbandry, which is well organized and features modern facilities, it is practiced in the most suitable areas of plains and hills. The trend has led, in recent years to the closure of less efficient farms and the concentration of production in the most advanced facilities that better meet the needs of processors in terms of hygiene and quality of milk. A good portion of Sicilian livestock is held, even today, in disadvantaged inland and mountain areas. It is frequently of traditional herds of semi-extensive type in which farms are distant from the market, small in size, with low production capacity and lacking both in structural (with hygienic-sanitary structures and conditions sometimes inadequate) and organizational terms. The spread of poorly specialized farms and of small size is dictated by the harsh environmental conditions, generally deficient in forage resources, and where the use of pasture were often the only power source. In such a context, the bond producing-territory is unequivocal, and the promotion of local food products is essential for the perpetuation of traditions and restrict livestock abandonment of native breeds to safeguard the island's biodiversity.

1.1. Farming situation in the Ragusa Province

Livestock farms in Ragusa province are focused in the Ragusano highland and in the Modica area. Overall, farms are oriented mainly towards milk production which is the carrier of local agricultural sector. Milk produced is sold directly to factories, or is transformed in the company for the production of local cheeses (i.e. Ragusano PDO, Cosacavaddu Ibleo, Provola Iblea, etc.).

[4] Source CLAL, 2013 (www.CLAL.it 2013)

AIA, has issued the following report for 2013 about milk productivity controls, noting that in the Ragusa province there were 343 farms, with an average of 22 animals per farm. The main breeds are reported in the Table 1.

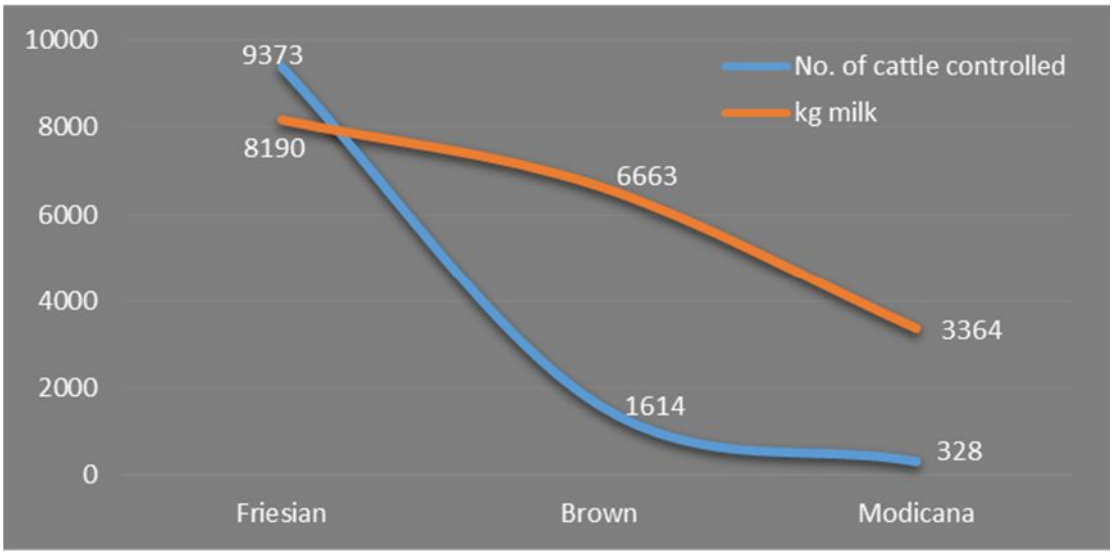
Herds in the Ragusa province consist mainly of specialized breeds for milk production such as Friesian (74%) and Brown (13%), but also dual-purpose breeds such as Red spotted (6%) and Modicana (3%). In fewer than in the past however, is the presence of mestizos. By AIA controls, in 2013 there were in production 14.606 heads of Friesian, 2,500 of Brown and 501 of Modicana.

Table 1: Milk productivity controls in Ragusa province (Source AIA 2013)

BREED	No. OF ANIMAL	%	FARMS	AVERAGE No. OF ANIMALS/FARMS
Friesian	14.606	74	295	50
Brown	2.500	13	149	17
Red spotted	1.203	6	96	13
Modicana	501	3	26	19
Meticci	983	5	95	10
TOTAL	**19.793**	**100**	**343**	**22**

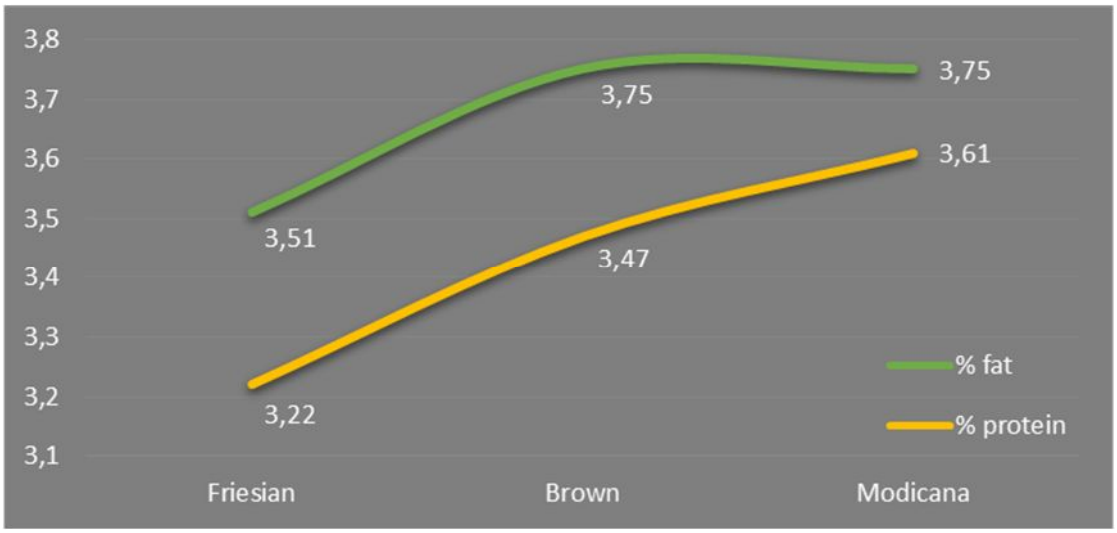
Considering the milk quantity and quality produced by these three races and the number of animals checked, the situation reported in the province is explained in the Figure 4. The Figure 5 shows instead the average percentage of fat and milk protein yield in the 3 races.

Figure 4: Number of animal checked and kg of milk produced in Ragusa province for Friesian, Brown and Modicana (Source AIA 2013, modified)



Among the products with protected origin, Ragusano cheese is closely linked to the homonymous province. Its typical characteristics derive from a complex of element by the environmental, historical-ethnographic, cultural, social, technological and economic origin related to territorial specificities.

Figure 5: Milk fat and protein percentage in Friesian, Brown and Modicana checked in Ragusa province (Source AIA 2013, modified)



Thanks to the 2nd May 1995 Degree, Ragusano PDO has obtained prior recognition of the designation of origin and then, by C.E. Regulation n.1263 of 1st July 1996, it benefited from the protected designation of origin that certifies not only the quality but also the production according to the methods handed down by tradition. In this way the production of Ragusano PDO was bound to a specific geographical area of production which covers the entire province of Ragusa and the towns of Noto, Palazzolo Acreide and Rosolini in the province of Siracusa.

2. Modicana cow

2.1. Morphological characteristics and history of Modicana cows

Having been farmed in Sicily over several centuries, the Modicana cattle breed has a red coat with shading ranging from wine-red to black (especially in the males). There is still no consensus on how this breed came to the island: many believe it arrived from the Mediterranean while others believe it came from the European continent with the Normans and Angevins. Either way, it has been raised in Sicily for a very long time, practically since the island was populated.

The breed Modicana named after the ancient county of Modica, whose territory is identified today with the province of Ragusa, which is considered his native area. Nowadays this breed is bred in the Sicilian territory, especially in the provinces of Palermo, Ragusa (Figure 6) and Enna.

Figure 6: Modicane cows grazing (Floridia Farm, C.da Scorsone)

According to Durst, the breed had its origin from *Bos taurus macroceros*, whereas for Sanson would have originated from *Bos taurus ibericus* (D'Urso, et al., 1983). Schicchi (1970) asserted that, at the time of Frederick II, after the plague decimated Sicilian cattle, many subjects in red cloaks from Scandinavian countries to rebuild cow's population. The

current Modicana breed is thought to have derived from a cross between these imported animals and the native breeds.

Other authors, like Santacroce (1953) and Tortorelli (1964), believe that the breed constitutes an improved log of the original Sicilian cattle population, selected in eastern Sicily, in the county of Modica.

By the beginning of the 20th Century, in the Ragusa province, the first attempts at improving the Modicana breed had been implemented, especially in regards to the animal's morphology.

Bulls from Ragusa were used throughout the island, thus the name Modicana was extended to the whole population derived from the original one. The peculiar morphological characters were fixed with D.M. (Ministerial Decree) 28 of July 1936 in the appropriate standards that can be summarized as follows:

- ✓ *Size and weight*: in adult bulls 1.60 m and 800 kg; bull calves m 1.40 and 550 kg; in adult cows 1.45 m and 550-600 kg.
- ✓ *Coat* of sallow colour that is wine-red with black accents in the head, neck, shoulders, forearms, thighs. It can present lighter shades, as light as fawn.
- ✓ Red dark *pigmentation* to stub axle, slate colour at the natural openings and bow tail. Tongue and palate speckled, the scrotum and perineum of the same colour of the coat. Claws slate.
- ✓ *Head* rather small, with a straight profile or slightly like a mutton; broad forehead, prominent orbital arches; long and wavy tuft; lively eyes, wide nostrils and crushed. Horns with a circular section, half-moon in the bulls, directed sideways and inwards in cows, medium length, yellowish at the base and black at the tip.
- ✓ *Trunk* tending to the rectangular shaped, with back-lumbar line tending to the horizontal. Withers broad, low rise; Chest broad, rounded; long back and kidneys; long and wide rump that appears slightly sloping.
- ✓ Robust *legs* in the bulls, but finer and solids in cows, with fair aplomb; large joints; shoulders well adherent to the chest; hocks wide and just opening; short and wide shins; tight and strong claws.
- ✓ *Udder* big, wide, well-shaped, with rather large nipples.

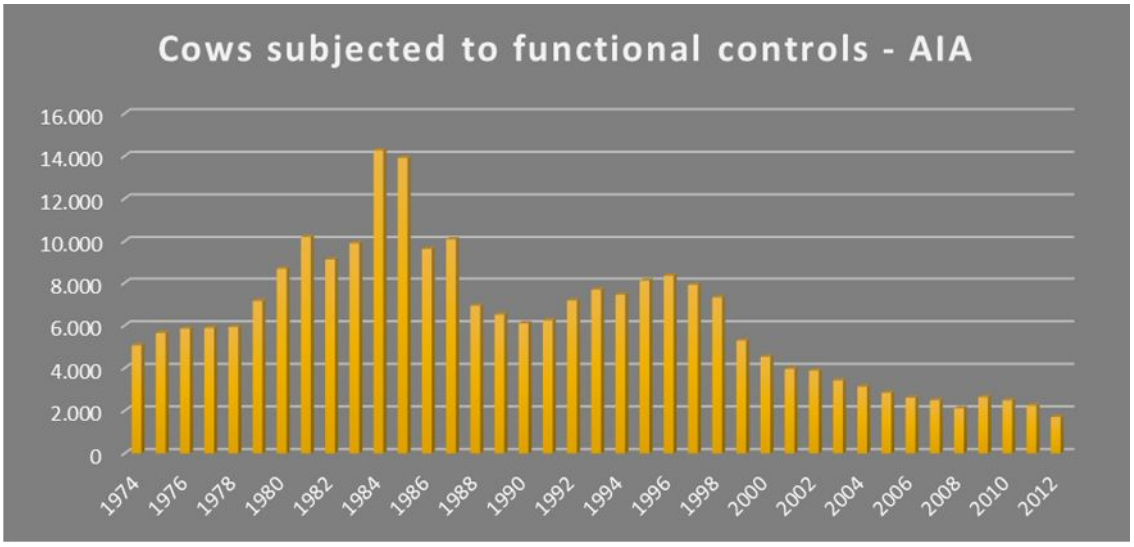
The minimum milk production for registration in the Herd Book, is set at 1,200 kg for primiparous and 1,800 kg for cows of 2nd and 3rd generation, and 200 days of lactation. In 1958 the breed was included in the Herd Book. In 1965 functional checks by the Regional Breeders Association commenced.

2.2 Population size and farming system

With reference to livestock species, over the years, there has been a steady decrease in consistence and the economic importance of many populations and local races, which over time were diversified and characterized by their genetic characteristics.

Their decline is linked to the introduction of mechanized architecture, their low productivity in both dairy (3,000 litres a year) and meat (less than 55%), and the fact that many farms no longer produce cheese from their own milk. This phenomenon has triggered a particular process, which just resulted in the introduction of livestock undertakings of technological innovation. As a result, there is an increase of the corporate fixed costs. This increase encourages the replacement of the autochthonous cow's races bred, with other, in order to have a productive capacity such as to offset the means of production used.

Figure 7: Animal subject to functional controls in Italian territory from 1974 to 2012 (source AIA)



This evolutionary model has affected the whole of Europe and in particular areas with intensive agriculture. This is evident when looking at the spread of more productive cosmopolitan breeds, such as the Italian Friesian and Italian Brown, at the expense of indigenous races. The highest number of animals reared and controlled (14,100) was recorded in 1984 (Figure 7). However, the following years saw a decline in the use of the Modicana race, probably due to the introduction of the cosmopolitan Friesian and Brown races. The replacement of native species, and specifically of the Modicana breed, has resulted in a decrease in the numbers of animals and, in 2012, only 1,709 Modicana cows are present nationwide.

The Modicana breed showed such a steep decrease in numbers, that today this race is included in the list of native farm animal breeds at risk of extinction.

Nonetheless, the Modicana is considered to be one of the best "triple-purpose" breeds, and in the past it was particularly valued for its work capabilities. It is extremely hardy and able to survive hot Mediterranean summers, feeding mainly on pastures, with a small amount of feed provided when grass is scarce. The cattle grazes free-range all year, and shelter is provided only for milking. Like all wild cattle breeds, the cows only produce milk when they have calves by their side.

Considering the Sicilian provinces where Modicana cattle is raised, it is evident how, unlike in the past, the animals are bred in the province of Palermo and that even there, there

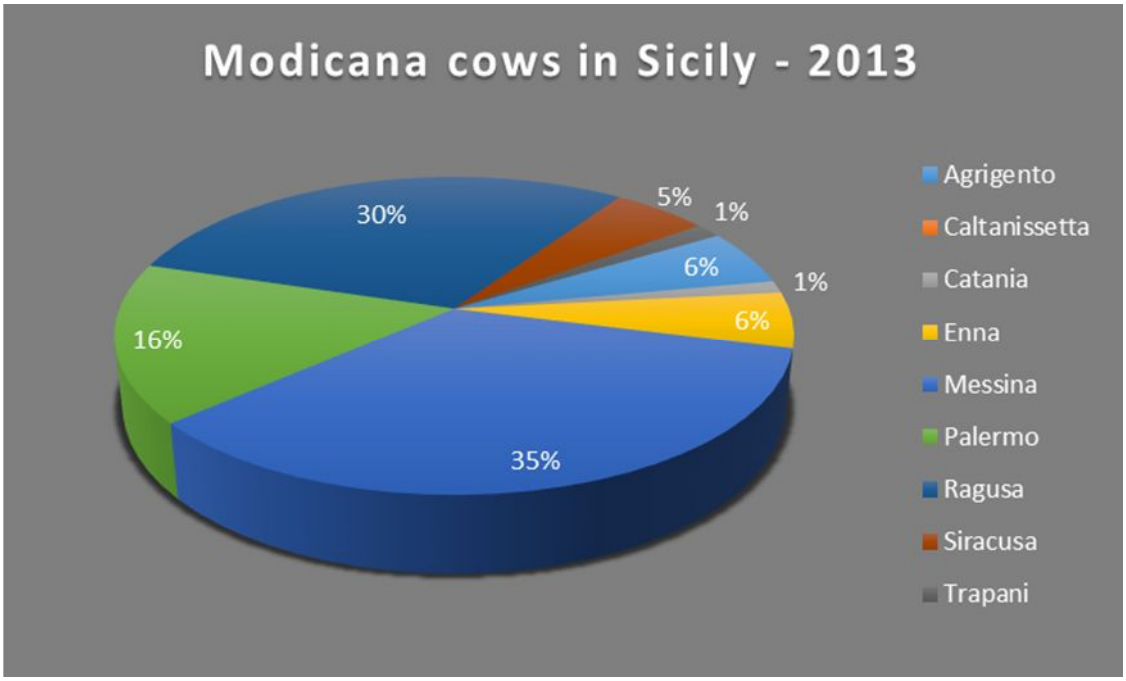
has been a constant decrease in the breed's population over the last decade (Table 2 and Figure 8).

Table 2: Animals subject to functional tests in each province, 2005-2013 (source AIA)

DISTRICT	2005	2006	2007	2008	2009	2010	2011	2012	2013
Agrigento	255	238	242	22	179	154	170	127	83
Caltanissetta	24	31	30	23	17	15	12	0	0
Enna	406	352	370	265	345	198	151	67	83
Messina	133	132	104	118	636	637	689	516	505
Palermo	1,340	1,166	1,13	841	925	824	582	522	237
Ragusa	484	489	499	498	499	518	508	522	436
Siracusa	83	84	65	70	76	86	87	81	78
Trapani	119	120	105	79	62	54	43	30	22
TOTAL	**2,844**	**2,612**	**2,488**	**2,116**	**2,639**	**2,486**	**2,242**	**1,709**	**1,462**

This finding highlight the serious threat of extinction that the breed is encountering.

Figure 8: Distribution of the animals in 2013 in Sicily (source AIA)



The breeding system of native Sicilian cattle breeds is mainly semi-wild type with extensive use of pasture, but rarely, semi-intensive farming is also used (Figure 9). The farms are small in size, both in the aspects of unit surface area and the number of animal raised. The vast majority of the farms devote their entire product to cheese-making. These farms are mainly family-led businesses, which enhance a territory difficult for the same means employed.

The animals are kept at pasture in the classic "*chiuse ragusan*e" or paddocks confined to shelters throughout the year. The shelters are used for milking or mostly on winter when the night temperature are lowered. In the winter, when weather conditions

make grazing difficult, lactating animals are kept indoor in the shelters and fed high quality fodder, to help them maintain a balanced and comprehensive diet. With the start of spring, as natural and cultivated forage become abundant, the animal are returned to the pasture, and milk production increases. In summer, the animal feed consists mainly of residual pasture, which as a consequence of the arid summer climate. This negatively affects milk production.

Figure 9: Cows in the barn and grazing (Floridia Farm)

Overall, in the territory of the Ibleo Mountains, livestock farms are oriented mainly towards cattle for milk production, and this is one of the most important agricultural sector for the economic development of the district. The milk produced is sold directly to the factories or is transformed in the farm for the production of local cheeses (i.e. Ragusano PDO, Cosacavaddu Ibleo, Provola Iblea). Cattle farming is practiced by family-run businesses, which either employ solely family members, or labour force from outside the EU.

The stables in most farms, being obsolete buildings, are insufficient and logistically inappropriate for agricultural activities, but also unsuited as lodging for workers. However, the sector is undergoing substantial restoration, with more and more farms in recent years being equipped with buildings suitable for animal rearing, fodder and machinery storage.

2.3 Milk production

Milk production of the Modicana cow normally follows a lactation curve that shows a rapid increase in daily milk production from delivery, with a peak between week 5 and 10. Subsequently there is a gradual decline of 5- 9% per month until the cow goes dry at the age of 0-10 months (Wood, 1967).

The graph (Figure 10) compares the lactation curves of Modicana and two specialised breeds. A substantial difference can be observed both in the duration of lactation and in the overall quantity of milk produced. A comparison in the chemical composition of bulk milk between the two main cosmopolitan dairy cow breeds and the Modicana breed is shown in Table 3.

Among the factors that most influence milk production of Modicana breed the delivery characteristics and size are the most representative. In regards to delivery characteristics, multiparous cows showed a higher total production of milk (+400 kg) over an equal lactation period compared to primiparous cows. Milk production has then been related to animal size, defined as height at the withers.

Milk production has been then compared to the size of the animals, distinguished according to the height at the withers in large (>140 cm) and small (<140 cm) cows (Alabisio, et al., 1999). However, no significant relationship between size and milk production was observed.

Figure 10: Comparison of lactation curve of Modicana, Friesian (Frisona) and Brown Swiss (Bruna)

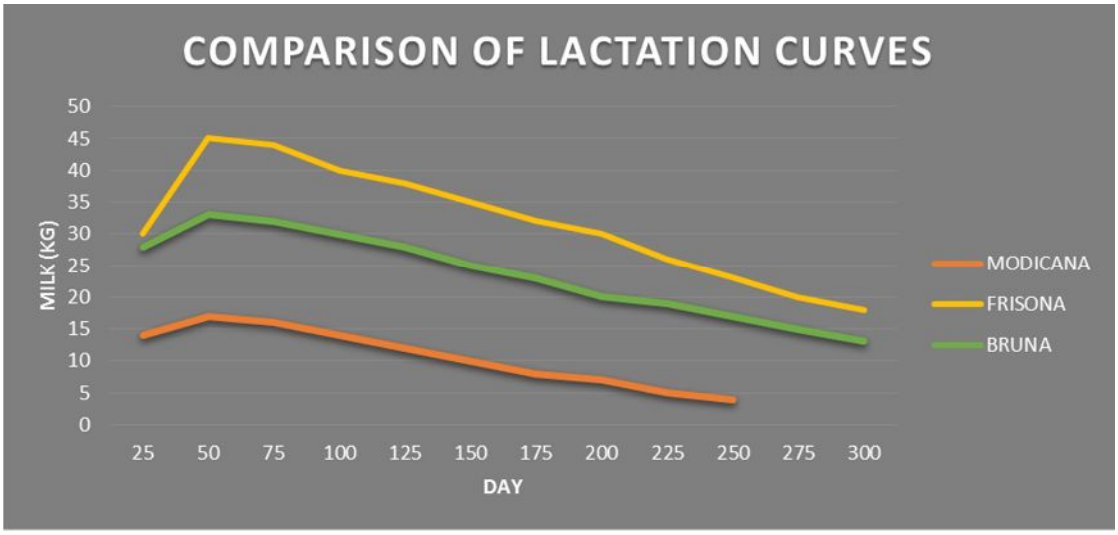


Table 3: Comparison of the chemical composition of the bulk milk of Modicana, Holstein and Brown Swiss (Guastella, 2014)

TRAIT	UNIT	MODICANA	BROWN SWISS	HOLSTEIN
Fat	g/100g	3.97 ± 0.11	3.94 ± 0.14	3.51 ± 0.17
Protein	g/100g	3.60 ± 0.14	3.55 ± 0.17	3.13 ± 0.09
Lactose	g/100g	5.04 ± 0.17	5.10 ± 0.11	4.83 ± 0.08
Calcium	mg/100g	129.08 ± 6.64	120.76 ± 4.51	109.73 ± 3.40
Phosphorus	mg/100g	100.72 ± 5.45	97.32 ± 5.38	87.40 ± 2.08
Magnesium	mg/100g	11.41 ± 1.07	13.16 ± 1.21	10.34 ± 0.45
Sodium	mg/100g	50.19 ± 5.74	51.67 ± 4.58	47.08 ± 4.18
Potassium	mg/100g	145.00 ± 11.94	139.11 ± 12.13	152.09 ± 4.89
Chloride	mg/100g	84.81 ± 11.34	96.25 ± 11.10	105.55 ± 8.08
Casein	g/100g	2.70 ± 0.20	2.71 ± .016	2.40 ± 0.08
Whey Protein	g/100g	0.58 ± 0.04	0.61 ± 0.08	0.56 ± 0.03
Non-Protein Nitrogen	mg/100g	28.81 ± 3.37	28.81 ± 3.37	76.68 ± 1.03
Casein index	%	78.03 ± 0.84	77.53 ± 0.67	76.68 ± 1.03

Given the type of farming, milk production is strongly dependent on seasonal trends. Production peaks in spring, when forage availability is highest, and ceases completely during the dry summer season, when grazing resources decline.

3. Milk and milk products

Milk is a very important product in human nutrition. This is due to its complex and heterogeneous composition, which allows it to be used in a number of way and makes it at the same time a complete and variable food. It is consists of water, which represents 87% of the total weight and of a variety of components, present both in the state of true solution (salts, water soluble vitamins, non-protein nitrogenous substances, sugars), both in colloidal state (proteins and proportion of the phosphate and calcium citrates) and both in the state of an emulsion (lipids and fat-soluble vitamins). Furthermore, it contains other substances of importance from a nutritional point of view, such as vitamins, enzymes, hormones and oligoelements. These constituents of milk are closely linked to each other by means of a close interdependence; their concentration depends on the species, breed, diet, stage of lactation of the cow, etc. (Salvatori del Prato, 1998).

Research has been focused on increasing the daily milk production and the improvement of protein component in order to enhance the milk yield and the attitude to make cheese. Thus, the composition of raw milk was one of the most important aspects to establish nutritional value, technological properties of milk and dairy products, and farmer's milk price.

The milk, from a chemical-physical point of view, is a complex mixture of various kinds of components, which can be divided into four fractions (Corradini, 1995):

- ✓ An *aqueous fraction* which contains dissolved lactose, other sugars, minerals, non-protein nitrogen compounds, free amino acids and water-soluble vitamins;
- ✓ A *globular fraction*, represented by fat particles (MFG, milk fat globule) consisting of triglycerides and coated by a natural membrane phospholipid and protein (MFGM, milk fat globule membranes);
- ✓ A *micellar fraction* composed of casein;
- ✓ A *colloidal fraction*, consisting of tricalcium phosphate and α and β lacto-globulin.

These different phases are in an unstable equilibrium. In fact, preservation of the milk at room temperature involves, within the first 12-24 hours, separation and ascent to the surface of the fat phase in the emulsion (outcrop or surfacing) and, subsequently, following the biological action of microorganisms, the separation of the casein suspension with clot formation. Milk is white, due to the casein micelles that disperse the light, while the yellowish shades can be attributed to lipid fraction and carotenoids[5]. In milk serum flavins are present that confer greenish-yellow pigments.

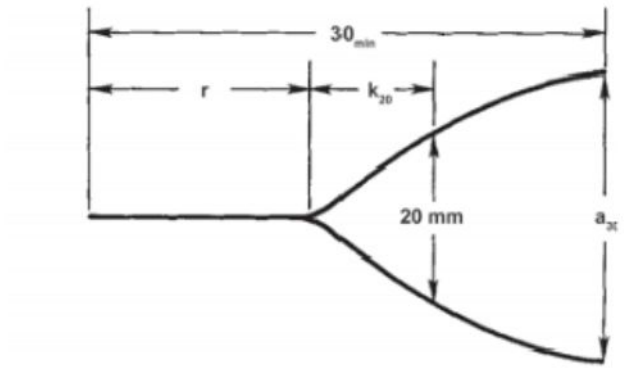
3.1 Milk quality criteria

The quality of a food is defined by multiple factors, such as its nutritional and sensory characteristics and its response to quality and health requirements expected by consumers (Hocquette, et al., 2005). Specifically for milk, the quality include: the chemical composition, i.e. nutritional requirements as the content of protein and fat, health and safety

[5] Art. 15 del Rdl del 9-5-1929, citato da APA Cuneo, 2004

standards and technological milk, understood as the ability of the milk to transform itself in products of high organoleptic quality (Bittante, et al., 2003). In terms of milk quality, a key aspect for our country it is related to technological properties for processing into traditional cheeses, which are influenced by the chemical composition of milk (Stefanon, et al., 2002). The relationship between milk quality and the processed product becomes even more important if reference is made to the medium or mature PDO cheese from raw-milk products, for which the allowed changes to the raw material are reduced if not prohibited (Verdier, et al., 1995). Technological milk quality is considerably important for the evaluation of the milk destined to cheese processing and can be expressed in terms of attitude to coagulation (Mariani, et al., 2002). The latter is commonly evaluated by analysing the lacto dynamographic (LDG) graphic, which considers the response of milk to the addition of rennet (Figure 11).

Figure 11: Sample track obtained with lacto-dynamograph after the addition of rennet to milk: RCT, k20, a30 (McMahon, et al., 1984)



As reported by Bittante et al. (2012), the three parameters that define the LDG are:

- ✓ Clotting time (min), the time from rennet addition to the formation of the first visible and measured floccules. In the Figure 11 is shown the interval between time 0 (addition of the rennet) and the opening of the track;
- ✓ Firming time (k20, min), the time required for the clot to reach a mechanical resistance such as to determine an amplitude of 20 mm path;
- ✓ Consistency of the clot to 30 minutes (a30, mm), width of the track 30 minutes after rennet.

3.2 Factors affecting milk quality

Milk composition is very variable, being conditioned by the complex metabolic activity of the animal, linked to animal, nutritional and environmental factors (Table 4). These include, in varying proportion, breed, age of the animal, healthy conditions, feeding characteristics, stage of lactation, milking technique, environmental conditions and seasons.

Table 4: Factors affecting milk quality

ENDOGENOUS FACTORS	
Genetic:	breed individual animals
Physiological:	animal's health condition stage of lactation
EXOGENOUS FACTORS	
Environmental:	feeding characteristics climate farm management rearing system frequency and milking techniques

These factors do not act independently from each other, but rather interact (Comba, et al., 1995).

3.2.1 Endogenous factors

Regarding genetic origin as a factor influencing milk quality, there is a hereditary individual variability of milk production and composition, which is characteristic of each individual animal of the same breed and reared in the same conditions. Naturally, milk composition varies depending the breed considered and in particular, the fat content varies greatly between breeds and between individuals within the same breed (Mariani, et al., 1987; Alais, 2000). In fact, both fat and protein percentage in milk are determined for a 40% by genetic (heritability) and for 60% by the environment, while the yield of milk, fat and protein (kg of fat and proteins produced in lactation) are determined for 25-30% by genetics and for 70-75% by environment (Salvatori del Prato, 1998). The components that have less variability are lactose and minerals which are indicators used for the detection of normal milk, also because lactose is a limiting factor for breast synthesis capacity and therefore for milk production.

Milk composition is also affected by health conditions of the animal, in fact, mastitis causes a reduction and alteration of milk production. These diseases cause an increase in milk somatic cell proportional to the intensity of the infectious phenomenon, resulting in an enrichment of cellular origin enzymes, in addition to the increase in immunoglobulins. Mastitis also causes a decrease in the capacity for synthesis of the mammary gland, with consequent decrease of fat, casein, lactose and an increase of the direct filtration products from the blood plasma (serum proteins, mineral salts, enzymes, etc.), determined by an increased permeability of the capillaries (Corradini, 1995). Mastitis also represent the main source of profound changes, not only in milk composition but also in clotting attitude (Mariani, et al., 1987). Finally, all changes in milk composition result in an increase in milk pH, goes from 6.60-6.65 in normal milk, to 6.8 in milk with altered composition.

The most significant effects of milk composition variation are associated with the animal's physiological state. During the course of lactation content changes occur for various components (Alais, 2000). Specifically, fat and total proteins tend to decrease until reaching minimum values in the second and fifth month of lactation, respectively. These components then gradually start increasing towards the end of the cycle. The productivity curve has an opposite pattern to the one of fat and protein, reaching its maximum between the first and third month. The changes that occur mainly during lactation are visible in the

first and last days of lactation itself. Immediately after delivery, and for the first 3-4 days, the animal produces colostrum, a liquid that has not the characteristics of real milk, being more rich of all components (especially immunoglobulin), with the exception of lactose, potassium, non-protein nitrogen and water. Its composition tends to change quickly and variation during the later stages of lactation mainly takes place in the proteins, phosphorus, calcium and thin residue that, after an initial decrease, tend to remain constant to increase at the end of lactation (Strzalkowska, et al., 2002). In this last phase, milk in addition to having a higher content of total dry matter for further fat content, protein and ash, also presents changes in minerals components, becoming richer in calcium and sodium and poorer in potassium and phosphorus. In addition to variation in the calcium/phosphorus equilibrium, at the end of lactation milk presents lower stability to thermal treatments.

3.2.2 Exogenous factors

Environmental factors, such as climate, may exert different influences on the production and composition of milk during lactation (Garcia, et al., 2001). Seasonal variation, for example resulting in an increase in fat, the residual fat, proteins and minerals during winter. Whereas for environmental temperatures, between 0 and 29°C, percentage composition of the milk constituents does not show significant changes. In contrast, at temperatures >30 degrees, milk production decreases, alongside protein content, fat residue and lactose. Extreme environmental conditions such as extreme heat can result in loss of milk production, and have effects such as higher frequency of milks hypo-acid. The effect of environmental factor is not, however, the same for all milk components, in fact, the effect on lactose and mineral content is almost insignificant, while the percentages of fat and protein may be modified respectively of 2-3 decimal points (i.e. from 3.5 to 3.7%) and of decimals 0.1-0.4 in conjunction with nutritional factors (Salvatori del Prato, 1998).

Even *feeding* holds a key role: although changes in milk composition can also be influenced through the techniques of breeding and management, nutrition is able to induce changes more rapidly in the desired direction (Fredeen, 1996). Feeding considerably influences milk quality especially in terms of fat content (Ubertalle, et al., 1998). Indeed, the fatty acid composition of milk depends on the type and quantity of precursors for the synthesis processes, which get to the breast from the diet. Consumption of insufficient amounts of sources of energy causes, for example, a mobilization of fat reserves in the form of free fatty acids (FFA) which are received from the udder and incorporated into milk fat with a resulting high molecular weight fat, and of oleic acid in particular (Pulina, et al., 2007). Finally, it must be remembered that a poor diet is reflected not only on the quality of milk and its rheological properties but especially on the animal's health status.

Research has shown that when animals are fed at pasture produce a milk richer of aromatic components and molecules important for health (Martin, et al., 2005). The lipid fraction of milk from animals grazing has, in addition to a higher CLA content, an ω-6/ω-3 lower ratio (2:1), mainly attributed to the increased presence of polyunsaturated fatty acids, such as the α-linolenic acid, that abound in the grass at a non-advanced vegetative stage (Abu-Ghazaleh, et al., 2001). Pasture also affects the amount of fat-soluble vitamins in the milk. In particular, it has been observed that vitamin E and vitamin A content are higher in milk from pasture fed than hay fed animals and decreases if animals are fed with plants

which are at an advanced stage of maturity (Kalac, et al., 2006; Noziere, et al., 2006). It has been proven that milk from animals grazing at high altitude had a higher CLA content and α-linolenic acid and *trans*-vaccenic than that coming from pastures located at a lower altitude, highlighting the influence of the different botanical composition on the fatty acid profile of milk (Collomb, et al., 2002).

Finally, *milking technique* and emptying of the breast can affect milk quality, especially if they are not performed correctly. Milk "dripping" leads to a higher lipid content, whereas partial milking may cause a reduction in fat percentage (Pulina, et al., 2007). In fact, lipid content increases during the milking process in relation to blood cell viscosity (Martini, et al., 1999).

4. Lipids in milk

Cow milk has a fat content of 3-5% (Table 5). Milk lipid fraction consists 98-99% of triglycerides, non-polar lipids, which are the main energy source in milk, determine overall flavor and rheological properties (Laben, 1963). The remaining 1-2% is formed by polar lipids: phospholipids, sterols, monoglycerides, waxes, squalene, carotene, fat-soluble vitamins and traces of free fatty acids.

In the first stages of milk caseification, fat generally does not take part in important activities, but is incorporated and becomes part of the casein reticulum influencing the structure and consistency of the cheese. Moreover, fat plays an important role in the maturation and seasoning process, influencing crucial organoleptic characteristics of the finished products (Salvatori del Prato, 1998). Quantity and composition of fat are strongly influenced by various factors, including lactation stage and feeding (grazing for example improves the fatty acid profile of the milk) (Bailoni, et al., 2005).

Table 5: Percentage of fatty acids and cholesterol in milk (Claeys, et al., 2014)

SATURATED FATTY ACIDS (SFA)	**55.7-72.8**
Monounsaturated Fatty Acids (MUFA)	22.7-30.3
Polyunsaturated Fatty Acids (PUFA)	2.4-6.3
Linoleic Acid	1.2-3
Linolenic Acid	0.3-1.8
Coniugate Linoleic Acid (CLA)	0.2-2.4
Cholesterol (mg/100 ml of milk)	13.1-31.4

Chemically, triglycerides are derived by the esterification of fatty acids (FAs) with a trivalent alcohol (glycerol). Therefore, depending on the number of esterified bonds, fats are divided into triglycerides (3 bonds esterified), diglycerides (2 bonds esterified) and monoglycerides (1 bond esterified). The last two are present in milk together with triglycerides respectively around 1.5 and 0.25% of total lipids. Regarding the FAs composition, milk fat is one of the most complex components, being composed of 150 different FAs, has a proportion of approximately 2/3 of SFA, 1/3 of UFA and a high proportion of volatile FAs with low molecular weight. Milk contains mainly FAs with an even number of carbon atoms between C4:0 and C20:0, while those at odd number are present only in traces. Almost all SFA have an even number of carbon atoms number, as the starting point in their synthesis is acetic acid (C2:0) (Table 6). Moreover, SFA comprised between C4:0, C10:0 and a part of C12:0 are volatile when in free state, i.e. not esterified with glycerol. These are very important, because they influence the milk organoleptic characteristics and the quality of dairy products, for example, they are responsible for aroma formation (Poveda, et al., 1999). Butyric and caproic acid in particular are the most characteristic part of the milk in ruminant because they are soluble in water and represents about 5% of the whole.

Among the SFA, palmitic acid is the main component (25-30% of total FAs), followed by stearic and myristic, with intermediate concentrations (~ 12% and 11% respectively). While among the UFA, oleic acid is present in high concentrations compared with others (~ 23%) (Malacarne, et al., 2001). In addition, there are small amounts of MUFA and PUFA,

diene and triene. The latter, in particular, falls within the group of the essential FAs for human nutrition.

Table 6: Fatty acid in bovine milk and their percentage distribution (modified from (Salvatori del Prato, 1998)

	NO. OF C ATOMS	%	PHYSICAL STATUS (MELTING TEMP °C)
SATURATED FATTY ACID (67%) $CH_3 - (CH_2)_N - COOH$			
SALUBLE VOLATILE:			
Butyric acid	C4:0	3-4	Liquid (-8)^
Caproic acid	C6:0	2-5	Liquid (-3)
INSOLUBLE VOLATILE:			
Caprylic acid	C8:0	1-1.5	Liquid - Solid (+16)
Capric acid	C10:0	2	Solid (+30)
Lauric acid	C12:0	3	Solid (+42)
Myristic acid	C14:0	11	Solid (+54)
Palmitic acid	C16:0	25-30	Solid (+62)
Stearic acid	C18:0	12	Solid (+70)
Arachidonic acid	C20:0	0.2	Solid (+75)
UNSATURATED FATTY ACID (33%)			
MONOENOIC:			
Palmitoleic acid	C16:1	2	Liquid (+0.5)
Oleic acid (cis9)	C18:1	23	Liquid - Solid (+16)
Vaccenic acid	C18:1	2-3	Solid (+43)
NO-CONJUGATE POLYUNSATURATED:			
(diene) Linoleic acid	C18:2	2	Liquid
(triene) Linolenic acid	C18:3	0.5	Liquid
(tetraene) Arachidonic acid	C20:4	0.2	Liquid
CONJUGATE POLYUNSATURATED:			
Diene	C18:2	0.8	Liquid
Triene and tetraene	C18:3-C18:4	traces	Liquid

^ The triglycerides melting point is close to that of the fatty acid, in the case of fixed acids.

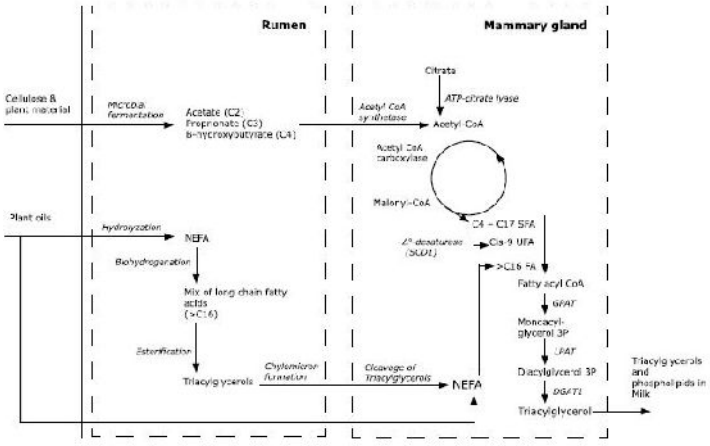
A major component of the lipid fraction is represented by phospholipids, which are distinguished from triglycerides, as they are polar lipids that are found mainly linked to fat globule membrane (about 60% of total milk phospholipids). They have a lipophilic part, consisting mostly by UFA, esterified to a glycerol molecule, and a hydrophilic part constituted by a molecule of phosphoric acid linked to a nitrogenous base (Carboni, et al., 1983). Among the major milk phospholipids there are lecithin, with choline as organic base, cephalins, which contain ethanol-amine and sphingomyelins, with choline and sphingosine as bases. Lectins are excellent emulsifying agents and play an important role in the constitution of milk fat globule membranes, helping to stabilize the suspension of the fatty substance. Milk fat contains, in small concentrations (0.5% of total FAs), also keto and oxyacids and lactones derived from oxyacids. These compounds are partly responsible for the milk aroma and dairy products characteristics. Their concentration increases with warming. The unsaponifiable portion of milk fat is composed of sterols, carotenoids and fat-soluble vitamins. The most abundant is cholesterol (~ 0.3% of fat, that is 0.1 g/l of milk), which is of great nutritional importance as a precursor to many hormones and vitamin D. It act as an emulsifier and stabilizer of fat, maintaining the functional integrity of cell membranes, because it adjusts fluidity and permeability. It also has a partial effect on the lipase inhibitor. Milk sterols, too, seem to be closely associated with lecithin, which regulate the hydrophilic power. Carotenoids provide milk its natural coloring and their content

varies with animal feeding and thus with the seasonal pattern. Isomers α and β carotenes, vitamin A and a low concentration of xanthophyll, squalene and lycopene are the ones most frequently found in milk. Carotenes, such as fats, are protected from tocopherols oxidation (Vit. E). They play an antioxidants role and their content in bovine milk is very variable (from 0.2 to 1.2 mg/l).

4.1 Fatty acid synthesis

The secreting cells of mammary glands synthesize milk lipids from FAs, of which 40% is synthesized de novo from the precursor β-hydroxybutyrate (which contributes to 15%) in the same secreting cells. The remaining 60% is made from FAs taken from blood flow (Secchiari, et al., 2002). Figure 12 gives a schematic overview of the two major synthesis pathways of milk FAs.

Figure 12: Schematic overview of major milk fatty acid synthesis pathways (Stoop, 2009)



The rumen microbial population is responsible of the degradation and fermentation of carbohydrates and proteins present in the diet and, consequently, of the production of volatile fatty acids (VFAs) such as acetic acid, propionic acid and butyric acid. Particularly, acetate and butyrate (previously converted into 3-hydroxybutyrate at ruminal walls level) are the precursors of medium and short chain FAs in milk and in adipose tissue (Chilliard, et al., 2000). Their equilibrium depends upon the diet composition and especially, from the fiber proportion in the feed ration. Conversely, production of propionate depends on the proportion of the concentrates inserted in the feed ration. In ruminants in general, adipose tissue, whose metabolism is oriented towards glucose synthesis, is the most active site for FAs synthesis, while in lactating animals is represented by mammary gland.

Particularly, SFA synthesis up to 16 carbon atoms occurs in the cytoplasm through the involvement of two enzymes: acetyl-CoA-carboxylase (ACC) and fatty acid synthase (FAS) (Chilliard, et al., 2000).

In mammary gland, therefore, elongation of the chain can continue to the formation of FAs with 14 or 16 carbon atoms, but the latter cannot be converted to stearic acid (C18:0), although a small proportion of C14:0 and C16:0 are desaturated to C14:1 and C16:1.

Therefore preformed medium and long chain FAs (with 16 or more carbon atoms) present in blood plasma, from the diet or by body reserves mobilization, may be used by tissues, across the action of the enzyme lipoprotein lipase (LPL), through the direct withdrawal of the non-esterified fatty acids (NEFA) from the bloodstream, or from those contained in chylomicrons and in the very low density lipoproteins (VLDL). In addition, the cells of the mammary gland allow to convert stearic acid to oleic acid by the enzyme SCD, which is able to introduce a double bond in Δ9 position. FAs thus formed can then be used in the mammary gland and adipose tissue for triglyceride and phospholipid synthesis. Long chain FAs are potent inhibitors of FAs synthesis originated by mammary synthesis, through a direct inhibitory effect on the activity of acetyl-CoA-carboxylase (ACC). A third factor that could cause this change is also the reduced availability of acetate and 3-hydroxybutyrate for mammary synthesis.

4.2 Lipid metabolism in the rumen

Studies about ruminal lipid metabolism have focused on identifying how FAs pass through the rumen and how they are subject to the microbial population actions (Jenkins, 1993; Doreau, et al., 1994).

From these studies, two important rumen microbial transformations were highlighted: lipolysis and biohydrogenation. Lipolysis involves the release of free FAs thus allowing the next biohydrogenation, which consists in reducing the number of double bonds present on the carbon chain. Microbial presence involves *de novo* FAs synthesis from the precursors of carbohydrates therefore lipids that reach the duodenum are composed of FAs whose origin can be either food or microbial. So the study of rumen lipid metabolism is particularly important because it (Buccioni, et al., 2002):

1. Allows to control antimicrobial effects of FAs, in order to perform ratio integration with lipid sources, without incurring in rumen fermentation disorders and digestive processes abnormal;
2. Allows to adjust the biohydrogenation in order to regulate the absorption of specific FAs that may enhance animals productive performance or improve milk nutritional quality.

Total lipid content of the bacterial dry matter in the rumen ranges from 10 to 15%. This variation is possible as lipids of bacterial origin are derived both from exogenous (increase of long-chain FAs in the diet) and endogenous (*de novo* synthesis) sources. Their contribution depends on diet lipid content and on bacterial species. MUFA instead represent 15-20% of bacterial FAs and are synthesized anaerobically, while PUFA are not commonly synthesized by bacteria, except for cyanobacteria.

The added lipids in ruminant diets can interfere with rumen fermentation, causing a reduced digestibility of non-lipidic energy sources. Ruminal digestion of structural carbohydrates, for example, can be reduced more or less by 50% with only 10% of added fat. This reduction is accompanied by a low production of methane, hydrogen and volatile FAs and a reduced acetate/propionate ratio. When lipid supplements inhibit ruminal fermentation and consequently limiting the fermentation in the last part of the intestine, it can also decrease fiber digestibility in the entire digestive tract. This does not occur when

fat is added to the ratio, which is less deleterious to non-structural carbohydrate digestibility. It was also observed that, an increase in fat levels in the ruminant diet leads to an increase in long-chain FAs, and a decrease in short- and medium- chain FAs (Carrol, et al., 2006).

Rumen UFA concentration is controlled by content, fat type added and lipolysis rate, biohydrogenation rate and carboxylate salt formation. High concentrations of triglycerides in the diet increase the total lipid content in the rumen. The corresponding increase of free UFA in the rumen pool might be increasingly reduced if lipolysis and biohydrogenation are decreased or if the formation of carboxylate salts are high.

The levels of lipolysis usually are sufficient to convert most of triglycerides from diet into free FAs. Some studies have revealed, moreover, that lipolysis and biohydrogenation levels are altered substantially by the inclusion of forages with high maturity levels, low in nitrogen and with food particles size in the rumen (if they are finely ground) (Buccioni, et al., 2002). In this last case, the size of the food particles is of fundamental importance, as it affects the bacteria adherence on the food particle surface and increase transit rate through the rumen barrier, decreasing exposure time to bacterial activity. Therefore, also animal diet composition could influence, as a source of fat, rumen fermentation. In fact, fats that normally inhibit fermentation and digestion, can reduce this capacity when hay content in the ration is too high. Considering UFA duodenal flow regulation, it can be emphasized that the rumen is a formidable barrier to the distribution of UFA in the small intestine. The hydrogenation of unsaturated FAs present in the diet by ruminal microbes, enriches the duodenal chyme of SFA, which are absorbed and deposited in various tissues of the body. Since 1970, there have been several attempts to protect the UFA from ruminal biohydrogenation in order to allow their passage from the duodenum. The first approach has been lipid encapsulation in a proteins matrix treated with formaldehyde (Ashes, et al., 1979). This technology has indeed demonstrated an increase in UFA absorption in ruminants and, consequently, an increase of unsaturation in milk. This has led, however, to believe that protected FAs are necessary because effectively resist to biohydrogenation, without interfering with rumen fermentation or intestinal lipid absorption. Among others techniques studied, the use of calcium salts of FAs, today is the most recognized technique (Ferlay, et al., 1993; Chilliard, et al., 2000), followed by the use of suitably treated whole seeds (extrusion, roasting, etc.), integument and cell walls of which represent sufficient protection in respect of lipids content (Antongiovanni, et al., 2002).

4.3 Variability of composition of milk fatty acids

Milk FAs composition varies according to the influence of different factors related to species, race (Malacarne, et al., 2001; Carrol, et al., 2006), individual dairy characteristics, feeding, breeding techniques and environmental conditions (Palmquist, et al., 1993; Ferlay, et al., 2006; Ferlay, et al., 2008).

Important are the parameters related to nutrition: ration type, mode of administration, energy concentration and energy level of ration, physical state of the forage and of the entire ration (quantity, quality and length of fiber, type, fitness and treatment of cereals, etc.). Even the use of oils and fats protected and calcium salts plays a determining

role in the change of the milk FAs composition (Malacarne, et al., 2001). Composition varies during the year also depending on the season, which determines a different concentration of various milk components. Therefore the different feeding strategies employed can affect nutritional aspects, sensory and technological quality of milk fat (Kraggerud, et al., 2008), which have an impact on dairy products, and especially on cheeses production, whose technological and organoleptic characteristics depend on the initial composition of milk FAs. A significant factor is also determined by the nature of forage (Lourenco, et al., 2008) as by botanical composition, maturity stage and preservation technique. All this, can impact FAs composition considerably, vitamins and carotenoids. The nature of forages is therefore a critical factor, just think of the difference between the pastures in mountain and in plains. Antongiovanni et al. (2002), in fact, showed that, mountain pasture is better than the plain one with regard to the content of butyric acid, lauric, myristic, palmitic, stearic, oleic and for the concentrations of linoleic acid and linolenic acid. Consequently, the milk products organoleptic characteristics are best, for example butter presents a better degree of spreadability. Fresh fodder, in particular, contains about 1-3% of FA, in particular, in spring and in autumn, and the majority of such acids is represented by α-linolenic acid. This entails the increase in long chain FAs concentration and reduction, at the same time, of short and medium chain FAs by *de novo* synthesis in the mammary gland (Khanal, et al., 2008; Palmquist, et al., 2008).

Particularly significant are also the changes related to the bovine physiological state. Content of some FAs varies during the lactation stage (i.e. oleic, linoleic and linolenic acids). Studies showed that lactation state is one of the main factors of change in milk FAs composition, since, as known, at the beginning of the lactation, the energy balance is negative and this leads to a fat mobilization from the body reserves and the incorporation of them into the fat milk as long-chain FAs (Secchiari, et al., 2002). The increase of this fraction leads to the reduction of the short chain FAs of *de novo* synthesis in mammary tissue. This phenomenon naturally tends to diminish with the progress of lactation and the achievement of a positive energy balance.

Regarding the different dairy production technologies, significant variations have not been observed (Collomb, et al., 2002; Lucas, et al., 2006), as no effect on single milk components was observed, with the exception of minerals and folates.

The relationship between FAs composition and milk proteins polymorphisms has also been investigated (MacGibbon, et al., 1997; Bobe, et al., 1999; Bobe, et al., 2004).

A study has shown that genetic polymorphism of αs1-cn has important effects on FAs composition in goat milk and in particular, at the level of medium-chain triglyceride and at Δ9 desaturase enzyme level (Chilliard, et al., 2006). Another study has suggested an association between the different phenotypes for κ-cn and β-Lg and bovine milk FAs, with special reference to the presence of some FAs synthesized at mammary gland level (Bobe, et al., 2004).

4.4 Nutritional importance of milk fatty acids

Although dairy products provide only 15-25% of total fat in human nutrition, they provide 25-35% of total SFA, which if consumed in excess, plays a decisive role in

development of cardiovascular diseases (Chilliard, et al., 2000; Ferlay, et al., 2008). Milk FAs composition have different effects on quality (including physical properties: melting, crystallization and fractionating of milk lipid), on organoleptic characteristics, as well as on nutritional properties.

Among the short chain FAs, butyric acid has a protective role of the colonic mucosa (Ballarini, 1998; Russo, et al., 1999; Parodi, 1999).

The medium-chain FAs have mainly metabolic and energy functions, although in some studies, it has been found the hypercholesterolemic effect of lauric, myristic and palmitic acids, due to the increase of LDL cholesterol, while stearic acid seems to be neutral and does not have an atherogenic effect, although it is a SFA (Mensink, et al., 1998). In particular, lauric and palmitic acids are considered to average harmfulness to human health, unlike myristic acid, considered to be the worst of all (Ulbricht, et al., 1991).

Regarding MUFA, several studies (Secchiari, et al., 2002) were aimed at the determination of their effects on human health and some of these are able to reduce serum cholesterol levels in a manner similar to PUFA, but unlike PUFA, ω-6 does not lower the level of HDL (Mensink, et al., 1998).

Milk PUFA in addition to having energy functions, metabolic and structural, have relevant health properties. Regarding the anti-microbial properties, some studies have shown the importance of conjugated linoleic acid (CLA) in the prevention of weight loss due to infections and stimulation of the immune system (Ballarini, 2000). Also it has anti-carcinogenic and anti-oxidants properties.

Another extra nutritional function relates to hormonal activities of milk fat, which can be direct or indirect. Direct activities are mediated by the action of natural hormones, fat-soluble and especially steroid type (estrogen, etc.) and by phytohormones present in animal nutrition. Indirect activities are linked to fat-soluble vitamins (vit. D) and to cholesterol, as basic biochemistry of steroid hormones (i.e. sex hormones, corticosteroids, etc.).

Other important lipids activities are the psycho-dietary, which in addition to making energy, actively participate in the cell membranes construction and play an essential role in nervous system, especially during the development phase. Cell membranes in fact consist mainly of lipids and proteins, and in particular, phospholipids account for about 60% of the membrane lipids. More than 1/3 of the FAs in phospholipids consists of essential FAs, with particular prominence of linoleic and α-linolenic acids. These membrane FAs, also known as structural, are extremely important in human nutrition and a balanced diet should cover much of the lipid requirements. The organs in particular during their development suffer from shortages of essential FAs, especially the brain which uses energy primarily from blood glucose. Consequently a deficiency in PUFA from food is particularly harmful when brain is developing, which occurs immediately after birth and in the first years of life. Regarding the anticarcinogenic activities of fats, some studies have shown that several fat components have this characteristic. The most important are CLA, sphingomyelins, butyric acid and ether lipids (alkyl-glycerol and alkyl-glycerol-phospholipids and their derivatives).

Linoleic acid, in particular, is an essential PUFA that with α-linolenic represent the founders respectively of the series of ω-6 and ω-3 PUFA (Antongiovanni, et al., 2002).

Linoleic and α-linolenic acids are contained in different amounts in meat and milk and are absorbed virtually unchanged in the intestine. They are extremely important because are the PUFA precursors and their biological derivatives polyunsaturated more active are: arachidonic acid C20:4 ω-6, eicosapentaenoic acid C20:5 ω-3 (EPA) and docosahexaenoic C22:6 ω-3 (DHA). Also acquit themselves important functions: linoleic acid enters is part of the lipid complexes that form a permeability barrier for the epidermis, while a deficiency of α-linolenic acid may be a significant risk factor against heart disease, cancer and neurological functions disorders in both children and adults.

4.5 Conjugate Linoleic Acid in milk

Over the years, interest in the study of beneficial effects on human health of some essential FAs has greatly increased. Among these, linoleic acid (C18:2 *cis*-9, *cis*-12) is particularly important because, not being synthesized by the body, it must be introduced through adequate nutrition. Linoleic acid is the precursor of CLA (conjugated linoleic acids). CLA include the set of position and geometric isomers of linoleic acid with conjugated double bonds (Parodi, 1997). Within CLA, rumenic acid (*cis*-9, *trans*-11) is the only FA that had unequivocally anti-carcinogenic activities in experiments carried out on animals (Banni, et al., 1999; Kelsey, et al., 2003; Destaillats, et al., 2005; Jenkins, et al., 2006). It has also proved to be active against different diseases, such as atherosclerosis, diabetes, obesity, showing high ability to interfere positively with the immune system (Melis, et al., 2003). Unlike other isomers that have an exogenous origin, rumenic acid can have an endogenous origin (mammary gland and adipose tissue) from vaccenic acid C18:1 *trans*-11, following the action of Δ9 desaturase enzyme (Stearoyl-CoA Desaturase - SCD) (Griinari, et al., 2000; AbuGhazaleh, et al., 2004; Kay, et al., 2004; Whitlock, et al., 2006). This is made possible due to the presence of the *Butyrivibrio fibrisolvens* bacterium in rumen that is able to convert linoleic acid (widely present in the forage) in CLA by enzymatic way. It was also shown that rumenic acid competes with linoleic acid reducing arachidonic acid formation, an eicosanoids precursor that play an important role in carcinogenesis (Secchiari, et al., 2002). Among CLA activities (mainly of the two isomers) there is also an anti-atherogenic and hypocholesterolemic properties. A decrease in LDL cholesterol levels in plasma has been observed with a resulting decrease of the arterose plaques formation in rats fed with diets enriched with this FA. Finally, the positive effects on diabetes are related to the best use of glucose in plasma and increased insulin efficiency.

In milk and dairy products, CLA presence is higher than that present in meat and about 80-90% is *cis*-9, *trans*-11 isomer (Jiang, et al., 1998; Zhang, et al., 2006a; Zhang, et al., 2006b). Naturally, CLA variation is affected by season (Thorsdottir, et al., 2004; Chamba, et al., 2006) and is also influenced by food, lactation stage and race (Delmonte , et al., 2000; Collomb, et al., 2002; Kelsey, et al., 2003; Bernal-Santos, et al., 2003).

Regarding the latter factor, although the ability to synthesize rumenic acid from its precursor seem to be common to many species, there are substantial differences within and between species. Particularly, the milk which contains the most amount of CLA and, therefore, of rumenic acid, is in sheep milk, followed by cattle and then goat (Mele, et al., 2002).

Consequently, researchers of livestock sector have tried to achieve nutrition and dietary strategies for ruminants aimed to the increase of these FAs in milk and its derivatives (Perfield, et al., 2004; Piperova, et al., 2004; Khanal, et al., 2005), acting in a particular way, on ration composition (Lawless, et al., 1998; Dhiman, et al., 1999; De Veth, et al., 2005; Loor, et al., 2005; Rego, et al., 2005).

4.6 ω-6 and ω-3 in milk

Within PUFA there are FAs of ω-6 series (linoleic acid LA C18:2, γ-linolenic C18:3 and arachidonic acid AA C20:4) and of ω-3 series (α-linolenic ALA C18:3, eicosapentaenoic EPA C20:5 and the docosahexaenoic DHA C22:6). These FAs are defined essential because they cannot be synthesized by mammals, but they must be compulsorily introduced with the diet. A source of significant amounts of ω-3 is represented by microalgae and marine organisms, with significant quantitative differences between the various species.

The α-linolenic instead it is mostly found in dark green vegetables (spinach, lettuce), linseed oil (57% of total FAs), in rapeseed, soy, wheat germ, nuts (which contain from 7 to 13% of ALA). In particular soybean oil, for its particular FAs composition, can be considered a major source of ω-3 FAs, present mainly as α-linolenic acid (Caponio, et al., 2003). The ω-6 FAs (LA, AA) have rather eggs, poultry, most vegetable oils, grains, the baked goods, fried foods and margarine as source of origin (Lòpez Huertas, et al., 2003). A change in the diet that favors the FAs production belonging to the ω-3 series determines, not only an increase of this series of FAs, but also a decrease of FAs of the ω-6 series (Secchiari, et al., 2002; Stam, 2003; Lòpez Huertas, et al., 2003; Gabaldo, et al., 2007).

As an energy source, ω-6 and ω-3 FAs are embedded in cell membranes, where through the reactions catalyzed by cyclooxygenase and lipo-oxygenase enzyme, they generate substances at action hormone-like such as prostaglandins, prostacyclins and leukotrienes (eicosanoids), involved in a wide range of physiological processes such as blood clotting, inflammatory and immunological reactions. Several studies have highlighted the antiatherogenic properties, antithrombotic and anti-inflammatory of the long-chain PUFA ω-3. It has been observed the beneficial effects of ω-3 are particularly evident at the level of cardio-vascular diseases, of cholesterol and triglycerides. These FAs also tend to reduce hypertension, due to a lowering of the overall level of blood pressure, improve rheumatoid arthritis, symptoms of depression and other mental illnesses. For this reason it has been considered the possibility to enrich different types of food (Berra, et al., 2003). Among these there is milk. Several studies have reported that by altering the FAs composition of the lipid fraction through the ration, animals fed with fresh forage have a higher content of PUFAs ω-3 (α-linolenic acid and EPA) in adipose tissue and in particular in phospholipids. Conversely, a cereal-based diets result in an increase of ω-6 PUFA. When it is not possible to use green forage, the only alternative is the addition of fish liver oil in the ration. This is rich in long chain PUFA and it is suitably protected in order to not worsen the animal performances and milk fat concentration.

5. Genetic improvement of milk fatty acids composition

Milk FAs composition varies considerably between individual cows and is affected both by feeding, by breeding and by genetics. Generally, the FAs that can be changed by feeding are the blood derived FAs (Moate, et al., 2007) while the *de novo* synthesis FAs can be best changed by breeding. Obviously, knowledge about the origin of FAs is important when there is the interest in changing the FAs composition of milk. Large variation between cows exists in milk FAs composition and part of this variation is due to genetic differences.

Milk fat biosynthesis is a complicated process regulated by many genes belonging to several pathways (Bionaz, et al., 2008). Genetic analyses of bovine milk FAs have shown heritable variation. For most FAs, only a relatively small part of the genetic variation is explained by the variants studied and this means more genes exists that affect the milk FAs composition. Identifying these genes or markers closely linked to these genes can help the further improve the FAs composition in milk. Identification of genomic regions, and preferably individual genes, responsible for genetic variation in milk fat composition will enhance the understanding of biological pathways involved in fatty acid synthesis and may point towards opportunities for changing milk fat composition via selective breeding.

In the last few years, researchers have focused on studying the effects of the polymorphisms of individual loci on productive characteristics of animals. The results are often conflicting. The positive effects observed on the production in some studies may be due to the association between the locus analysis and other loci on the chromosome, rather than the unique effect of allelic locus itself. Therefore, it is believed that a better estimate of the various allelic effects of the locus considered is obtained by directing the analysis to the same gene cluster, starting not from simple genotypes, but from different haplotypes observed in every breed. It was shown that the same haplotypes tend to have similar effects in different races and this seems to confirm the importance of gene clusters than single polymorphisms contained therein, to explain the effects associated with it. Also with regard to the quantity and quality of milk fat, the candidate gene approach has been experienced, however, unlike what has been shown for the protein fraction, selection application experience is still lacking.

Several studies have allowed the identification the position of quantitative trait loci (QTL) controlling milk production on bovine chromosomes (BTA). It has been mapped QTL for milk fat percentage on BTA 3, 6, 14, 20 and 26 (Georges, et al., 1995; Zhang, et al., 1998; Plante, et al., 2001; Ashwell, et al., 2004). Others study identified QTL associate with milk fat yield on BTA 1, 9, 10, 14, 19 and 26 (Georges, et al., 1995; Zhang, et al., 1998; Plante, et al., 2001; Bennewitz, et al., 2003). Another study mapped QTL for milk fat content to BTA 19 (Bennewitz, et al., 2003). The findings from numerous studies looking at QTL effects on milk production traits in dairy cattle were summarized as QTL maps form by Polineni et al. (2006) and Khatkar et al. (2004). Discoveries of numerous QTL for milk production traits in dairy cattle prompted researchers to identify causal genes for the QTL of interest.

Milk fat synthesis mechanisms involve various enzymes that take part in the collection of precursors from blood (lipoproteinlipase), in the lengthening and desaturation of the FA carbon chains (acetyl-CoA carboxylase; FA synthase, Δ- 9 desaturase), in the FA

transport into the cytoplasm (FA Binding Protein), in the esterification of glycerol for the triglycerides and phospholipids formation (Acyl-transferase). Since the lipid metabolism control is regulated by expression and activation of numerous genes giving rise to multiple enzymes, some of which play a key role as Acetyl-CoA-carboxylase complex (ACC) and fatty acid synthase (FAS), lipoprotein lipase (LPL), acyltransferase (GPAT, LPAT, DGAT), fatty acid desaturase (Δ4, Δ5, 6and Δ9 stearoyl Co-A desaturase, SCD). These genes have been considered as potential candidate genes. In addition to these, there are all the enzymes involved in the energy metabolism of the glandular cell in order to provide reductive equivalent and ATP necessary for the biochemical reactions and redox that are the basis of the milk fat synthesis. Important role in determining the availability of blood precursors for mammary synthesis of milk fat is also played by the hormonal balance. The hormones more involved are insulin, epinephrine, GH and leptin. Their action is based mainly on the animal's capacity to mobilize lipid reserves and, therefore, to make available FAs contained in them both for their oxidation and for increase the availability of preformed FAs to the mammary gland according to the animal's energy balance.

The knowledge of physiological and biochemical mechanisms that underlie milk fat secretion has enabled the development of nutritional strategies to improve fat milk quality. In addition, since the composition of FAs depends on the action of numerous enzymes, a thorough knowledge of lipid metabolism can help to identify potential candidate genes that could significantly influence the content of particular FAs.

In the current analysis, the polymorphisms in some genes associated with milk production and composition has been evaluated. The genes being studied were localized in the BTA 6, 14, 19 and 26. Detail, these are ATP-Binding Cassette G2 (ABCG2) in BTA 6, Diacyl-Glycerol o-Acyl-Transferase 1 (DGAT1) in BTA 14, Signal Transducer and Activator of Transcription 5A (STAT5A) in BTA 19 and Steroyl-CoA Desaturase (SCD) in BTA 26.

Studies in different cattle populations have reported effects of SNPs in these genes on the routinely collected traits milk-fat percentage, milk-fat yield or both (Brym, et al., 2004; Brym, et al., 2005; Cohen-Zinder, 2005; Dybus, et al., 2005; Weikard, et al., 2005; Khatib, et al., 2006; Roy, et al., 2006; Morris, et al., 2007).

5.1 Genetic parameters and effects of ABCG2 on milk composition

ATP binding cassette sub family G member 2 (ABCG2) gene, encodes a transporter protein that facilitates transport of drugs through the cell membrane by binding ATP.

ABCG2 product is expressed in the apical membrane of alveolar mammary epithelial cells in cows and is known that it has important effects on the milk yield traits. In mouse, it has been demonstrated that, ABCG2 is responsible for the active secretion of substrates into milk (Cohen-Zinder, 2005).

Also other members of the ABC subfamily G are sterol transporters. In a bacterial expression model, ABCG2 was shown to facilitate the efflux of a labelled sterol, confirming that ABCG2 may have a role in sterol transport into milk (Janvilisri, et al., 2003). It has also been shown to facilitate secretion of riboflavin into milk (van Herwaarden, et al., 2007) and is involved in folate homeostasis (Assaraf, 2006).

It is located in BTA 6 where a variety of QTL were mapped. Different studies about cattle population suggest that, in this chromosome, there are distinct QTL regions affecting milk composition, as casein cluster and a number of QTL for milk fat and production traits (Khatkar, et al., 2004).

According to the bovine genome assembly[6] in BTA6, one QTL with a predominant effect on milk protein and fat content is located in the interval 37.2-38.2 Mb (QTL region 1), proximal to marker BM143 (44.17 Mb). This region 1 includes ABCG2 which belongs to the superfamily of ATP-binding cassette (ABC) transporters.

It has been suggested that, in ATP-dependent processes, ABCG2 plays an important role in the secretion of xenobiotics, cytotoxic drugs across the plasma membrane and some carcinogens and micronutrients such as cholesterol and vitamin K3 into milk (Cohen-Zinder, 2005; Litman, et al., 2000; van Herwaarden, et al., 2006).

Research paper, Jonker et al. (2005) describes how ABCG2 is not expressed in virgin mice but is greatly induced during late pregnancy and increases during lactation. Moreover, the expression level significantly increases during lactation compared to the dry period (Farke, et al., 2008).

There is strong evidence that a polymorphism in this gene is associated with effects on milk yield and composition in the Holstein cattle (Cohen-Zinder, 2005), dairy cattle (Olsen, et al., 2007) and Indian cattle (*Bos indicus*) and buffalo (*Bubalus bubalis*) breeds (Tantia, et al., 2006).

In cows, a single nucleotide polymorphism (SNP) in exon 14 of ABCG2 has been described widely (Y581S) by different authors and seems to be associated with higher fat and protein percentages and lower milk yield. This polymorphism causes a substitution of tyrosine-581 to serine and it has been proposed as a QTL on BTA6 (Cohen-Zinder, 2005; Olsen, et al., 2007). Animals having A allele compared with animals having C allele on gene region with the amino acid alteration on exon 14 at 62596 nucleotide (NCBI Acc. Num. AJ871176), displayed a decrease in milk yield and an increase in milk fat and protein percentage (Cohen-Zinder, 2005; Lillehammer, et al., 2009). When both the two alleles are adenine on 62569 nucleotide, the synthesised amino acid is tyrosine (TAT) while when the nucleotide is cytosine (TCT), the synthesized amino acid is serine (Tantia, et al., 2006; Ron, et al., 2006).

Ron et al. (2006) studied the allele frequencies of the polymorphism Y581S for the ABCG2 gene in 35 different bovine breeds. The $ABCG2^{A}$ allele was predominant in all populations while the $ABCG2^{C}$ allele was detected (with low frequencies) only in 12 breeds analysed belonging to *Bos taurus*. The detection of $ABCG2^{C}$ only in *Bos taurus* breeds may indicate that $ABCG2^{A}$ is the ancestral allele, and that the Y581S substitution occurred after the separation of *Bos indicus* and *Bos taurus* lineages over 200,000 years ago (Loftus , et al., 1999).

Another study conducted by Kowalewska-Łuczak et al. (2009) in Jersey cows, shows similar results obtained by other researchers. Allele A was more frequent and in 181 animals analysed, the frequencies of the genotypes and alleles were; AA-0.61, AC-0.39 and A0.80, C-0.20 respectively. The associations between the different ABCG2 genotypes and

[6] http://www.animalgenome.org/cattle/

milk production traits (daily milk yield in kg, fat and protein percentage content), showed statistically significant differences only in the case of fat content in milk. Animals with AC genotype had a higher fat content (+0.20 %). In regards to other milk production traits, their values were found to be higher in individuals with AC genotype, even if these differences were not statistically confirmed.

Also Komisarek et al. (2009) in Polish Holstein-Friesian, Yildirim et al. (2010) in Turkish Holstein cows, and Ateş et al. (2014) in South and East Anatolian Red (SAR and EAR) reported a predominance in the frequencies of ABCG2^{A} allele (Table 7). The evaluated polymorphism effects on milk production were similar to those above reported.

Table 7: Allele frequencies of genotyped animals in different breeds

AUTHOR	BREED	NO. OF ANIMALS	A FREQUENCIES	C FREQUENCIES
Komisarek et al. (2009)	Polish Holstein-Friesian	453	0.99	0.01
Yildirim et al. (2010)	Turkish Holstein	207	0.98	0.02
Ateş et al. (2014)	SAR	49	0.63	0.37
	EAR	40	0.64	0.36

The effects of ABCG2 variations are economically favourable for most selection indexes used in dairy cattle breeding programs (Miglior, et al., 2005). The ABCG2 gene can be studied as the candidate gene effecting milk traits.

5.2 Genetic parameters and effects of DGAT1 on milk composition

Among lipogenic enzymes, acyl-transferases are responsible for triglyceride synthesis. They are delegated to the selective transfer of FA in the three positions of the glycerol (sn-1, sn-2 and sn-3). The enzyme diacyl-glycerol acyl-transferase (DGAT) plays a fundamental role in the metabolism of cellular diacyl-glycerol and is important in higher eukaryotes for physiological processes involving triacylglycerol metabolism, such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation (Cases, et al., 1998). It catalyzes the last step of triglyceride synthesis. The reaction involves the esterification of diacyl-glycerol with the acyl-CoA in sn-3 position. DGAT is expressed in many tissues, especially in the intestine and liver. In the mouse intestine and adipocytes and in the bovine mammary glands, DGAT utilizes a wide range of FAs and diacyl-glycerol. In addition to the formation of triglycerides, DGAT is involved in several processes, such as energy storage, intestinal fat absorption, lipoprotein assembly, fat storage, production of oocytes and muscle energy metabolism.

Until now, two acyl-CoA:diacylglycerol acyltransferases – DGAT1 and DGAT2 – have been identified (Cases, et al., 1998; Cases, et al., 2001). The knockout of mouse DGAT1 gene indicates its crucial role for lactation. Females deficient in this enzyme are characterized by a complete lack of milk production, most probably because of impaired triglyceride synthesis in the mammary gland (Smith, et al., 2000).

No differences were noted between DGAT1 and DGAT2, even if they belong to gene families that do not show homology (Cases, et al., 2001). DGAT1 is located in an area of the BTA14 where there is a QTL associated with the improvement of milk fat production. This QTL is between the microsatellite markers BULGE13 and BULGE9. Grisart et al. (2001) suggest that DGAT1 is an associated candidate gene for this QTL. The DGAT1 coding sequence is 8.6 kb in size. It consists of 17 exons which have an average length of 121.8 bp. The gene and the respective protein of the bovine, show high level of similarity with the human ones, 89.5% and 92.5% respectively (Grisart, et al., 2001).

In exon 8 of DGAT1, a non-conservative dinucleotide substitution changing lysine to alanine at position 232 (K232A) of the encoding protein has been reported (Grisart, et al., 2001; Winter, et al., 2002).

This polymorphism showed a strong effect on milk fat content and other characteristics of milk in Jersey breeds, Ayrshire and Holstein (Thaller, et al., 2003; Weller, et al., 2003). It was reported that the K allele has a higher Vmax reaction than allele A, it also expresses a higher milk fat content (+0.35%) and increased milk production (+10 kg) (Grisart, et al., 2004; Winter, et al., 2002).

Moreover, the DGAT1^{K} allele was associated with a larger fraction of C16:0; and smaller fractions of C14:0, unsaturated C18, and CLA (Schennink, et al., 2007). Direct evidence for the causality of the K232A polymorphism was provided by biochemical data that revealed that the K-encoding allele is characterized by higher triglycerides producing than the A allele (Grisart, et al., 2004).

Analysis of the sequences in database (NCBI Acc. Num. AW446985) highlighted the existence of alternative splicing. This implies the presence of two transcripts that differ in their length. The longer utilizes part of the eighth terminal intron, precisely 6 bp upstream of the polymorphic site K232A. Amplifying the region comprising the alternative splicing site, it was possible to assess whether that the shorter variant is associated with the K allele of the locus K232A (Grisart, et al., 2004).

A research carried out at Isfahan University in Iran has analyzed the influence of K232A polymorphism in a Friesian population (Pirzad, et al., 2014). It showed a significant association between this polymorphism and milk production traits (Table 8). The research was conducted with 408 animals belonging from 5 different farms. DGAT1 K232A polymorphism results showed significant differences ($p \leq 0.05$). Milk yield from animals with genotype AA was higher than animals with KA and KK genotype. On the contrary, AA was lower ($p \leq 0.05$) than KA and KK genotypes in fat percentage.

Table 8: K232A polymorphism effect Holstein dairy cows (Pirzad, et al., 2014)

	GENOTYPES		
Traits	AA	KA	KK
Milk yield (kg)	9,606 (±141)	9,296 (±127)	9,043 (±242)
Fat (%)	3.06 (±0.04)	3.2 (±0.043)	3.3 (±0.08)
Protein (%)	2.93 (±0.01)	2.97 (±0.01)	3.00 (±0.03)
SCS	1.87 (±0.09)	1.86 (±0.08)	1.7 (±0.16)

The K232A polymorphism has also been analyzed on seven Italian bovine breeds (Scotti, et al., 2010). Analysis of allele frequencies showed the majority presence of A allele than K variant in all breeds tested (Table 9).

Table 9: Genotype and allele frequencies of DGAT1 K232A polymorphism obtained in 7 cattle Italian breeds (Scotti, et al., 2010)

BREEDS	NO.	GENOTYPE FREQUENCIES (NO. OF ANIMALS)			ALLELE FREQUENCIES	
		p.232KK	p.232AK	p.232AA	p.232K	p.232A
Italian Holstein	116	0.056 (6)	0.405 (47)	0.542 (63)	0.254	0.746
Italian Brown	115	0.000	0.000	1.000 (105)	0.000	1.000
Italian Simmental	95	0.000	0.011 (1)	0.989 (64)	0.005	0.995
Valdostana Red Pied	95	0.000	0.011 (1)	0.989 (64)	0.005	0.995
Rendena	62	0.000	0.016 (1)	0.984 (61)	0.008	0.992
Reggiana	128	0.000	0.334 (44)	0.656 (84)	0.172	0.828
Modenese	50	0.000	0.000	1.000 (50)	0.000	1.000

The effects of the DGAT1 polymorphism were confirmed in another study conducted on 57 Red Spotted cows in Poland (Table 10) (Lešková, et al., 2013).

Table 10: K232A polymorphism effect Red Spotted cows (Lešková, et al., 2013)

TRAITS	GENOTYPES		
	AA	AK	KK
Milk yield (kg)	5427	5598	5390
Fat (%)	3.93	4.05	4.12
Protein (%)	3.37	3.41	3.21

Other studies confirmed this strong effect on milk fat content and other milk characteristics in the New Zealand, Dutch, German, Israeli Holstein-Friesian, Brasilian and in Jersey cattle (Grisart, et al., 2002; Spelman, et al., 2002; Thaller, et al., 2003; Weller, et al., 2003; Komisarek, et al., 2004; Lacorte, et al., 2006; Komisarek, et al., 2009).

Other studies, however, pointed out that the DGAT1 K232A mutation is not solely responsible for the milk production QTL on bovine chromosome 14 (Bennewitz , et al., 2004). Moreover, alleles of the DGAT1 promoter derived from the variable number of tandem repeat polymorphisms were associated with milk fat percentage in animals that were homozygous for the allele 232A in DGAT1 (Kühn, et al., 2004). The presence of additional genetic polymorphisms in DGAT1 and elsewhere on bovine chromosome 14 indicates that the DGAT1 K232A polymorphism cannot completely explain the variability in milk production traits attributed to the QTL on the same chromosome, and thus new polymorphisms are awaiting their discoveries.

Analysis of the DGAT1 gene sequence has showed the existence of other polymorphisms: two SNPs (A→G; C→T) in the intron 12, called N984+8 (AG) and N984+26 (CT) respectively and one SNP (C→T) in the 3'UTR region called NT-1501 (Grisart, et al., 2001).

5.3 Genetic parameters and effects of STAT5A on milk composition

Signal transducers and activators of transcriptions (STAT) are a family of latent cytoplasmic transcription factors with 7-members that include STAT1, 2, 3, 4, 5A, 5B and 6 (Darnell, et al., 1994; Schindler, et al., 1995).

In mammals, STAT proteins are a group of cytoplasmic transcription factors that mediate the actions of many peptide hormones and cytokines within target cells. The DNA-binding capacity of STATs is induced by phosphorylation of a tyrosine residue at the C-terminus of the protein, which leads to dimerization and nuclear localization. STAT5 is known as a main mediator of growth hormone (GH) action on target genes (Argetsinger, et al., 1996). It was initially discovered as a PRL-induced transcription factor and can activate transcription of milk protein genes in response to prolactin (Wakao, et al., 1994). Then it was called mammary gland factor (MGF).

Initially, STAT5 gene was identified in sheep but subsequently two forms of STAT5 (STAT5A and STAT5B), encoded by two different genes, have been identified in mouse, human, rat and cattle (Hou, et al., 1995; Kazansky, et al., 1995; Liu, et al., 1995; Mui, et al., 1995; Ripperger, et al., 1995; Lin, et al., 1996; Silva, et al., 1996; Darnel, 1997; Goldammer, et al., 1997). Genes encoding STAT5A and STAT5B are highly homologous, being about 90% identical in coding sequence; the two isoforms differ by few amino acids in the carboxylic end of the protein molecule (Moriggl, et al., 1996). Moreover they exhibit differences both in their DNA binding specificities (Boucheron, et al., 1998; Verdier, et al., 1998) and with respect to their tissue distribution (Liu, et al., 1995; Mui, et al., 1995).

In cattle, STAT5A gene has been mapped to chromosome 19q17 within 40 Kbp, STAT locus also contains STAT3 and STAT5B genes (Seyfert, et al., 2000; Moleenar, et al., 2000). The STAT5A gene consists of 19 exons encoding 794 amino acids chain (Seyfert, et al., 2000).

This gene is suggested as candidate associated with milk protein yield and percentage in dairy cattle. Three functional domains of STAT5A, namely the DNA binding domain, SH2 domain and C-terminal trans-activating domain, encoded by exons 9-19, were selected to be screened for polymorphisms.

Lechner et al. (1997) reported that a STAT5 polymorphism may influence milk protein gene expression during lactation and milk production traits in dairy cattle through the interaction and functional synergism with receptors for glucocorticoid and insulin.

Several nucleotide sequence polymorphisms of the bovine STAT5A gene have been detected: McCracken et al. (1997) found TG repeats of different length within STAT5A intron 12. Antoniou et al. (1999) described two SSCP variants of the gene fragment that encodes SH2 domain in bovine STAT5A protein.

Brym et al. (2004) detected a new SNP (A/G) located in intron 9 at position 9501 and reported significant differences for milk traits. The animals tested belonged to different breeds (Polish Friesian, Jersey, Polish Red, Simmental, Limousine and Charolaise). Cows with GG genotype showed a higher milk yield in the first lactation compared to ones with AA genotype. Moreover, cows with AA and AG genotypes showed higher protein content compared to GG genotype in both lactations considered. For fat content, cows with the AA

genotype showed the highest level in the 1st and 2nd lactations. Interestingly, cows with genotype AG showed significantly higher protein yields in comparison to cows with the AA genotype. Also, some minor associations in the 2nd lactation were found at the significance level.

Flisikowski et al. (2002) reported a SNP in exon 7 of the bovine STAT5A gene also investigated by other authors (Dario, et al., 2009a; Dario, et al., 2009b; Selvaggi, et al., 2009; Sadeghi, et al., 2009; Selvaggi, et al., 2011).

Moreover, Flisikowski et al. (2003a) reported the deletion of CCT in intron 15.

A polymorphism in the exon 16 of STAT5A gene has been reported by different authors. Exon 16 codes for domain SH2, responsible for dimerization of the STAT5 transcription factors to the activation proteins (Pellegrini, et al., 1997). Mutations in this region might result in changes in the transactivation properties of the STAT5A protein, and thus influence the level of expression of the genes regulated by this transcription factor. A T/C substitution at position 12743, causing an amino acid changes (Valine to Alanine) in the STAT5A protein in 686 position (V686A) was discovered. This polymorphism is located very close to tyrosine 694, which plays a key role in the phosphorylation, activation and dimerization of STAT5 (Flisikowski, et al., 2003b; Flisikowski, et al., 2004). The V686A mutation showed a change in the DNA-binding properties of STAT5A. In the DNA-protein binding assays nuclear proteins extracted from livers of animals with CC genotype always showed less DNA protein complexes than those with TT genotype (Flisikowski, et al., 2003b). The lower DNA-binding capacity of CC genotype confirms that STAT5A transcription factors take part in the formation of the DNA-protein complex.

Flisikowski et al. (2003b), in 108 young bulls of different breeds (Friesian, Charolaise, Limousine, Aberdeen, Angus, Hereford and Simmental) reported that the TT genotype was the most frequent and the CC genotype was found in low frequency only in Polish Friesian and Charolaise. The T and C alleles' frequency was 0.84 and 0.16, respectively.

The same authors (Flisikowski, et al., 2004), analysed the association between the V686A mutation with milk production traits in 150 Polish Friesian. The T and C alleles' frequency was 0.85 and 0.15, respectively. The frequency of CC genotype was very low (0.02) so only TC and TT genotype were considered. They reported that, both milk and fat corrected milk (FCM) yields and lactose were higher in animals with TC genotype than TT genotype. Furthermore, daily yield of value corrected milk (VCM), total solids, non-fat-solids, proteins, and lactose were higher in TC cows compared to those genotyped as TT. No association with protein and fat content in milk and milk fat yield was found. All these results, suggest the beneficial influence of the C allele on the majority of milk production traits. However, another study conducted by Selvaggi et al. (2013) showed a positive effect of the T allele in some milk production traits, contrarily to what previously reported. The study was carried out on 95 cows belonging to Jersey. A high frequency of T allele (0.85) than C allele (0.15) was reported. In this study, the genotype frequencies were TT – 76.68%, TC – 23.16% and CC – 3.16%. The difference in milk yield, kg of fat, protein percentage and protein content was not significant between the TT and TC genotype. In addition, milk from TT animals showed a higher fat content in comparison with that of TC animals. In any case,

it can be hypothesized that it is the low frequency of the C allele that does not permit a complete comparison between the genotype.

The results presented are not sufficient to postulate a definitive relation between the analysed polymorphism with milk production traits and further investigation should be done on bigger cattle populations of different breeds.

5.4 Genetic parameters and effects of SCD1 on milk composition

Stearoryl-CoA desaturase (SCD) belongs to the desaturase acyl super-family. It is a key enzyme in the MUFA metabolism and plays a central role in regulating FAs metabolism (Heinemann , et al., 2003). It catalyzes the insertion of a double bond in cis-Δ9 position in a broad spectrum of medium and long chain FAs (Palmquist, et al., 1993). SCD is a protein located on the endoplasmic reticulum membrane of microsomes and catalyzes the desaturation in cis-Δ9 position of certain FAs, including stearic acid and vaccenic acid, thus generating oleic acid (C18:1 *cis*-9) and rumenic acid (CLA *cis*-9, *trans*-11) respectively (Ntambi, et al., 2001). Palmitoleic and oleic acids are the most represented in the membrane phospholipids compounds, for which, SCD is responsible for the saturated/unsaturated FAs ratio in the triglyceride composition and in the phospholipid membrane. It also plays a two adjacent cells (Ntambi, 1995; Heinemann , et al., 2003).

The gene encoding SCD was mapped to BTA 26 (Campbell , et al., 2001), where some other QTLs for fat yield and other milk traits have been also identified. Bovine SCD gene is 17088 bp long and consists of 6 exons and 5 introns. The amino acid sequence is characterized by the presence of 3 regions rich in histidine, which are repeated in all species with a high rate of similarity. This suggests that these 3 regions have been actively involved in the catalysis of the reaction (Ntambi, 1995; Behrouzian, et al., 2003; Heinemann , et al., 2003). The SCD protein is 359 amino acids long as in other species and shows a high homology with goat (93.9%), sheep (93.6%), human (87.2%) and mouse (80.5%) (Taniguchi, et al., 2004).

The study of gene structure has revealed the presence of a polymorphism, extended too many breeds (Holstein, Jersey and Brown Suisse) (Medrano , et al., 1999), consisting of 3 SNPs in the fifth exon. The first two SNPs are silent mutations, while the third involves the replacement of an amino acid, valine to alanine, in the third histidinic region (Medrano , et al., 1999; Taniguchi, et al., 2004). The three SNP in the fifth exon are inherited simultaneously (linkage disequilibrium) and form two haplotypes, A and V. From this experimental evidence enzymatic changes in the activity of the two proteins have been hypothesized.

A study conducted on 360 Italian Friesian cows, revealed that individuals carrying the A haplotype showed a significantly higher content of MUFA in milk and a more elevated desaturase activity (measured as C14:1/C14:0 ratio) if compared to individuals homozygous for the V haplotype (Mele, et al., 2007).

Another study conducted on 975 purebred Poland Holstein cows Kulig et al. (2013), showed the effects of g.10329C>T SNP in exon 5 of SCD1. Animals with the A haplotype (T allele) showed a higher milk fat content with lower frequency compared with V haplotype

(allele C). The V haplotype is characterized by a greater production and the heterozygous variant showed higher values for milk traits considered (Table 11).

Table 11: SCD1 polymorphism effects in Poland Holstein cows (modified by Kulig, et al., 2013)

TRAITS	GENOTYPE (NO. OF ANIMALS)		
	TT (60)	CT (391)	CC (524)
Milk yield (kg)	216.98	313.05	297.71
Fat content (kg)	9.74	10.79	8.99
Protein content (kg)	9.03	11.19	9.64

Study conducted by Taniguchi et al. (2004), have identified other 5 SNPs in the 3'UTR region of the cDNA: at 1905 nucleotide (G/A), at 3143 nucleotide (C/T), at 3351 nucleotide (A/G), at 3537 nucleotide (A/G) and at 4736 nucleotide (A/G).

SCD gene is one of the few, together with that DGAT1, for which a genetic polymorphism associated with significant changes in the production of fat in milk and meat has been demonstrated. This fact leads to consider these two genes as possible candidates for the identification of molecular markers to be used for selection purposes.

6. Mineral composition of milk

Minerals in milk include metal ions, inorganic and organic anions, and makes us for approximately 1% of total milk (Table 12).

Table 12: Mineral content (mg/100ml) of bovine milk (Claeys, et al., 2014)

MINERAL	µg/100ml
Ca	112-123
P	59-119
K	106-163
Mg	7-12
Na	58
Cl	100-119
Fe	0.03-0.1
Zn	0.3-0.55
Cu	0.01-0.08

Minerals are found mainly in solution, while calcium and magnesium are in large part inside of the casein micelles. The most important trace elements are zinc, iron and copper.

Minerals in milk play an important role in the structure and stability of the casein micelle. Small variations in their composition or division may have important consequences on casein micelles (Gaucheron, 2005).

Most of the saline components are constituted by inorganic or mineral salts, from dissociable substances, and small amounts of organic salts, i.e. acids which contain organic radicals, such as citric acid. They are found in the form of cations (calcium, sodium, potassium, magnesium) and anions (inorganic phosphorus, citrate, and chlorine).

Milk mineral components are substantially different from those of blood serum, which means that the mammary gland plays an active role in this process. Mineral composition in milk is relatively constant even it can present observable slight variance. Calcium and phosphate appear to be higher in protein-rich milk in such as that of the Frisian. Variation also exists during the different stages of lactation. Colostrum is rich in mineral components, which decrease gradually until it reaches normal milk concentrations. The concentrations of sodium, calcium and magnesium, are the first to decrease while there is the progressive increase of potassium (Gaucheron, 2005). In the last months of lactation however there is a high concentration of phosphorus and chlorine. Changes in mineral component load may also occur if there is mastitis, especially for sodium and chloride concentrations. Instead, nutrition should not affect the mineral content. Deficiencies tend, over time, to reduce milk production, but the saline composition tends to remain constant at the expense of mineral reserves.

Mineral salts in milk, are present in true solution and colloidal solution. This is linked to the structural elements of the milk in a dispersed or colloidal form (proteins and fats). The two forms are in moving equilibrium that is, if there is a decrease of the soluble salts, a part of the colloidal salts tends to go into solution and conversely. This distinction regards especially salts taking part in the caseins micelle formation. Some mineral salts

incorporated in the colloidal phase are found in milk at a higher concentration to their water solubility (phosphates). Others minerals are found in milk at the soluble state. Ions of potassium, sodium and chlorine are diffusible whereas calcium, inorganic phosphate and magnesium are bound to the casein micelles. Although 1/3 of calcium, half of inorganic phosphate, 2/3 of magnesium and over 90% of citrate are in the aqueous portion of milk (Gaucheron, 2005).

In cow's milk, salt concentration is on average about 0.80 to 0.85% and in particular calcium has values comprised between 0.9 to 1.38 (g/kg), while phosphorous has values between 0.75-1.7 (g/kg). In the serum of curdled milk, instead, calcium is present in proportions of around 0.48 (g/kg) and phosphorus 0.53 (g/kg).

Calcium and phosphorus are present in milk in different forms. Calcium is divided into:

- ✓ ion Ca (10% of the total Ca);
- ✓ soluble Ca (25% bound to soluble calcium citrates and phosphates although these to a lesser amount because less soluble);
- ✓ colloidal Ca (20% bond with casein and 45% bound to phosphorus in apatite bridges).

Phosphorus, however, is divided into organic (20% as colloidal P linked to serine amino acid and 15% as emulsion of fat linked to membranes) and inorganic (25% as colloidal in apatite bridges of casein 40% as calcium phosphate soluble).

Calcium and phosphorus are in equilibrium with each other, but this balance can be changed by pH and milk temperature (Salvatori del Prato, 1998). The variation in phosphorous equilibrium does not include all forms present, but it is restricted only to inorganic forms. In fact, phosphorus esterified to serine, is not modified either by acidification or by heating. The pH reduction of milk causes the passage in the soluble fraction of the casein of milk due to the loss, on the part of micelles, of contents of micellar calcium phosphate, magnesium and few citrate. This occurs at pH value of 5.2. The calcium remains present until pH 3.5, beyond which solubilizes (Le Graete, et al., 1993). These changes are irreversible. Heating as well as cooling damages the milk's ability to be converted into cheese.

Excessive heating causes a lowering of ionic calcium, instead increasing colloidal form, until arriving in extreme cases to a precipitation of citrate and calcium phosphate. These changes are irreversible at temperatures above 120 degree. Low temperature causes casein demineralization because of calcium and phosphorus colloidal decrease in favor of soluble phase, with negative influence on micelles mineralization, less aggregation and less curd firmness (Gaucheron, 2005). Alterations, if not drastic, however, are partially reversible, in fact, milk that is chilled and then heated before coagulation, regains a normal reactivity to rennet.

In addition to pH and temperature, also chelators or sodium chloride may change the balance of minerals in milk. Chelators are substances, (citrates, oxalate) which if present in addition, cause the reduction of micellar calcium phosphate and accordingly the structure of micelle, with an increase of calcium and inorganic phosphorus in the aqueous fraction of

milk. This is a negative effect on the yield, because micelles, if too small, can result in longer coagulation times.

Addition of NaCl causes a decrease in pH and then a rise in Ca^{2+} concentration. The increase in ionic strength causes an increase in the dissociation of the ion pairs and consequently hydration of the micelles increases. The ion-salt balance in milk are very complex, but their alterations especially regarding calcium and phosphorus, are of major importance in the operations preceding the cheese manufacturing itself. A more or less demineralized curd will be more or less consistent and capable of retaining water and fat. For example a soluble calcium reduction causes a series of consequences milk processing, such as: increase of times of yield, increase of firming times, increase of clot fragility, decrease of spontaneous bleeding of the clot.

7. Animal nutrition and milk composition

Changing milk composition through breeding and nutrition strategies has been the main focus for the dairy sector in many parts of the world. Nutritional approaches have distinct effects on milk composition such as: ration type, administration mode, energy concentration and energy level of ration, quantity, quality and fiber length, type, processing of cereals, use of protected substances (oils, fats, calcium salts), etc.

A number of comprehensive reviews examining the impact of nutrition, including the significant role the rumen plays in the extensive metabolism of dietary on milk composition have been published (Chilliard, et al., 2004; Lock, et al., 2004a; Lock, et al., 2004b; Palmquist, et al., 2005).

Ration composition varies during the year, depending on the season, which determines a different concentration of the various milk components. Therefore, different feeding strategies may influence nutritional, sensory and technological quality of milk, which have an impact on dairy production, especially on cheese production.

Specialized breeding of dairy cows is based on intensive type models that provide for the use, in a confined environment, of high-energy concentration rations even if it is not rare to see extensive and semi-extensive breeding systems based on free grazing of animals. Proper management of extensive farming dairy cow, requires knowledge of quantity and quality of pasture ingested by the animal. Unlike confined environment where animals receive controlled rations, the variables to take into consideration increase especially when pasture is the exclusive source of animal food. The ingested amount of grazing by cows, is the main limiting factor in extensive farming (Leaver, et al., 1968). The voluntary intake is regulated mainly by physical mechanisms, such as rumen space and using only grazing often animals are unable to satisfy their energy needs (Balch, et al., 1965). Ingestion gradually increases with the improvement of forage digestibility and reaches the higher limit when there is grass that has the highest digestibility (Freer, 1981).

Pastures are an important source of food for cattle during the period of availability and can often be the most appetizing form of forage with a great nutritional value. Pasture utilization varies according to several factors:

- ✓ *Vegetative stage of forage species* affects primarily the amount of forage obtained and the nutritional value. The nutritional value of forage species, in dry matter, is highest at young stages of development and remaining high until the beginning of flowering then decrease more or less quickly depending on the botanical species. Climatic and pedological conditions also can accelerate or retard the maturity stage and the aging phenomena of the plants (Bittante, et al., 1990). In young grasses, dry matter protein content is 20-23% of crude protein, which is considerably higher than the protein content of the plants at the time of full flowering and maturation (8-13%). Young grasses, in addition, contain little crude fiber (18-22% on dry matter) represented by almost pure cellulose while mature fodder contains 30-35% of crude fiber. With the grass' maturation, as well as to a gradual increase of cellulose content, lignification occurs, which causes a progressive decrease in the digestibility of all nutrients, particularly of crude fiber. Considering the soluble carbohydrates content, mainly sugars, there is a variability in relation to species

(higher in grasses and less in legume species) and vegetative stage, which ranges from 3-4% to 30% of dry matter. Young grasses are also rich in vitamins, especially carotenes and water soluble vitamins complex. A diet based on good pasture provides animals with all the necessary vitamins, with the exception of vit. D (Bittante, et al., 1990).

- ✓ *Floristic composition*: natural grazing is constituted by a large number of herbaceous species, variously assorted, depending of the adaptability of each in function of climatic and pedological conditions. Chemical composition, nutritional properties and palatability are specific to each species, therefore, the pasture nutritional value depends strictly on its floristic composition. Sometimes, plants with high nutritional value which grow in the presence of other plants that are toxic to the animal (Bittante, et al., 1990).
- ✓ *Climatic conditions*: temperature and rainfall affect the pasture quality and quantity affecting both the flora composition, that the development of each plant (Gusmeroli, 2004).
- ✓ *Pedological factors*: are mainly related to chemical and structural soil composition, also influences qualitative and quantitative flora composition (Gusmeroli, 2004).
- ✓ *Management*: grazing techniques can be free (wild, semi-wild, free) and controlled. In the first one, animals do not have (or limited) movement restrictions grazing. The second one includes rationed grazing systems, rotations, where the herds are confined. In free grazing, animals choose where and how to eat, while controlled grazing implies the adoption of a grazing plan (Gusmeroli, 2004).

Natural pastures are always characterized by the presence of many spontaneous species, each of which has specific biological growth cycles. The natural pastures' floristic composition varies both within and between forage seasons (Licitra, et al., 2001).

Pastures are generally considered important both from a naturalistic point of view (for the large variety of species that contain) and from the nutritional point of view. Wild plants significantly influence milk characteristics.

The Ibleo territory has always been characterized by presence of many small farms, often family-run, with a limited number of animals whose diet is essentially made from pasture. The Iblei pastures are characterized by presence of many spontaneous species. It has been shown the presence of more than 100 wild species belonging to 26 different botanical families; some of which (about 30%) presents a percentage of biomass, estimated during the whole season forage, less than 0.5% (Licitra, et al., 1995).

7.1 Influence of pasture on milk composition

Influence of animal feeding on milk quality has been widely studied. The possibility to change milk chemical composition by altering pasture composition has been demonstrated. An important aspect is related to technological properties for processing into cheese. Relationship between milk quality and processed product becomes even more

important if referred to PDO products to medium and long maturation. Here, the changes to the raw material must be reduced if not forbidden and therefore milk defects can be reflected and amplified in cheese (Coulon, et al., 1995; Verdier, et al., 1995).

Animal feeding effect on fat, protein and lactose depends on the choice of nutritional strategy, characteristics, quality and hygiene of the food. Lactose is the main regulator of osmotic glandular secretion, it depends only minimally to nutrition because it is synthesized from blood glucose. Its fluctuations are minimal and more related to udder health than diet (Sutton, 1989; De Peters, et al., 1992; Kennelly, et al., 1998).

7.2. Milk proteins and nutrition

Milk protein content is under genetic, food and physiological control and numerous studies have highlighted the importance of these factors. Milk proteins are positively influenced by diet energy concentration and starch level (Sutton, 1989; De Peters, et al., 1992; Kennelly, et al., 1998).

Beauchemin et al. (1997) have reported the origin and effect of starch amount in the ration on milk production and composition. They also indicate that using low ruminal degradability starch, such as corn, increases milk production with improving of protein content.

Diet starch concentration is also be able to alter the relationship between casein fractions (Summer, et al., 2002) increasing αs1- and αs2-cn percentage. Auldist et al.(2000) and Mackle et al. (1999) reported a positive effect of energy availability on bovine casein fractions which, however, is independent of the phenotype for β-Lg and κ-cn. In particular, in a study on grazing cows conducted in spring and summer, Auldist et al. (2000) reported that animals that ingested forage *ad libitum* produced a significantly greater amount of α-cn, β- cn and κ-cn compared to animals that had limited availability of ingestion, regardless of the season.

Studies conducted by Grandison et al. (1984) indicate that changing the rearing management with pasture use there is an increase in milk casein content. Specifically, there is a greater content in αs- and β-cn, while a significant increase in κ-cn was not observed.

Finally, Auldist et al. (2000) reported that cows with different genetic potential, fed at pasture using two experimental schemes, free-grazing and rationed grazing, showed that a higher intake of pasture has a positive effect on milk production, particularly on protein and casein content and in α-, β-, γ- and κ-cn fractions.

Several authors studied the effect of ration energy level, forage/concentrate ratio and reserve carbohydrates on cheese properties (Andrighetto, et al., 1996; Beauchemin, et al., 1997; Kennelly, et al., 1998; Toso, et al., 2002). Results have shown a significant variation of the two main parameters of coagulation (clotting time and curd firmness, a30) in function of those parameter.

7.3 Milk lipids and nutrition

Nutritional effect on milk fat content and on FAs composition is very important. This because the influence of the food on fat content is due both to the presence of long-fiber fodder which favors ruminal fermentation (Sutton, 1989; Piva, et al., 1993; Mansbridge, et al., 1997; Kennelly, et al., 1998); and food integration with lipid sources (Palmquist, et al., 1993; Ashes, et al., 1997; Jones , et al., 2000).

In regards to the fiber effect, it is believed that fat decrease observed with low fodder/concentrates ratio diets is due to the lower production of acetate in rumen and thus in a deficiency in mammary gland of precursors for FAs short and medium chain synthesis. The lower rumen production of acetate would be caused either by a minor presence of fermentable fiber or by a decrease in rumen pH to below 6.2, the limit value to ensure the optimal activity of cellulolytic micro-flora. In support of this, Beauchemin et al. (1997) reported that the decrease of fat concentration, which is observed using high doses of concentrate, can be avoided adding substances with buffer action on ruminal pH.

It was suggested that the reduction of milk fat content may depend on the presence of some conjugated FAs which form intermediate stages during their rumen biohydrogenation (Griinari, et al., 1998). The FAs biosaturation process, of linoleic acid in particular, has been of particular interest for its implications on the presence of CLA in milk and their positive effects on human health. Using vegetable oils in the ration, rich in MUFA and PUFA, is a strategy to change the milk FA profile and improve milk composition for human consumption (Ashes, et al., 1997). Particular interest was generated by the possibility of increasing the FAs content of omega 3 series through the addition of fish oil in the ration (Jones , et al., 2000). This determines a significant increase of PUFAs in milk but can have a contraindication regarding the milk taste. Alternatively, a significant increase in the milk omega-3 content, can be obtained using linseed oil (Ward, et al., 2002). In both cases, the transfer coefficient of long-chain FAs in milk is rather low, probably because of the biosaturation process that takes place in the rumen and the lack of lipoprotein lipase to hydrolyze specific vegetal or animal triglycerides.

Milk CLA content depends on rumen production of vaccenic acid and of the Δ9 desaturase activity. Diets with high amounts of linoleic acid (by using vegetable oils or concentrates starch) reduce ruminal pH and cause an increase in milk CLA (Jones , et al., 2000; Secchiari, et al., 2001).

Even grazing influences the CLA amount in milk and cheese. In particular, when the pastures are lush and rich in PUFA, the biohydrogenation may be activated on an abundant substrate and consequently the production of CLA is also favored (Bauman, et al., 1999). Thus, milk products derived by grazing animals, contain higher levels of biologically active molecules than those derived from animals reared in the barn. Cheese organoleptic properties are strongly related to the type of spontaneous species present in pastures. This is the case for cheese Ragusano PDO and Iblei pastures, where the organoleptic characteristics are strongly influenced by a high number of secondary compounds, the determination of which has allowed the identification of positive relationship that exists between grazing and quality of dairy products. Milk CLA content is five times higher when cows graze during the forage season, than when they are fed in the stable (i.e. with 50% alfalfa silage and corn silage and 50% of concentrates) (Antongiovanni, et al., 2002). Some

researchers have found that increasing the amount of silage administered to cows, increases the milk CLA content, but in spite of this, such a concentration will never reach the values of milk CLA produced by cows fed on pasture (Antongiovanni, et al., 2002).

The interaction between diet and rearing conditions on milk qualitative characteristics is considerable. It is evident the role that the power supply and its management plays in determining also other milk quality characteristics, both for fresh consumption and milk products.

EXPERIMENTAL PART

8. AIM OF THE STUDY

The purpose of this doctoral thesis is to study the effects of genetic polymorphisms at lipogenic loci and their interaction with feeding system on milk production in order to enhance qualitative, technological and nutritional traits of cow milk. The attention given to the interaction between the studied polymorphisms and the feeding system adopted, will allow to assess how farming systems influence the quality of dairy products in Sicilian autochthonous cattle carrying the identified genetic profiles.

The Modicana cows, here analysed, were characterized under the genetic profile for some polymorphisms, described in literature, at four lipogenic genes (ABCG2, DGAT1, STAT5A and SCD1). On all genotyped animals, individual milk yield, gross milk composition (protein, lactose, fat), fatty acids and mineral composition were recorded, in individual samples, in order to evaluate the effect of genetic polymorphisms and interaction with feeding system on the quantitative and qualitative characteristics of milk.

9. MATERIALS AND METHODS

9.1 Animal and farms identification

Initially, inspections in 4 farms were performed in order to select the farms in which to carry out research activities. The farms would have to meet the following requirements:

- ✓ make use of good technical production standards that reflect the typical farming system used in Sicily (semi-intensive and extensive farming using grazing);
- ✓ numerosity of animals raised (since the polymorphisms at milk protein and lipogenic loci are not detectable to the naked eye, it is necessary to have a large test group on which to perform the analysis);
- ✓ availability to scientific collaboration.

Based on the above criteria, two farms were selected from which blood and milk samples were collected to be tested. Feeding trials were also going to be performed in such farms. The chosen companies are:

Farm A: located in Contrada Cilone in Ragusa District (S.T. Ragusa – Chiaramonte Gulfi). The farm is of medium to large size and has diversified its activities between breeding and agro-tourism services. It is located on the Altipiani Iblei to 600 MASL and comprises about 110 cows of Modicana breed in production and its offspring (Figure 13).

Figure 13: Modicana cattle grazing to the Altopiani Iblei (Farm A)

In addition to milk production, the farm is dedicated to cheese manufacture, in particular local products such as Ragusano PDO, provolone and ricotta Ragusana (Figure 14).

Figure 14: Instrument for traditional cheese making and traditional Ragusano PDO cheese

In this farm, the breeding system is semi-intensive type. Feeding consists of the administration of hay in barn, feed and in some hours of daily grazing.

Farm B: located in the Ragusa Municipality in Contrada Donnafugata. The farm is a family business for the production of Modicana cow's milk. The herd consists of about 80 cows in production with its offspring (Figure 15), all enrolled in the Herd Book. The farm is organic certified. The production is aimed at the sale of milk to companies or consortia from the territory for the production of Ragusano PDO cheese.

Figure 15: Modicana cows after milking (Farm B)

The farm's products have long been recognized and protected for its specific organoleptic characteristics, and for their production and aging characteristics.

Breeding is semi-extensive type and the feeding is primarily based on pasture (about 8 hours per day) with daily feed integration in barn.

9.2 Sample collection

9.2.1 Blood collection

A total of 188 blood samples were collected from Modicana cows from both selected farms in the Ragusa province, 118 samples from farm A and 70 from farm B. The farms adopt the semi-extensive (farm A) or the extensive farming system (farm B) characteristic of the Iblei area.

9.2.2 Milk collection

A total of 188 milk sample were collected from the herds of the two selected farms. Milk samples were collected in the morning (5:45 am) and in the evening (4:30 pm) by milking. The collected milk was proportionally mixed for a total of 60 ml and aliquoted into two 15 ml polypropylene tubes for the analytical determination of the composition and in two 2 ml microcentrifuge tubes for the determination of the composition of FAs with the gas chromatography. Aliquots were stored at minus 20 °C until time for carrying out the analysis.

9.3 Genomic DNA extraction

9.3.1 Preparation of Buffy Coat and DNA extraction

The blood samples (10 ml), collected using Na_2EDTA as an anticoagulant, were centrifuged at 3,000 rpm at 4° C for 20 minutes in order to separate leukocytes from red blood cells and plasma. The separated white blood cells were first re-suspended in 7 ml of deionized water, to achieve the lysis of residual erythrocytes (by osmotic shock) and after 45 seconds in 7 ml of NaCl (1.8%) to restore the normal osmotic pressure. The samples were centrifuged again at 3,000 rpm (4° C) to separate the pellet of nucleated cells, which served as the starting matrix for the DNA extraction.

The extraction of DNA from white blood cells it was performed using the EUROGOLD DNA Blood Mini Kit, following the protocol provided by the manufacturer.

This system provides a digestion in the presence of proteinase K, cell lysis and repeated purification steps by filtration on columns containing specific membranes. The columns consist of silica membranes that bind DNA during the purification steps and hold it during subsequent washing steps with ethanol, guaranteeing an effective removal of pollutants.

9.3.2 Determination of yield and quantity

The concentration of extracted DNA was measured by using the NanoDrop 1000 Spectrotometer, a UV-visible spectrophotometer. This instrument quantifies, in a very accurate way, the DNA by measuring the optical density of the sample: the absorbance is done automatically at 260 nm (DNA) and 280 nm (proteins) of wavelength. The concentration can be determined as follows:

DNA concentration (µg/ml) = absorbance$_{260}$ x 50 x dilution factor

The $A_{260/280}$ ratio of pure nucleic acids is about 2.0. If the ratio is appreciably lower in either case, it may indicate the presence of contaminants, such us proteins that absorb strongly at the range 260-280 nm. All DNA samples were brought to a concentration of 30-50 ng/µl.

9.4 PCR-RFLP for loci associate with milk composition

Genomic DNA was amplified in a specific region of the studied loci. Genotypes have been determined using PCR-RFLP method. Each investigate polymorphism caused an amino acid substitution in the protein sequence.

In the Table 13 are described, for each gene analyzed, the polymorphisms investigated and the related amino acid substitution.

Different PCR-RFLP protocols have been performed for each gene. PCR amplifications were performed generally in a 30 µl reaction volume. Primers pairs are reported in the Table 14.

ABCG2 was analysed at the 14th exon according to Cohen-Zinder (2005) and Komisarek et al. (2009). The solution included 30-50 ng/µl of genomic DNA, 1 x PCR buffer, 2 MM of $MgCl_2$, 0.5 units of Taq polymerase (Bioline), 5% of DMSO, 1 µM of forward and reverse primer (Metabion International AG) and 200 µM of each dNTPS (Promega).

DGAT1 was analysed at the 8th exon according to Grisart et al. (2002) and Komisarek et al. (2011). The solution included 30-50 ng/µl of genomic DNA, 1 x PCR buffer, 2 MM of $MgCl_2$, 0.5 units of Taq polymerase (Bioline), 5% of DMSO, 1 µM of forward and reverse primer (Metabion International AG) and 200 µM of each dNTPS (Promega).

STAT5A was analyzed at the 16th exon according to Flisikowski et al. (2004). The PCR mix included 30-50 ng/µl of genomic DNA, 1 x PCR buffer, 2 MM of $MgCl_2$, 0.5 units of Taq polymerase (Bioline), 1 µM of forward and reverse primer (Metabion International AG) and 200 µM of each dNTPS (Promega).

SCD1 was analysed at the 5th exon according to Taniguchi et al. (2004) and Komisarek et al. (2009). The solution included 30-50 ng/µl of genomic DNA, 1 x PCR buffer, 2 MM of $MgCl_2$, 0.5 units of Taq polymerase (Bioline), 5% of DMSO, 1 µM of forward and reverse primer (Metabion International AG) and 200 µM of each dNTPS (Promega).

The amplification reaction was performed in the GeneAmp 9600 Applera Biosystem using the conditions reported in the Table 14.

Amplified fragment were digested for 2 hours at 37°C with 5 units of restriction enzyme (New England BioLabs Inc.) described in the Table 14. Table 14 also reported the digestion products size.

Amplified and digested fragments were subjected to electrophoretic separation in 3% GelRed Nucleic Acid Gel Stain (Biotium) agarose gel (Bio-Rad) then visualized and recorded using the ChemiDoc™ System with Image Lab™ Software (Bio-Rad Laboratories, Inc.).

Table 13: Gene and polymorphism considered in this research

GENE	CHROMOSOME	GENE REGION	ACC. NUM.	SEQUENCE POLYMORPHISM	AMINO ACID SUBSTITUTION	REFERENCES
ABCG2	BTA6	Exon 14	AJ871176	A/C	Y581S	Cohen-Zinder (2005) Komisarek et al. (2009)
DGAT1	BTA14	Exon 8	AY065621	AA/GC	K232A	Grisart et al. (2002) Komisarek et al. (2011)
STAT5A	BTA19	Exon 16	AJ237937	T/C	V686A	Flisikowski et al. (2004)
SCD1	BTA26	Exon 5	AY241932	C/T	A293V	Taniguchi et al. (2004) Komisarek et al. (2009)

Table 14: PCR-RFLP conditions for the analysed polymorphisms

SNP	PRIMERS (5'-3')	AMPLIFICATION REACTION	PCR PRODUCT SIZE (bp)	RESTRICTION ENDONUCLEASE	DIGESTION PRODUCT SIZE (bp)
ABCG2-Y582S	F-AACAGCCTCAGCTCCAGAGAGATAT R-CGGTGACAGATAAGGAGAACATACT*	Cycle 1: 96°C x 30'' Cycle 2-30: 94°C x 30''; **58°C x 30''**; 72°C x 40'' Cycle 31: 72°C x 5'	292	*PstI*	A (Y): 292 C (S): 268, 24
DGAT1-K232A	F-TGCCGCTTGCTCGTAGCTTTGCC R-ACCTGGAGCTGGGTGAGGAACAGC	Cycle 1: 96°C x 30'' Cycle 2-30: 94°C x 30''; **58.5°C x 30''**; 72°C x 40'' Cycle 31: 72°C x 5'	378	*BglI*	AA (K): 282, 96 GC (A): 245, 96, 28
STAT5A-V686A	F-AGCCCTACAGCTCCAATCCT R-GGGTGTACC–CGCTGCTTAG	Cycle 1-34: 94°C x 1'; **61°C x 1'**; 72°C x 1'	281	*MslI*	T (V): 163, 67, 51 C (A): 163, 118
SCD1-A293V	F-GCCCTGTGAGAGTGGAAAATCAGGT R-TCTTGCTGTGGACTGCTGACTTACG	Cycle 1: 96°C x 30'' Cycle 2-30: 94°C x 30''; **60°C x 30''**; 72°C x 40'' Cycle 31: 72°C x 5'	333	*HinPII*	C: 306, 27 T: 333

* an intentional mismatch incorporating the restriction site to a sequence

9.5 DNA sequencing of DGAT1 and SCD1 gene

DNA sequencing was carried out by chain termination method (Sanger, et al., 1977) through the capillary automatic sequencer ABI PRISM® 3130 (Applied Biosystem, Foster City – USA).

The 378 bp and 333 bp corresponding to the polymorphic region of DGAT1 (Exon 8) and SCD1 (Exon 5) were sequenced.

The samples were amplified in two PCR reactions, in a GeneAmp 9600 Applera Biosystem, using the same primers also used in the PCR-RFLP protocol. Amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), in order to eliminate the portions of nucleic acids not involved in amplification, and then eluted in 20µl of pure water. The amplicons taken into consideration have been subjected to sequence amplification using ddNTPs triphosphate labeled with fluorophores (BigDye Terminator, Applied Biosystems). The terminating chains thus obtained were purified with the use of the CENTRES-SEP COLUMNS (Princeton Separation, Inc.) then sequenced.

The output sequences, visualized using the Sequencing Analysis software (Applied Biosystems), were first manually corrected comparing electropherograms obtained with the respective reference sequences (GenBank Acc. Num. AY065621 and AY241932 to DGAT1 and SCD1, respectively) then aligned with the aid of the software CLUSTALX 1.8 (Thompson, et al., 1994).

9.6 Analytical determination of milk composition

Gross composition (yield, fat, protein and lactose) was determined in individual milk samples using an automated Fourier transform infrared absorption spectrophotometric analyser (Combi-foss 6000, Foss Electric, Hillerød, Denmark).

9.7 Gas chromatography (GC) for the determination of the milk fatty acid composition

Individual milk samples were analysed for fatty acids composition determined on freeze-dried samples ground to pass a 1-mm screen. Lipid extraction and esterification were performed as described by Palmquist et al. (2003). Nonadecanoic acid (19:0) was added as internal standard. Fatty acid methyl esters were analysed on a Trace Thermo Finnigan GC equipped with a flame ionization detector, on a 60 m × 0·25 mm i.d. fused-silica capillary column (SP-2340, Supelco, Inc., Bellefonte PA, USA). The temperatures of injector and detector were maintained at 220 °C and 250 °C, respectively. The oven temperature was initially set at 160 °C for 1 min, and then increased at a rate of 3 deg/min to 210 °C for 3 min. Helium was the carrier gas. Split flow was 20 ml/min. Injected volume was 1 µl, split ratio, 17.

9.8 Determination of milk mineral composition

Collected individual milk was also used for mineral composition determination (Na, K, Mg, Ca). Mineral component analysis was carried out using the service of a private lab, MEDISAN S.A.S., Catania.

9.9 Feeding management

On the basis of the genotypes at lipogenic loci, 36 cows in farm A and 38 cows in farm B were selected, at their third to fourth lactation, homogeneous for day of lactation (farm A, 75.3 ± 20.1 days; farm B, 88.7 ± 31.3 days) and milk yield (farm A, 10.1 ± 1.3 kg/d; farm B, 9.0 ± 1.8 kg/d).

Feeding management in the two farms was performed as follows:

Farm A: the cows were fed following a semi-intensive system, consisting in a daily administration of 5 kg of hay, 4-5 kg of concentrate and 2-3 hours of grazing on natural pasture.

Farm B: the cows were fed following a typical extensive system of the Iblean area, consisting in 8 hours on natural pasture and 5-6 kg of hay supplement administrated indoors.

9.10 Statistical analysis

Individual data for milk production and composition (fat, protein, lactose, fatty acid profile, minerals) were analysed using the GLM procedure of SPSS (SPSS for Windows, SPSS Inc., Chicago IL, USA). The analysis included main effect of genotype at each polymorphic locus (STAT5A or SCD1 or DGAT1 loci), feeding system and interaction genotype × feeding system. For each genotype the model used was as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ijk}$$

where;

α_i is the genotype (STAT5A or SCD1 or DGAT1) with 3 levels (respectively for STAT5A or SCD1 or DGAT1: TT, TC, CC; TT, CT, CC; GC/GC, GC/AA, AA/AA),

β_j is the feeding system with 2 levels (semi-intensive and extensive),

$(\alpha \times \beta)_{ij}$ is the interaction between genotype and feeding system,,

ε_{ijk} is the random error.

10. RESULTS AND DISCUSSION

10.1 Analysis of the genetic variability at the lipogenic loci examined

The analysis of genetic variability at lipogenic loci (ABCGG2, DGAT1, STAT5A and SCD1) allowed the identification of the various genotypes in Modicana cows reared in the two farms. The distribution of the individual genotypes, with genotype and allele frequencies, are reported in Table 15, Table 16, Table 17 and Table 18.

10.1.1 Genotype and allele frequencies of ABCG2 gene

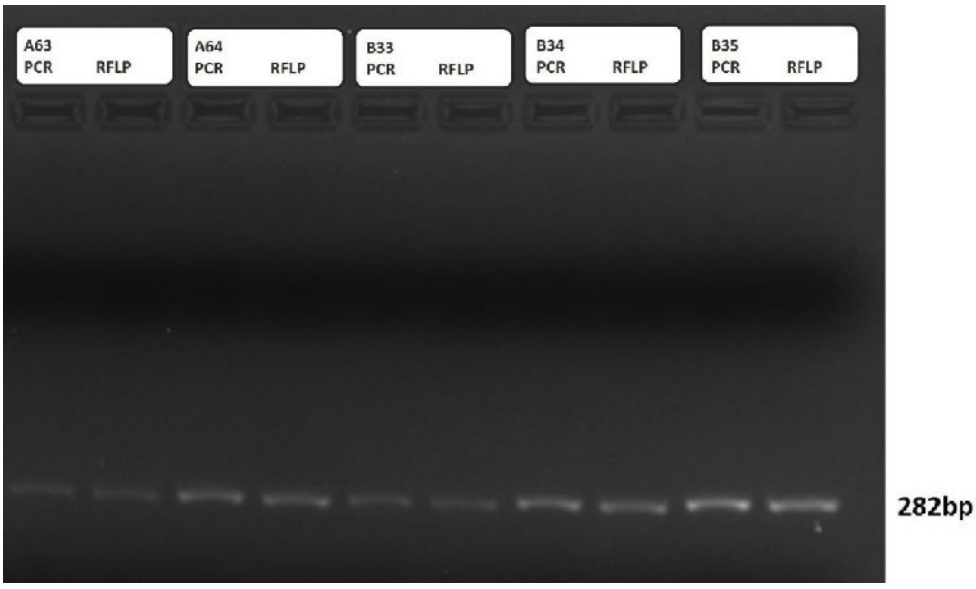
Genotype and allele frequencies at ABCG2 gene, Y581S polymorphism are reported in Table 15. The genotype has been characterized for 188 Modicana breed subjects.

Table 15: Genotype, genotype and allele frequencies at the ABCG2 locus in Modicana breed

		GENOTYPE *FENOTYPE*			**GENOTYPE FREQUENCIES**			**ALLELE FREQUENCIES**	
		AA	Aa	aa	AA	Aa	Aa	A	a
	No.	*SS*	*SY*	*YY*	*SS*	*SY*	*YY*		
FARM A	118	118	0	0	1	0	0	1	0
FARM B	70	70	0	0	1	0	0	1	0
TOTAL	**188**	**188**	**0**	**0**	**1**	**0**	**0**	**1**	**0**

The entire population tested was monomorphic for AA genotype at this locus. Figure 16 shows PCR products and PCR-RFLP pattern visualized by agarose electrophoresis in Modicana cows.

Figure 16: Electrophoresis agarose gel at 3% of the PCR products of the ABCG2 gene and the fragments digested with the enzyme PstI



Different studies have reported that the AA genotype is predominant in several bovine populations analysed, so far. Ateş et al. (2014) found that the ABCG2^{A} allele frequency was 64% in the Turkey indigenous cattle breeds analysed. The A allele frequency was 99% in Polish Holstein-Friesian bulls (Komisarek, et al., 2009) and 98% in Turkey Holstein cows (Yildirim, et al., 2010).

According with Ron et al. (2006), the higher frequency of the A allele suggested that the ABCG2^{A} could be an ancestral allele and that the Y581S substitution occurred after the separation of the *Bos indicus* and *Bos Taurus* lineages.

10.1.2 Genotype and allele frequencies of DGAT1 gene

Genotype and allele frequencies of DGAT1 gene K232A polymorphism are reported in Table 16. The genotype has been characterized for 175 of 188 Modicana breed subjects.

Table 16: Genotype, genotype and allele frequencies at the DGAT1 locus in Modicana breed

		GENOTYPE *FENOTYPE*			**GENOTYPE FREQUENCIES**			**ALLELE FREQUENCIES**	
		GC/GC	GC/AA	AA/AA	GC/GC	GC/AA	AA/AA	GC	AA
	No.	*AA*	*AK*	*KK*	*AA*	*AK*	*KK*	*A*	*K*
FARM A	105	89	13	3	0,85	0,12	0,03	0,91	0,09
FARM B	70	56	14	0	0,80	0,20	0,00	0,90	0,10
TOTAL	**175**	**145**	**27**	**3**	**0,82**	**0,16**	**0,02**	**0,91**	**0,09**

The genotype distribution of the DGAT1 locus in the sample complies with the Hardy-Weinberg equilibrium ($\chi 2$ =1.63 n.s.).

In the Figure 17, the agarose gel electrophoresis of the RFLP analysis of some samples of Modicana bovine are shown.

Figure 17: Electrophoresis agarose gel at 3% of DGAT1 fragments digested with the enzyme BglI

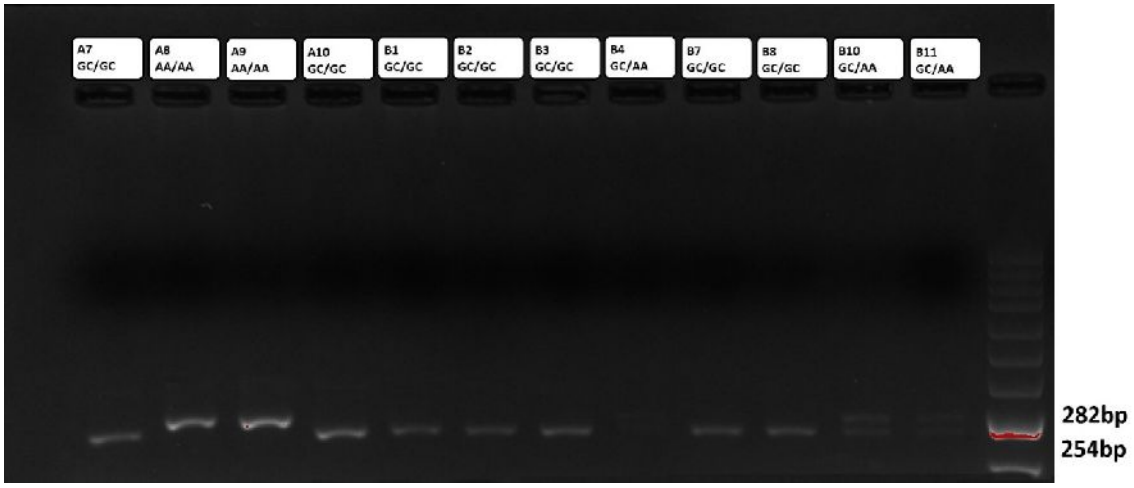


Table 16 shows the genotype at DGAT1 locus individuated in the analysed population. The GC allele (g.10433-10434GC) coding for the amino acid Alanine in position 232, is the most frequent in this sample of Modicana (91%). The AA/AA genotype associated to the Lysine (232K) amino acid is extremely rare in the Modicana sample and absent in farm B. The GC/GC genotype associated to the Alanine amino acid (232A) is instead largely predominant (82%) in the analysed sample of Modicana cows with a frequency of 0.85 and 0.80 in farm A and B respectively. This finding is consistent with those already reported in the main Italian breeds by Scotti et al. (2010) and in the Red Spotted by Lešková et al. (2013).

Other studies confirmed also that the GC allele (232A) is predominant and it was reported in a wide range of *Bos taurus* breeds (Spelman, et al., 2002; Kaupe, et al., 2004; Sanders, et al., 2006; Gautier, et al., 2007; Näslund, et al., 2008). Only in Holstein cows a more balanced distribution between the 2 alleles has been observed (60% for GC and 40% for AA) (Schennink, et al., 2008; Banos, et al., 2008).

Moreover, Gautier et al. (2007) and Conte et al. (2010) suggested that the frequency of the ancestral K allele (AA/AA genotype) is lately increase particularly in Holstein breed due to the pronounced selection in favour of fat content.

Furthermore, Gautier et al. (2007) proposed that K232A mutation may have happened early in the history of cattle domestication and the frequency variation observed reflects the different milk selection objectives in various countries and breeds

10.1.3 Genotype and allele frequencies of STAT5A gene

Genotype and allele frequencies of STAT5 gene V686A polymorphism are reported in Table 17. The genotype has been characterized for 187 of 188 Modicana breed subjects.

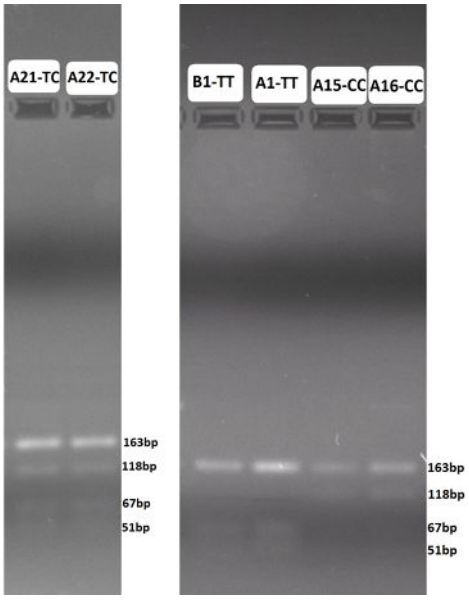
Table 17: Genotype, genotype and allele frequencies at the STAT5A locus in Modicana breed

		GENOTYPE *FENOTYPE*			**GENOTYPE FREQUENCIES**			**ALLELE FREQUENCIES**	
		TT	TC	CC	TT	TC	CC	T	C
	No.	*VV*	*VA*	*AA*	*VV*	*VA*	*AA*	*V*	*A*
FARM A	117	64	33	20	0,55	0,28	0,17	0,69	0,31
FARM B	70	33	27	10	0,47	0,39	0,14	0,66	0,34
TOTAL	**187**	**97**	**60**	**30**	**0,52**	**0,32**	**0,16**	**0,68**	**0,32**

In the Figure 18, the agarose gel electrophoresis of the RFLP analysis of some samples of Modicana bovine are shown.

Table 17 shows the genotype frequency of the polymorphism in exon 16. The TT genotype is more frequent in the population analysed with a frequency of 52% while the CC genotype is found be with a lower frequency in both farms (20% and 10% in farm A and B, respectively). The T allele (g.12743T) coding for the amino acid valine in position 686, is more frequent in this Modicana sample (0.68 vs. 0.32 of C allele). This finding is in line with what observed by Selvaggi et al. (2013) in Jersey Breeds and by Flisikowski et al. (2003b) in different cattle breeds and by Flisikowski et al. (2004) in Polish Friesian.

Figure 18: Electrophoresis agarose gel at 3% of STAT5A fragments digested with the enzyme MslI



The genotype distribution of the STAT5A locus in the sample are not complies with the Hardy-Weinberg equilibrium ($\chi 2$ =13.01 P≤0.001).

10.1.4 Genotype and allele frequencies of SCD1 gene

Genotype and allele frequencies of SCD1 gene A293V polymorphism are reported in Table 18. The genotype has been characterized for 165 of 188 Modicana breed subjects.

Table 18: Genotype, genotype and allele frequencies at the SCD1 locus in Modicana breed

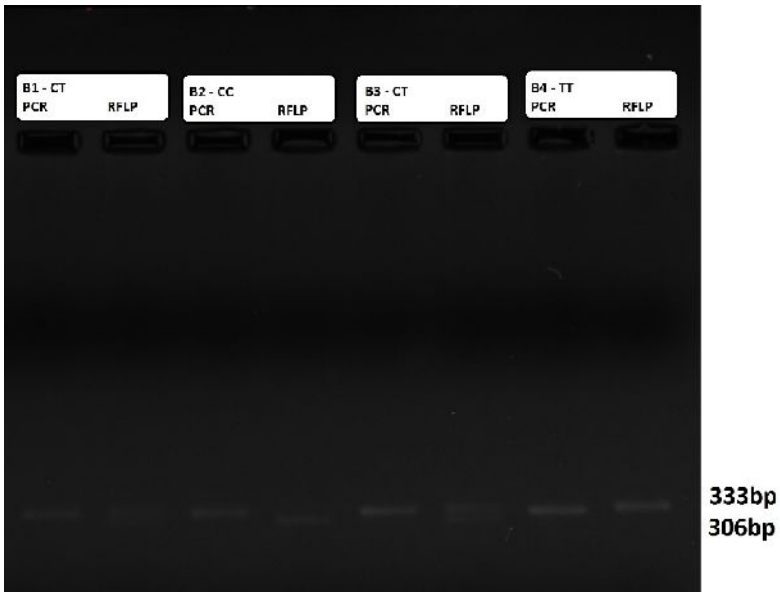
		GENOTYPE *FENOTYPE*			**GENOTYPE F REQUENCIES**			**ALLELE FREQUENCIES**	
		TT	TC	CC	TT	TC	CC	T	C
	No.	*AA*	*AV*	*VV*	*AA*	*AV*	*VV*	*A*	*V*
FARM A	96	81	4	11	0,84	0,04	0,11	0,86	0,14
FARM B	69	22	31	16	0,32	0,45	0,23	0,54	0,46
TOTAL	**165**	**103**	**35**	**27**	**0,62**	**0,21**	**0,16**	**0,73**	**0,27**

The genotype distribution of the SCD1 locus in the sample are not complies with the Hardy-Weinberg equilibrium ($\chi 2$ =35.14 P≤0.001).

In the Figure 19, the agarose gel electrophoresis of the PCR products and subsequent digestion (RFLP) analysis of some samples of Modicana bovine.

Table 18 reports the genotype frequencies observed in the Modicana cows analysed. All three possible genotypes were identified (TT, TC and CC). The TT genotype, associated with the amino acid Alanine (A), is largely predominant in the tested sample (62%). However, significant differences are observed in the distribution of genotypes in the two farms. In farm A, the TT genotype is the most frequent (84% vs. 32% of the farm B) while in farm B heterozygote genotype (TC) is mainly frequent (45% vs. 32% of the farm A).

Figure 19: Electrophoresis agarose gel at 3% of the PCR products of the SCD1 gene and the fragments digested with the enzyme HinP1I



The T allele (g.10329T) coding for the amino acid Alanine in position 293, is the most frequent in this sample of Modicana (73%), unlike of what happens in Polish Friesian where the allele frequency was 0.26 for T and 0.74 for C (Kulig, et al., 2013). In Italian Brown cows, Conte et al. (2010) reported the higher frequency of the C allele (293V, 82%) in contrast with Milanesi et al. (2008) (293A 75%).

The T allele (293A) was the most frequent in different breeds: in Holstein-Friesian, Schennink et al. (2008) described a T frequency of 0.73; in Japanese Black cattle, Taniguchi et al. (2004) found a T frequency of 0.59; in Italian Friesian, Mele et al. (2007) reported the higher frequency of T allele (0.57); in Jersey, Moioli et al. (2007) reported a frequency of 0.94 for the T allele. The higher T allele frequency was also observed in a sample of Italian Friesian bred in the province of Ragusa (0.74 - Marletta, 2015. Personal communication).

The variations observed in the frequencies of wild-type allele for SCD1 (293V) may reflect the different selection objectives followed as regard milk composition in different breeds and country (Gautier, et al., 2007).

10.2 Sequencing

A sub-sample of 12 subjects distributed in the two farms has been sequenced at DGAT1 and SCD1 loci in order to confirm the observed polymorphisms and for SNP searching. The amplified traits were of 378 and 333 bp for DGAT1 and SCD1, respectively. The obtained sequences were aligned with those in the database (Acc. No. AY065621 and AY241932, respectively) but no new SNPs polymorphism has been identified.

10.3 Milk yield and composition

The data collected from the physical-chemical analyses from individual milk, sampled during the trials, are shown in the tables below where the analysis of variance of milk yield and composition for the different genotypes at DGAT1, STAT5A and SCD1 loci and their interaction with feeding system are shown.

Evaluation of the results does not allow to find substantial differences in terms of yield and composition within the different phenotypes in milk of Modicana cows examined for DGAT1, STAT5A and SCD1 in both systems, extensive and semi-intensive. Differences in milk yield and composition can probably be due to the differences in the diets of cows in the two farms rather than to genetic differences.

Feeding system effects:

Animals rearing in a semi-intensive system showed a higher milk yield and protein content compared with animals fed with pasture, according with Sutton (1989), De Peters et al. (1992) and Kennelly et al. (1998). Lactose resulted higher in animals rearing in an extensive system. It is known that lactose fluctuations are minimally affected by nutrition and its difference may be more related to the rearing system than the diet itself (Sutton, 1989; De Peters, et al., 1992; Kennelly, et al., 1998). Surprisingly, fat was not affected by feeding system. In fact, the low use of concentrate in the extensive system, compared to the semi-intensive system, should have resulted in a fat increase, due to higher fiber content in the diet. This is not occurred, in our experimental conditions, probably due to a high individual variability of fat content in milk.

10.3.1 Analysis of the milk yield and composition with different genotype detected in the DGAT1 locus

Table 19 reports the effect of milk yield and composition in relation to the identified genotypes at DGAT1 locus, the feeding system and their interaction. Considering that for AA/AA genotype (232K), there were found only 3 animals (genotype frequency of 0.02), only GC/GC and GC/AA are considered in the analysis and discussion.

DGAT1 genotype and feeding system interaction:

Identified genotypes showed no statistically significant effects on milk yield, in contrast with results reported by several authors (Winter et al. 2002; Grisart et al. 2004; Scotti et al. 2010; Pirzad et al. 2014) which reported a considerable increase on milk production associate AA allele (232K). At the same time, protein and fat contents were not affected by AA allele as described by Thaller et al. (2003), Weller et al. (2003), Scotti et al. (2010), Lešková et al. (2013) and Pirzad et al. (2014) in different breeds.

The strong increase of milk fat content in AA allele reported in literature was not detected in our experimental conditions.

Table 19: ANOVA of milk yield and composition: effects of DGAT1 K232A polymorphism, feeding system and their interaction

	DGAT1 GENOTYPE						
	AA/GC - *KA*		GC/GC - *AA*		SIGNIFICANCE (P)		
	E	I	E	I	G	A	GxA
Milk (kg/d)	9.41	9.37	8.08	10.3	0.858	0.005	0.247
Fat (%)	3.93	3.91	3.95	4.06	0.381	0.781	0.698
Protein (%)	3.44	3.84	3.59	3.70	0.451	0.001	0.064
Lactose (%)	4.64	4.30	4.57	4.40	0.976	<0.001	0.169

^E and I are referred to the farm feeding system: E - Extensive (farm B), I - semi-Intensive (farm A)

Komisarek et al. (2004) described an increase on fat, linked to the AA allele in Jersey in agreement with Spelman et al. (2002) for New Zealand Jersey. They suggested that this difference depends by the selection objective that for many years was directed on the milk yield increasing while the New Zealand Holstein put more emphasis on the fat and then on the milk protein content. For this reasons their population present a higher frequency of the AA allele (232K).

10.3.2 Analysis of the milk yield and composition with different genotype detected in the STAT5A locus

Table 20 reports the effect of milk yield and composition in relation to the identified genotypes at STAT5A locus, the feeding system and their interaction.

STAT5A genotype and feeding system interaction:

Identified genotype showed no statistically significant effects on the milk yield and composition in our Modicana samples. On the contrary, Flisikowski et al. (2004) and Selvaggi et al. (2013) suggested a beneficial influence of the allele C on milk yield, protein and fat content.

Table 20: ANOVA of milk yield and composition: effects of STAT5A V686A polymorphism, feeding system and their interaction

	STAT5A GENOTYPE								
	CC - *AA*		TC - *VA*		TT - *VV*		SIGNIFICANCE (P)		
	E	I	E	I	E	I	G	A	GxA
Milk (kg/d)	9.08	10.5	8.20	9.90	7.49	10.2	0.343	<0.001	0.404
Fat (%)	3.43	4.05	4.13	4.09	3.96	3.99	0.159	0.157	0.216
Protein (%)	3.45	3.78	3.64	3.65	3.52	3.77	0.942	0.004	0.101
Lactose (%)	4.57	4.45	4.58	4.38	4.60	4.35	0.904	0.001	0.687

^E and I are referred to the farm feeding system: E - Extensive (farm B), I – semi-Intensive (farm A)

Interaction between identified genotypes and feeding system on milk yield and composition did not showed a statistically significant effect.

10.3.3 Analysis of the milk yield and composition with different genotype detected in the SCD1 locus

Table 21 reports the effect of milk yield and composition in relation to the identified genotypes at SCD1 locus, the feeding system and their interaction.

SCD1 genotype and feeding system interaction:

SCD1 A293V polymorphism showed no statistically significant effects on milk yield and composition, except for protein.

Table 21: ANOVA of milk yield and composition: effects of SCD1 A293V polymorphism, feeding system and their interaction

	SCD1 GENOTYPE								
	CC - *AA*		CT - *AV*		TT - *VV*		SIGNIFICANCE (P)		
	E	I	E	I	E	I	G	A	GxA
Milk (kg/d)	8.78	9.78	8.12	11.5	8.87	10.6	0.786	0.005	0.449
Fat (%)	3.86	4.02	4.03	4.34	3.95	4.11	0.695	0.311	0.955
Protein (%)	3.53	3.75	3.67	4.11	3.52	3.72	0.044	0.001	0.503
Lactose (%)	4.70	4.25	4.57	4.34	4.50	4.35	0.744	<0.001	0.071

^E and I are referred to the farm feeding system: E - Extensive (farm B), I – semi-Intensive (farm A)

Kulig et al. (2013) described a significant increase of fat and protein content associate to the presence of T allele. Other researchers obtained the same results in different breeds. Komisarek et al. (2009) reported an association between the T allele and a highest fat content, Alim et al. (2012) with a highest milk, fat and protein yield and Macciotta et al. (2008) with an increment on daily yield and protein content.

In our conditions, only protein content showed a significant trend ($P<0.05$) according to Macciotta et al. (2008), Alim et al. (2012) and Kulig et al. (2013). Protein content resulted higher in heterozygous animals than in homozygous. This significant difference between homozygous and heterozygous genotypes is difficult to associate to any biological mechanism. The individual variability for this parameter within each genotype could probably explain this significant result. Also Kulig et al. (2013) reported a higher EBV for milk, fat and protein yield in heterozygous animals.

Interaction between identified genotypes and feeding system on milk yield and composition did not showed a statistically significant effect.

10.4 Milk fatty acids composition

The data collected from the analyses of the individual cows in lactation sampled during the trial are shown in the tables below where the analysis of variance of the FAs composition for the different genotypes at DGAT1, STAT5A and SCD1 loci and their interaction with the feeding system are shown.

On average, milk collected from animals reared in an extensive system (E), showed a different FAs composition compared to the milk collected from the semi-intensive system

(I). Table 22 shows FAs composition, grouped by category, of milk collected from the 2 farms. It is also reported the content of CLA and of FAs in milk that resulted highly influenced by the feeding system.

Table 22: Milk fatty acids composition collected from the 2 farms

Fatty Acid	Unit	Extensive system	Semi-Intensive system
SFA[7]	% of tot FAs	62.8	67.6
MUFA[8]	% of tot FAs	25.3	23.2
PUFA[9]	% of tot FAs	4.5	3.0
OBCFA[10]	% of tot FAs	4.6	5.1
trans[11]	% of tot FAs	2.7	1.1
C16	g/100g FAs	26.95	31.56
C18:1*c9*	g/100g FAs	20.43	17.86
C14	g/100g FAs	10.54	12.08
CLA*c9t11*	g/100g FAs	0.92	0.42

^Extensive system (farm B), Semi-Intensive system (farm A)

As predictable, milk from animals reared in an extensive system with an exclusive use of natural pasture presented a lower content of SFA and a higher fraction of MUFA, PUFA and *trans* FAs compared to milk collected from animals reared in a semi-intensive system. This is in line with what reported in numerous studies (Palmquist, et al., 1993; Rego, et al., 2004; Khanal, et al., 2008; Soyeurt, et al., 2008; Heck, et al., 2008; Elgersma, 2015).

Feeding system affected also OBCFA that resulted lower in milk from extensive system. In general, feeding pasture increases milk BCFA (branched-chain FAs) and decreases linear OCFA (odd-chain FAs) and what observed in our milk sample results in agreement with Cabrita et al. (2007) and Khanal et al. (2008).

As regards CLA, the average content is 0.92 and 0.42 g/100g of total FAs in the milk collected from extensive and semi-intensive system, respectively. It is higher in animal feeding with natural pasture according to Elgersma et al. (2004) and Oldemiro et al. (2016).

It has been also calculated the FAs desaturation indices for the medium-chain (C14 and C16), for the long-chain (C18, CLA) and the total FAs index. The desaturation index represents the concentration of the unsaturated product proportional to the sum of the unsaturated product and the saturated precursor. Different study reported a significant variation in milk UFA content among breeds and cow fed with the same diet and suggested that also genetic variation may play an important role in the composition of milk FAs (Beaulieu, et al., 1995; DePeters, et al., 1995; Lock, et al., 2003).

Feeding system variation depends on the dietary supply of FAs. Natural pasture certainly offer nutrient rich in PUFA compared with conserved forages (Dewhurst, et al.,

[7] Sum of C4, C6, C8, C10, C12, C14, C16, C18, C20, C21, C22, C24
[8] Sum of C12:1, C14:1c9, C16:1c9, C18:1c6, C18:1c9, C18:1c11, C20:1c11
[9] Sum of C18:2, CLAc9t11, C18:3gamma, C18:3alfa, C20:3, C20:4, C20:5n3, C22:4, C22:5n3
[10] Sum of C15iso, C15, C15anteiso, C17iso, C17, C17anteiso
[11] Sum of trans FAs C14:1t, C16:1t, C18:1t9, C18:1t11

2001). Supply PUFA through the diet determines a decrease of *de novo* synthesis FAs and an increase of long-chain FAs in milk fat (Chilliard, et al., 2001).

The effects of the feeding system is almost always evident on the phenotypic means for FAs composition which are always favorable in animals bred with semi-extensive system. This means a higher content of CLA and ω-3 in milk that is translate in better antioxidative properties, anticancerogenic, antiatherogenic and antidiabetic linked to CLA and antiatherogenic properties, antithrombotic and anti-inflammatory related to long-chain PUFA ω-3. At the same time, the content of *trans* and medium-chain FAs decrease. The negative effects of *trans* and medium-chain FAs on milk nutritional value are known: if their content is high, they increase the level of total and LDL-cholesterol and consequently the risk of atherosclerosis. Moreover, the ω-3/ω-6 ratio increase with a general improving of the milk composition and reduction the risk of disease.

10.4.1 Analysis of the fatty acid composition with different genotypes detected in the DGAT1 locus

Table 23 shows milk FAs composition in relation to the identified genotypes at DGAT1 locus, the feeding system and their interaction. Considering that for AA/AA genotype (232K), there were found only 3 animals (genotype frequency of 0.02), only GC/GC and GC/AA are considered in the analysis and discussion.

DGAT1 effects on milk FAs composition:

Identified genotypes showed no statistically significant effects on the composition of FAs except for C22:4 ($P<0.05$). This acid is generally highly sensible to statistical analysis, probably because of its very low individual variability. It is very difficult to find a scientific and biological explanation for this significant result other than a mathematical artefact result.

Contrarily of what reported by Schennink et al. (2007) and Kala et al. (2016), increasing of SFA content in animals with heterozygous genotypes (AA/GC) and at the same time a minor content of MUFA, PUFA, OBCFA and *trans* FAs compared to animals with genotype GC/GC, has not been detected. In our sample of Modicana, lysine amino acid (genotype GC) do not seems associated with upper proportion of SFA and C16:0 and a lower proportion of C14, C18:1*c9* and CLA as suggested by Schennink et al. (2007) and Kala et al. (2016).

Schennink et al. (2007) found that GC allele was associated with a smaller fraction of C16:0 and a larger fraction of unsaturated C18 and CLA. This can be explained by increased activity of DGAT1 or by an altered specificities of the enzyme. As described, it cannot be demonstrated in our milk samples because no statistically association has been found.

Effects of the interaction between DGAT1 K232A polymorphism and feeding system:

Effects of DGAT1 K232A polymorphism on milk FAs composition resulted similar in extensive and semi-intensive system. However, significant DGAT1 by feeding system interaction was found for some FAs, especially CLA, the content of which in milk seem to be effected either by genotype or feeding system. Genetic role in regulation on CLA in milk has been supposed by Bauman et al. (2001), White et al. (2001) and Secchiari et al. (2003) that reported a great individual variability of CLA content in animals fed with the same diet.

Interaction between identified genotypes and feeding system on the milk FAs composition did not show a significant effect in almost all the FAs considered (Table 23). A significant effect ($P<0.05$) was observed only for C4, CLA and C22:4.

In our condition, a significant interaction between genotype and feeding was evident for both CLA content and CLA ratio that resulted higher in animals with GC/GC genotype fed with pasture, but not in animals fed with hay and concentrate. This is in line with Duchemin et al. (2012) that described in Netherland Holstein-Friesian an increase of unsaturated C18 e UFA in animals with GC/GC genotype associated with the consumption of fresh forage compared to animals fed with silage. Also Schennink et al. (2008) reported a higher CLA index associated with GC/GC genotype. As suggested by Duchemin et al. (2012), this may be explained assuming that feeding system could have resized the polymorphisms effects due to the different composition of the FAs present in the pasture.

The increase of CLA content in the milk of animals with GC/GC genotypes fed with pasture can be explained by greater activity or specificity of DGAT1 in fixation of FA to the third position of triacylglycerol (Cases, et al., 1998; Palmquist, 1996). On the contrary, Bouwman et al. (2011 and 2012) reported a higher content of CLA in animals with GC/GC genotype (232A) but fed with silage.

Butyric acid (C4:0) was affected by the interaction between the genotype and the feeding system. In our conditions, heterozygous animals fed with natural pasture (extensive system) showed a higher content of this SFA. This result is not confirmed in the literature. Bouwman et al. (2012) did not find a difference in the C4:0 content in milk collected from animals fed with pasture and silage. This significant difference between homozygous and heterozygous genotypes and the interaction with the feeding system is difficult to associate to any biological mechanism. The individual variability for this parameter within each genotype and the FAs composition of the pasture could probably explain this result.

No significant effect was found for desaturation index related to the interaction between genotype and feeding system except for CLA ratio, already discussed. Schennink et al. (2008) found a lower C14 and C16 index and higher C18 and CLA index associate with the GC/GC genotype. On the contrary Conte et al. (2010) in Italian Brown reported an increase of all desaturation indices associate to AA/GC genotype with the exception of the total index. In our conditions milk FAs ratio and total index did not show a genetic component and the differences may be attributed only to the composition of the pasture.

Regarding the CLA index, in our experimental condition, it resulted associated with the interaction between the genotype and the feeding system. It was higher in animals with GC/GC genotype fed with natural pasture in agreement with Schennink et al. (2008). This significant finding demonstrates that the DGAT1 K232A polymorphism may affect the CLA ratio by both genetic and feeding system.

Table 23: ANOVA of milk FAs: effects of DGAT1 K232A polymorphism, feeding system and their interaction

	DGAT1 GENOTYPE						
	AA/GC - *KA*		GC/GC - *AA*		SIGNIFICANCE (P)		
FATTY ACID	E	I	E	I	G	A	GxA
C4	2.393^{d}	1.760^{a}	2.112^{c}	1.892^{b}	0.517	0.000	0.042
C6	1.956	1.489	1.701	1.527	0.263	0.000	0.083
C8	1.358	1.092	1.183	1.085	0.214	0.004	0.172
C10	3.011	2.699	2.789	2.723	0.602	0.247	0.448
C11	0.298	0.386	0.274	0.334	0.163	0.001	0.472
C12	3.291	3.585	3.161	3.476	0.474	0.091	0.951
C121	0.166	0.255	0.160	0.237	0.222	0.000	0.549
C14	10.424	11.814	10.557	11.805	0.834	0.003	0.864
C15*iso*	0.387	0.456	0.343	0.455	0.453	0.000	0.222
C14:1*t*	0.039	0.011	0.010	0.009	0.097	0.166	0.194
C15a*nteiso*	0.770	0.814	0.765	0.838	0.943	0.045	0.615
C14:1*c9*	0.710	1.329	0.666	1.103	0.203	0.000	0.254
C15	1.586	1.588	1.550	1.611	0.414	0.410	0.439
C16	27.452	32.069	26.633	30.439	0.266	0.000	0.668
C17 *iso*	0.441	0.483	0.447	0.499	0.453	0.006	0.786
C16:1*t*	0.101	0.034	0.121	0.034	0.297	0.000	0.124
C17*anteiso*	0.384	0.714	0.489	0.711	0.095	0.000	0.074
C16:1*c9*	1.263	1.662	1.180	1.584	0.116	0.000	0.983
C17	0.813	0.692	0.804	0.713	0.766	0.007	0.702
C18	10.251	8.047	10.026	8.556	0.690	0.000	0.409
C18:1*t6*	0.112	0.096	0.130	0.088	0.611	0.000	0.088
C18:1*t9*	0.201	0.208	0.214	0.221	0.259	0.536	0.990
C18:1*t11*	1.928	0.840	2.223	0.743	0.659	0.000	0.090
C18:1*c6*	0.711	0.488	0.950	0.539	0.211	0.001	0.298
C18:1*c9*	20.380	19.822	20.500	21.226	0.373	0.917	0.430
C18:1*c11*	0.510	0.476	0.508	0.505	0.769	0.492	0.555
C18:2	1.693	1.678	1.637	1.652	0.316	1.000	0.825
C20	0.219	0.225	0.201	0.234	0.648	0.220	0.416
C18:3*gamma*	0.017	0.028	0.020	0.024	0.853	0.002	0.130
C20:1*c11*	0.170	0.062	0.145	0.064	0.679	0.000	0.382
C18:3*alfa*	1.057	0.518	1.170	0.504	0.383	0.000	0.162
CLA*c9t11*	0.805^{b}	0.476^{a}	0.989^{c}	0.427^{a}	0.254	0.000	0.010
C21	0.057	0.040	0.055	0.040	0.976	0.002	0.776
C22	0.096	0.105	0.110	0.104	0.783	0.917	0.534
C20:3	0.067	0.093	0.078	0.079	0.592	0.050	0.074
C20:3*c11,14,17*	0.019	0.013	0.025	0.011	0.590	0.000	0.084
C20:4	0.100	0.174	0.229	0.156	0.732	0.997	0.325
C24	0.070	0.070	0.065	0.071	0.831	0.622	0.646
C20:5*n3*	0.086	0.044	0.093	0.036	0.905	0.000	0.142
C22:4	0.055^{d}	0.044^{c}	0.028^{a}	0.037^{b}	0.005	0.908	0.047
C22:5*n3*	0.203	0.008	0.183	0.007	0.727	0.000	0.463
C14 ratio	0.779	1.441	0.729	1.197	0.091	0.000	0.258
C16 ratio	1.309	1.714	1.225	1.635	0.364	0.000	0.974
C18 ratio	22.397	22.314	22.555	23.741	0.353	0.517	0.457
CLA ratio	1.241^{a}	1.075^{b}	1.437^{c}	1.021^{b}	0.190	0.000	0.024
Desat. Index	0.316	0.306	0.321	0.320	0.367	0.628	0.658

^E and I are referred to the farm feeding system: E - Extensive (farm B), I – semi-Intensive (farm A)
^{a,b,c,d} values within row without a common superscript letter are significantly different (P<0.05)

10.4.2 Analysis of the fatty acid composition with different genotypes detected in the STAT5A locus

Table 24 shows milk FAs composition in relation to the genotypes identified at STAT5A locus, the feeding system and their interaction.

STAT5A effects on milk FAs composition:

Identified genotypes showed no statistically significant effects on milk FAs composition except for C17*iso*, C18:1*t11*, C18:1*c9*, C24:0, C22:4 and *trans* ($P<0.05$). Moreover, C15*iso*, C17*anteiso*, C17, C18:3*alfa* and C20 show an almost significant trend ($P<0.10$).

Results obtained regarding the effects of the STAT5A V686A polymorphism on OBCFA, are in contrast with Pegolo et al. (2016) that in Italian Brown reported a relevant influence of this polymorphism on some BCFA. They described a negative influence of the T allele on C17*anteiso* while in Modicana cows it was observed a positive correlation (0.61 vs. 0.58 g/100g of FAs in animals with TT and CC genotype, respectively).

Pegolo et al. (2016) also reported that C17*iso* seems to be positively affected by the C allele. In our condition, the content of C17*iso* results significant affected by the genotype. It was higher in homozygous animals and lower in the heterozygous animals. This significant difference between homozygous and heterozygous genotypes is difficult to associate to any biological mechanism. The individual variability for this parameter within each genotype could probably explain this significant result.

OBCFA content resulted higher in milk collected from animals with TT genotype compared with CC genotype (6.7% vs. 4.8% for TT and CC genotype, respectively ($P<0.05$).

Animals with TT genotype showed a higher content of C18:1*t11*, C18:1*c9*, C18 ratio and a lower C24:0, C22:5*n3*, C20:5*n3* and *trans* content compared to animals with CC genotype. In particular *trans* vaccenic acid (C18:1*t11*) showed, in both the feeding system, an increasing trend from the CC genetic group to the TT genetic group. These results cannot be confirmed by other researchers because literature includes very few studies regarding the relationship between STAT5A V686A polymorphism and milk FAs composition.

Effects of the interaction between STAT5A V686A polymorphism and feeding system:

No relevant interaction effects have been found between identified genotypes at STAT5A locus and feeding system on the milk FAs composition.

Table 24: ANOVA of milk FAs: effects of STAT5A V686A polymorphism, feeding system and their interaction

	STAT5A GENOTYPE								
	CC - *AA*		CT - *AV*		TT - *VV*		SIGNIFICANCE (P)		
FATTY ACID	E	I	E	I	E	I	G	A	GxA
C4	2.040	2.000	2.190	1.950	2.230	1.870	0.960	0.067	0.538
C6	1.630	1.620	1. 800	1.600	1.780	1.520	0.779	0.077	0.555
C8	1.120	1.140	1.270	1.140	1.230	1.090	0.568	0.175	0.581
C10	2.496	2.917	3.033	2.845	2.816	2.697	0.358	0.800	0.305
C11	0.258	0.314	0.295	0.359	0.276	0.333	0.242	0.003	0.980
C12	2.856	3.693	3.411	3.658	3.138	3.471	0.286	0.005	0.398
C12:1	0.153	0.236	0.165	0.248	0.162	0.234	0.602	0.000	0.801
C14	10.224	12.401	11.069	12.237	10.186	11.633	0.118	0.000	0.599
C15*iso*	0.369	0.473	0.323	0.442	0.374	0.449	0.098	0.000	0.345
C14:1*t*	0.010	0.009	0.015	0.012	0.018	0.008	0.859	0.437	0.737
C15*anteiso*	0.743	0.901	0.728	0.803	0.806	0.814	0.113	0.002	0.059
C14:1*c9*	0.657	0.955	0.699	1.227	0.666	1.060	0.263	0.000	0.506
C15	1.549	1.625	1.516	1.603	1.597	1.584	0.695	0.201	0.377
C16	27.966	30.562	26.826	32.287	26.437	30.445	0.379	0.000	0.425
C17*iso*	0.453	0.503	0.423	0.470	0.462b	0.498c	0.049	0.003	0.887
C16:1t	0.113	0.031	0.125	0.029	0.109	0.038	0.731	0.000	0.086
C17*anteiso*	0.437	0.720	0.426	0.669	0.501	0.716	0.075	0.000	0.621
C16:1*c9*	1.319	1.339	1.110	1.674	1.232	1.604	0.733	0.001	0.077
C17	0.894	0.701	0.760	0.659	0.813	0.725	0.067	0.000	0.442
C18	10.116	8.958	9.745	7.984	10.347	8.642	0.233	0.000	0.849
C18:1*t6*	0.121	0.096	0.119	0.079	0.132	0.090	0.192	0.000	0.639
C18:1*t9*	0.200	0.221	0.222	0.213	0.205	0.219	0.801	0.414	0.405
C18:1*t11*	1.796	0.628	2.210	0.654	2.228e	0.869c	0.031	0.000	0.295
C18:1*c6*	0.697	0.560	1.045	0.490	0.833	0.500	0.297	0.000	0.122
C18:1*c9*	21.83[b]	19.61[a]	19.05[a]	19.54[a]	21.14[b]	21.34[b]	0.021	0.472	0.343
C18:1*c11*	0.491	0.495	0.513	0.466	0.511	0.519	0.495	0.619	0.455
C18:2	1.744	1.701	1.574	1.616	1.680	1.694	0.221	0.943	0.886
C20	0.226	0.253	0.184	0.215	0.216	0.228	0.079	0.097	0.772
C18:3*gamma*	0.021	0.026	0.019	0.025	0.018	0.025	0.686	0.012	0.962
C20:1*c11*	0.157	0.058	0.160	0.052	0.141	0.066	0.968	0.000	0.459
C18:3*alfa*	1.053	0.501	1.137	0.441	1.179	0.543	0.095	0.000	0.384
CLA *c9t11*	0.838	0.393	0.975	0.384	0.955	0.467	0.215	0.000	0.311
C21	0.064	0.043	0.052	0.038	0.054	0.039	0.392	0.000	0.818
C22	0.112	0.118	0.118	0.096	0.095	0.103	0.461	0.774	0.327
C20:3	0.074	0.088	0.068	0.084	0.081	0.079	0.818	0.197	0.405
C20:4	0.105	0.158	0.343	0.164	0.110	0.156	0.112	0.664	0.145
C24	0.085[d]	0.074[c]	0.058[a]	0.066[b]	0.067[b]	0.71[c]	0.033	0.959	0.346
C20:5*n3*	0.100	0.036	0.090	0.034	0.090	0.039	0.594	<0.001	0.497
C22:4	0.057[d]	0.040[c]	0.021[a]	0.038[c]	0.030[b]	0.037[c]	0.011	0.566	0.027
C22:5*n3*	0.187	0.005	0.176	0.006	0.198	0.010	0.446	<0.001	0.704
trans	2.230	0.980	2.690	0.990	2.690	1.220	0.046	<0.001	0.254
C14 ratio	0.722	1.033	0.763	1.328	0.731	1.151	0.275	0.000	0.496
C16 ratio	1.367	1.382	1.152	1.725	1.279	1.657	0.723	0.001	0.077
C18 ratio	23.992	21.855	21.014	22.061	23.207	23.816	0.027	0.829	0.274
CLA ratio	1.305	1.065	1.425	0.981	1.388	1.011	0.970	0.000	0.385
Desat. Index	0.330	0.298	0.305	0.300	0.328	0.321	0.065	0.131	0.538

^E and I are referred to the farm feeding system: E - Extensive (farm B), I – semi-Intensive (farm A)
[a,b,c,d] values within row without a common superscript letter are significantly different (P<0.05)

10.4.3 Analysis of the fatty acid composition with different genotypes detected in the SCD1 locus

Table 25 shows milk FAs composition in relation to the genotypes identified at SCD1 locus, the feeding system and their interaction.

SCD1 effects on milk FAs composition:

Identified genotypes showed no statistically significant effects on the composition of FAs except for C8:0 ($P<0.05$).

Mele et al. (2007) in Italian Holsten described an association between milk of animals with alanine amino acid in position 686 (TT genotype) and greater content of UFA than milk of CC genotype (with valine amino acid). As reported cannot be statistically confirmed in our sample.

Regarding desaturation index, Schennink et al. (2008) in Dutch Holstein-Friesian reported the highest indices for C16, C18 and CLA associate to the A allele and Mele et al. (2007), Moioli et al. (2007) and Schennink et al. (2008) found a negative effects of the C allele in C14 index. Modicana animals with CC genotype showed a lower C16, C18, CLA and desaturation index and a higher C14 but with no statistically significant so the association found in literature cannot be confirmed in our conditions.

Various researches reported also a positive association among the T allele of SCD1 and C14 index that represents the best indicator for the SCD activity Mele et al. (2007), Schennink et al. (2008), Kgwatalala et al. (2009). In this study, the association with C14 index with the T allele has not been confirmed. Probably, in our sample, SCD1 is not the unique genetic factor that controls the desaturation activity in line with Conte et al. (2010).

Schennink et al. (2008) found that the presence of C allele in Dutch Holstein-Friesian increased the content of C10:0, C12:0, C14:0 C16:0 and CLA*c9t11*. In our experiment a similar but not significant trend was found for these FAs. Results in the effect of the A293V polymorphism for the medium-chain FAs are also confirmed by Moioli et al. (2007) in Piemontese e Valdostana and by Bouwman et al. (2011) in Dutch dairy cattle.

Effects of the interaction between SCD1 A293V polymorphism and feeding system:

Interaction between identified genotypes and feeding system on the milk FAs composition did not showed a statistically significant effect in almost all the FAs considered. A statistically significant effect ($P<0.05$) was observed only for the contents of C4, C6, C8, C18:1*t11* and C20:3.

In milk from cows reared in the extensive system, C4:0, C6:0, C8:0 and C18:1 *t11* acids gradually decreased from CC to heterozygous to TT genotype ($P<0.05$). The same trend was not found in the semi-intensive system. This may be explained assuming that feeding system could have resized the polymorphisms effects due to the different composition of the FAs present in the pasture as suggested by Duchemin et al. (2012).

As reported in not in concordance with Duchemin et al. (2012) that found a negative association on the interaction between C allele and feeding system with the C18:1*t11* content in milk in fed with pasture compared with animals fed with silage.

Effects of SCD1 A293V polymorphism on milk FAs composition is similar in extensive and semi-intensive system. Significant SCD1 by feeding system interaction was found for some FAs of which in milk seem to be affected either by genotype or feeding system.

Table 25: ANOVA of milk FAs: effects of SCD1 A293V polymorphism, feeding system and their interaction

	SCD1 GENOTYPE								
	CC - *VV*		CT - *VA*		TT - *AA*		SIGNIFICANCE (P)		
FATTY ACID	E	I	E	I	E	I	G	A	GxA
C4	2.446^{e}	1.847^{a}	2.249^{d}	1.877^{a}	1.984^{b}	2.093^{c}	0.692	0.051	0.023
C6	2.05^{d}	1.56^{a}	1.802^{c}	1.61^{ab}	1.58^{ab}	1.66^{b}	0.194	0.084	0.022
C8	1.45^{c}	1.14^{ab}	1.18^{b}	1.15^{ab}	1.10^{a}	1.12^{ab}	0.016	0.138	0.031
C10	3.397	2.802	2.857	2.887	2.525	2.882	0.120	0.747	0.052
C11	0.326	0.385	0.279	0.386	0.259	0.337	0.064	0.005	0.818
C12	3.721	3.677	3.193	3.808	2.897	3.661	0.142	0.068	0.169
C12:1	0.182	0.259	0.155	0.278	0.156	0.238	0.159	0.000	0.506
C14	11.165	12.195	10.488	12.237	10.181	12.031	0.559	0.014	0.740
C15*iso*	0.352	0.489	0.347	0.451	0.361	0.439	0.621	0.000	0.381
C14:1*t*	0.031	0.012	0.012	0.010	0.010	0.009	0.222	0.374	0.434
C15*anteiso*	0.764	0.865	0.749	0.790	0.771	0.808	0.639	0.115	0.619
C14:1*c9*	0.739	1.323	0.673	1.186	0.659	1.041	0.219	0.000	0.602
C15	1.584	1.646	1.538	1.680	1.549	1.576	0.540	0.183	0.729
C16	26.980	30.391	26.983	33.680	26.548	31.136	0.659	0.001	0.679
C17*iso*	0.415	0.507	0.447	0.456	0.460	0.478	0.797	0.079	0.157
C16:1*t*	0.127	0.030	0.116	0.029	0.107	0.037	0.701	0.000	0.250
C17*anteiso*	0.429	0.780	0.504	0.627	0.436	0.676	0.427	0.000	0.132
C16:1*c9*	1.026	1.499	1.195	1.771	1.309	1.606	0.203	0.001	0.578
C17	0.732	0.721	0.803	0.654	0.849	0.686	0.599	0.038	0.235
C18	9.981	8.697	10.209	7.652	9.984	8.301	0.890	0.006	0.780
C18:1*t6*	0.108	0.089	0.131	0.079	0.133	0.085	0.466	0.000	0.237
C18:1*t9*	0.211	0.197	0.208	0.189	0.213	0.219	0.555	0.558	0.715
C18:1*t11*	2.49^{e}	0.74^{ab}	2.15^{d}	0.66^{a}	1.91^{c}	0.80^{b}	0.121	0.000	0.038
C18:1*c6*	1.019	0.449	0.818	0.387	0.894	0.547	0.763	0.002	0.634
C18:1*c9*	18.190	20.268	20.663	18.909	21.684	20.186	0.204	0.722	0.175
C18:1*c11*	0.506	0.471	0.521	0.457	0.494	0.498	0.972	0.402	0.716
C18:2	1.495	1.703	1.663	1.442	1.742	1.651	0.343	0.721	0.154
C20	0.187	0.247	0.194	0.210	0.221	0.214	0.844	0.286	0.222
C18:3*gamma*	0.016	0.028	0.017	0.021	0.022	0.021	0.811	0.116	0.082
C20:1*c11*	0.129	0.062	0.137	0.047	0.182	0.060	0.294	0.000	0.322
C18:3*alfa*	1.119	0.467	1.153	0.410	1.142	0.516	0.731	0.000	0.778
CLA*c9t11*	0.969	0.384	0.943	0.363	0.915	0.430	0.967	0.000	0.640
C21	0.047	0.043	0.052	0.037	0.061	0.036	0.799	0.035	0.220
C22	0.128	0.109	0.092	0.097	0.102	0.096	0.361	0.682	0.847
C20:3	0.06^{a}	0.10^{d}	0.07^{ab}	0.09^{cd}	0.08^{bc}	0.07^{a}	0.808	0.247	0.011
C20:3*c11,14,17*	0.020	0.011	0.024	0.009	0.024	0.011	0.761	0.001	0.701
C20:4	0.294	0.172	0.117	0.142	0.210	0.151	0.762	0.627	0.881
C24	0.058	0.071	0.063	0.063	0.073	0.066	0.721	0.802	0.373
C20:5*n3*	0.085	0.036	0.089	0.038	0.098	0.035	0.580	0.000	0.426
C22:4	0.045	0.044	0.031	0.036	0.029	0.035	0.171	0.652	0.895
C22:5*n3*	0.174	0.010	0.184	0.002	0.196	0.006	0.857	0.000	0.727
C14 ratio	0.806	1.202	0.737	1.283	0.724	1.122	0.646	0.000	0.874
C16 ratio	1.064	1.565	1.240	1.824	1.359	1.639	0.249	0.001	0.492
C18 ratio	20.035	22.797	22.711	21.380	23.860	22.658	0.168	0.946	0.131
CLA ratio	1.359	0.928	1.390	0.914	1.394	0.980	0.795	0.000	0.949
Desat. Index	0.292	0.311	0.320	0.291	0.337	0.307	0.237	0.347	0.156

^E and I are referred to the farm feeding system: E - Extensive (farm B), I – semi-Intensive (farm A)
^{a,b,c,d,e} values within row without a common superscript letter are significantly different (P<0.05)

10.5 Milk mineral composition

Mineral composition (Na, K, Mg, Ca) of the milk samples, collected during the lactation of individual cows, is reported in Table 26.

Table 26: Mineral composition of the milk collected from the Modicana cows rearing in the 2 farms exanimated in relation of different genotype (average value ± SD; 110 samples for Farm A (I) – semi-intensive system, 70 samples for Farm B (E) – extensive system)

MINERAL	UNIT	FARM A (I)	FARM B (E)
Na	mg/l	50.43 ± 1.35	51.60 ± 2.76
K	mg/l	150.95 ± 1.89	151.85 ± 2.90
Mg	mg/l	4.10 ± 0.40	4.44 ± 0.73
Ca	mg/l	114.93 ± 8.51	112.46 ± 9.59

Milk collected from animals reared in an extensive system showed a higher content of Na, K and Mg. The content of Ca was instead higher in milk collected from animals reared in a semi-intensive system. As reported is partially in accord with Gabryszuk et al. (2008) which observed a higher concentration of Ca, Na, Mg in milk collected from cows reared in an intensive system while K content was similar in both system, extensive and intensive.

The differences found in mineral concentration depend on various factors: production system, nutritional status of the animals, lactation phase, environmental, vegetative stage of the pasture, chemical soil composition, individual variability, etc. (Gabryszuk, et al., 2010; Zamberlin, et al., 2012).

The analysis of variance of mineral composition for the different genotypes at DGAT1, STAT5A and SCD1 has been calculate and it showed in Table 27, Table 28 and Table 29.

Table 27: ANOVA of mineral constituent in milk with DGAT1 (marginal means expected at a confidence interval of 95% and P<0.05)

	DGAT1 GENOTYPE		
	GC/GC	AA/AA	SIGNIFICANCE (P)
Na	51.2	52.4	0.332
K	151.9	152.8	0.241
Mg	4.6	4.1	0.071
Ca	113.1	111.5	0.726

^36 GC/GC, 12 GC/AA

Table 28: ANOVA of mineral constituent in milk with STAT5A (marginal means expected at a confidence interval of 95% and P<0.05)

	STAT5A GENOTYPE			
	TT	TC	CC	SIGNIFICANCE (P)
Na	51.5	51.1	51.8	0.912
K	151.2	153.4	153.1	0.022
Mg	4.5	4.6	4.2	0.513
Ca	113.3	110.7	112.9	0.660

^32 TT, 25 TC, 8 CC

Table 29: ANOVA of mineral constituent in milk with SCD1 (marginal means expected at a confidence interval of 95% and P<0.05)

	SCD1 GENOTYPE			
	TT	TC	CC	SIGNIFICANCE (P)
Na	51.2	51.6	51.2	0.599
K	151.7	152.3	152.5	0.169
Mg	4.6	4.4	4.4	0.663
Ca	111.4	113.7	112.0	0.265

^24 TT, 25 TC, 14 CC

Milk mineral composition is not statistically different among the genotypes considered at the DGAT1, STAT5A and SCD1 loci. For DGAT1 K232A polymorphism, Bovenhuis et al. (2016) found a no significant effects of this polymorphism on Ca content in Danish Holstein Friesian, Danish Jersey and Dutch Holstein Friesian.

The only significant effect was reported for the content of K that resulted associate with STAT5A V686A polymorphism and a difference between the homozygous and the heterozygous was reported. The heterozygous animals (TC genotype) showed a higher content of K. This significant difference between homozygous and heterozygous genotypes is difficult to associate to any biological mechanism. The low individual variability for this parameter within each genotype could probably explain this significant result.

These results cannot be confirmed by other researchers because literature includes very few studies regarding the relationship between polymorphism and milk mineral composition. In our condition, the milk mineral composition may be explained by indirect rather than direct effects of DGAT1, STAT5A and SCD1 polymorphisms.

11 CONCLUSION

In the present study, it was investigated for the first time in Modicana breed, novel information about polymorphisms at key genes involved in lipid metabolism. Moreover the interaction between these polymorphisms and the feeding system was analyzed. In particular, it was studied how the presence of pasture in the diet can interfere with the effects of polymorphisms at the studied loci on milk yield, gross composition and fatty acid profile.

Animals have been characterized under genetic profile for the lipogenic polymorphisms at ABCG2, DGAT1, STAT5A and SCD1 loci.

The entire population resulted monomorphic at ABCG2 Y581S polymorphism while for the other loci, a genetic variability has been reported. Modicana cattle showed an unbalanced allele distribution of 3 genes with the higher allele frequency present at 91% for GC (232A) at DGAT1, 68% for T (686V) at STAT5A and 73% for T (293A) at SCD1.

As expected, feeding systems influenced significantly milk yield and protein content that resulted higher in animals fed with natural pasture but unexpectedly fat content was not affected probably because of the high individual variability. However, when feeding systems are considered in interaction with the studied polymorphisms at lipogenic loci, results do not allow to find significant differences within the different phenotypes in milk from Modicana cows in both systems, extensive and semi-intensive. Therefore variation on milk yield and composition can be probably due to the different diets of cows in the two farms rather than to genetic differences.

Identified genotypes for DGAT1 K232A polymorphism in Modicana did not show effect on milk yield and composition even if it is known that the AA allele (232K) identified at this locus generally increase milk fat composition, especially in terms of medium-chain fatty acids. As well, the polymorphism identified at STAT5A locus did not affect the milk yield and composition in our experimental condition though it seems that the substitution of C to T at STAT5A gene can be considered as a causative mutation for milk production traits. Finally, in our conditions, SCD1 A293V polymorphism affected milk protein content but heterozygous animals showed a higher protein content in both system, extensive and semi-intensive. The difference between homozygous and heterozygous genotypes is difficult to associate to any biological mechanism and probably this could be explained as an individual variability for this parameter within each genotype.

Despite, in our experimental conditions, it has demonstrated any effects of the interaction between polymorphisms at lipogenic loci and feeding systems, such approach needs more evidence in literature and lays the foundation for expanding this field of research.

As predictable, feeding system widely affected milk fatty acids composition. Animals reared in an extensive system with an exclusive use of natural pasture showed a lower content of SFA and a higher fraction of MUFA, PUFA and *trans* fatty acids. It is known and extensively studied that milk derived by grazing animals, contains higher levels of biologically active molecules than milk derived from animals reared in a more intensive system. Natural pasture is a rich source of PUFA that determines a decrease of *de novo* synthesis of fatty acids and an increase of long-chain fatty acids in milk. This can be easily

translated in a better composition of milk with an increase of the nutritional and healthy value.

Then, considering the interaction between the feeding systems and the polymorphisms at lipogenic loci on milk fatty acids composition, results showed that some fatty acids were significant affected by this interaction.

The interaction between DGAT1 K232A polymorphism and feeding systems on milk fatty acids, affected the content of C4, CLA and C22:4. The increase of CLA content and CLA ratio in the milk of animals with GC/GC genotypes fed with pasture seems to demonstrate the greater activity or specificity of DGAT1 in fixation of fatty acid to the third position of triacylglycerol linked to the presence of the GC allele. Also C4:0 resulted higher in heterozygous animals (AA/GC genotype - KA) fed with pasture but this is difficult to associate to any biological mechanism and probably the individual variability for this parameter within each genotype and the fatty acids composition of the pasture could probably explain this result.

Genotypes identified for STAT5A V686A polymorphism affected the composition of odd and branched fatty acids, the content of which resulted higher in animals with TT genotype. The content of C17*iso* results affected by the genotype. It was higher in homozygous animals and lower in the heterozygous animals. This significant difference between homozygous and heterozygous genotypes is difficult to associate to any biological mechanism. Moreover animals with TT genotype showed a higher content of C18:1*t11*, C18:1c9, C20:4 and C22:5n3 and a lower C20:5n3 content. In particular *trans* vaccenic acid (C18:1*t11*) showed, in both the feeding system, an increasing trend from the CC genetic group to the TT genetic group. These results cannot be confirmed by other researchers because literature includes very few studies regarding the relationship between STAT5A V686A polymorphism and milk fatty acids composition. This finding could be considered as a preliminary knowledge on the influence of this polymorphism on milk fatty acids composition and more studies are needed in order to confirm the effects on the OBCFA. When the interaction between the identified genotype and the feeding system is considered, no relevant effect have been detected.

Genotypes identified at SCD1 A293V polymorphism affected only the content of C8:0. When the interaction between identified genotypes and feeding systems on the milk fatty acids composition was considered, the effects were observed for the contents of C4, C6, C8, C18:1*t11* and C20:3. In milk from cows reared in the extensive system, C4:0, C6:0, C8:0 and C18:1*t11* acids gradually decreased from CC to heterozygous to TT genotype. The same trend was not found in the semi-intensive system. This may be explained assuming that feeding system could have resized the polymorphisms effects due to the different composition of the fatty acids present in the pasture.

Finally, mineral composition have been considered in relation to the identified genotype. The only significant effect was reported for the K content that is associated with STAT5A V686A polymorphism and a difference between the homozygous and the heterozygous was reported. The heterozygous animals (TC genotype) showed a higher content of K even if a low variability was reported within each genotype that could probably explain this significant result.

Further studies are required to describe in detail the relationships between the polymorphisms at lipogenic loci and the feeding systems in Modicana cows and in different

breeds in order to better understand the variations in milk production traits, within and between the breeds, not only through a genetic or nutritional point of view but as a dynamic interaction between these two aspects.

The association between polymorphisms at lipogenic loci and their interaction with feeding system on milk production traits could usefully be used in gene-assisted selection programs for the milk production improvement. Moreover, this could be also used for the characterization of local breeds and products linked with them in order to valorize the local biodiversity.

The research lays the foundation to address the issue from a nutrigenomics perspective in order to assess how the farming systems used in Sicily influence milk quality on the basis of already identified genetic polymorphisms.

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