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Master's Degree in

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COMPARATIVE STUDY OF FATTY ACIDS PROFILE IN A1 AND A2 MILK, FRESH AND RIPENED CHEESE

Thesis Type: Experimental

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Riassunto

I latticini sono ampiamente consumati in tutto il mondo e sono componenti essenziali della dieta in quanto forniscono importanti macro e micronutrienti. Nel tempo e' nata una controversia tra la salubrita' del latte A1 e quella del latte A2. La differenza tra le due varianti del latte è nella composizone della beta-caseina.

Il presente lavoro di tesi si sviluppa nell'ambito del progetto I-Milka 2- PSR MARCHE 2014 e ha come scopo la valutazione dell'impatto della biodiversità genetica dei bovini da latte con genotipo A2A2 (a2) della beta-caseina contro gli altri genotipi (a1) sul profilo degli acidi grassi del latte e dei rispettivi formaggi (freschi o stagionati). Durante il nostro studio abbiamo analizzato 26 campioni di ogni tipo di latte (58 campioni in totale), oltre a formaggi freschi e stagionati A1 e A2**. I risultati mostrano che ilcontenuto di acidi grassi saturi (SFA) dei latti A1 variava dal 58,6 al 72,5%, mentre nei latti A2 era compreso tra il 55,2 e il 69,9%. Gli acidi grassi omega 3 e omega 6 variavano in un intervallo di 4,3-7,0 e 4,2-7,2 rispettivamente per il latte a1 e a2. Gli acidi grassi trans erano presenti in un valore medio del 3,5 e 3,4% rispettivamente per i latti a1 e a2. L'analisi dei componenti principali (PCA) mostra che il profilo degli acidi grassi non varia tra il latte a1 e a2 (varianza totale del 38,14%) e il formaggio fresco (variazione totale del 46,17%).**

Il formaggio stagionato ha un basso contenuto di acido butirrico (C4:0) e acido caproico (C6:0) ma non esiste alcuna relazione sul suo effetto sulla beta-caseina.

Summary

Dairy products are widely consumed all over the world and is essential to the diet of several millions of people worldwide as it provides important macro and micronutrients. There has been controversy regarding A1 and A2 milk for many years. The question A2 milk is healthier than A1 milk is still open. The difference between the two milk variants is only a single amino acid deviation of beta-casein.

The framework of this thesis is included in the project I-Milka 2- PSR MARCHE 2014. The aim of this thesis is to evaluate the impact of the genetic biodiversity of dairy cattle with A2A2 (a2) genotype of beta-casein against the other genotypes (a1) on the fatty methyl esters profile of milk and cheese (fresh or during ripening). During our study we analzed 26 samples of each type of milk (58 samples in total) across one and half year, as well as A1 and A2 fresh and ripened cheese. The results shows that the **SFA of a1 milks ranged from 58.6 to 72.5 %, while from 55.2 to 69.9% for a2 milks. Omega 3 and omega 6 fatty acids varied in a range of 4.3-7.0 and 4.2-7.2 for milk a1 and a2, respectively. Trans fatty acids were present in an average value of 3.5 and 3.4% for a1 milks and a2 milks, respectively**. **Principal component analysis (PCA) show that the fatty acid profile did not vary between a1 and a2 milks (total variance of 38.14%) as well as fresh cheese (total variance of 46.17%).**

The ripened cheese has low butyric acid (C4:0) and caproic acid (C6:0) but there is no relation exist on its effect on beta-casein.

1. Introduction

1.1 Milk

Every day, billions of people worldwide consume dairy products, which are essential for living a healthy life and the overall development of the body. Milk is a highly nutritious, white liquid food, secreted by the mammary glands of mammals. Cows' milk consumption varies worldwide, with an average of 10–212 kg per person per year. Milk is an excellent complex food as it is an essential source of nutrients and micronutrients. Milk contains 18 of 22 essential nutrients, including a variety of bioactive peptides and fatty acids such as caseins, whey proteins, milk polar lipids (MPL), α-linolenic acid (ALA), conjugated linoleic acids (CLA), palmitic acid (16:0), lactose and other minor constituents (i.e., calcium, phosphorous, magnesium, and vitamin D) which have a significant impact on human metabolism and health (Kaskous, 2020). The average composition of milk is reported in Table 1.

Table 1. The average composition of different milk lactating animals (Kaskous, 2020).

Lactating animals	Dry Matter %	Lactose %	Protein %	Fat %	Ash %
Cow	13	4.9	3.4	4.0	0.7
Goat	13.2	4.1	3.4	3,5	0.8
Sheep	19.3	4.8	5.5	7.4	0.9
Buffalo	17.2	4.8	4.2	7.6	0.8
Camel	10.6	4.0	2.3	3.5	0.7

1.1.1 A1-milk and A2-milk

Milk proteins are a heterogeneous group of polymeric compounds that have a wide range of different molecular structures and properties. They occur as caseins, whey proteins, fat globule membrane proteins, enzymes, minor proteins, and nitrogen compounds (Kamiński et al., 2007) (Table 2).

Table 2. Compositional protein quality in cow's milk, according to Barth and Behnke (1997).

Protein components	Protein subclasses	Concentrations (g/kg)
Caseins	α_{s1}-casein (A, B, C, D, E)	10.3
	α_{s2}-casein (A, B, C, D)	2.7
	β-casein (A1, A2, A3, B, C, D, E, F, G, H^1, H^2, I).	9.6
	k-casein (A, B)	3.5
	γ-Casein	0.8
Whey proteins	α-Lactalbumin (A, B, C)	1.2
	β-Lactoglobulin (A, B, C, D, E, F, G)	3.4
	Serum albumin	0.4
	Immunoglobulins (A, G1, G2, M, E)	0.7
	Lactoferrin	0.1
	Transferrin	0.1
	Other minor proteins	0.1
Enzymes	Lysozyme, lactoperoxidase, and 60 others	traces
Peptide hormone	Prolactin, growth hormone, insulin growth factor (IGF)	traces
Non-protein-nitrogen	Urea, creatine, creatinine, peptide, uric acid, hippuric, orotic acid, free amino acids, nucleic acids.	1.1

Cow's milk contains a total of 80% of casein protein (Jenness, 1961). The second significant fraction of casein is beta-casein which ranges up to 45% (Cavallo et al., 1996). β-casein has several variants that are genetically determined (Farrell et al., 2004). Farrell et al. (2004) reported that β-casein existed as three polymorphs A, B, and C. Moreover, it was discovered that β-casein A could be separated into three additional variants named β-casein A1, A2, and A3 (Aschaffenburg, 1963).

1.1.1.1 The biochemical structure of A1 β-casein and A2 β-casein

Beta-casein consists of 209 amino acids (Brooke-Taylor et al., 2017) and the only difference between A1 and A2 types of milk is at the 67th position. In the A1 type of milk, histidine is present at the 67th position, so when ingestion of A1 milk occurs, enzymatic cleavage takes place in the gut beta-casomorphin-7 (BCM-7) is produced, which is responsible for gastric disturbances. At the same time, proline is present in the A2 type of milk, hindering the cleavage so that BCM-7 is not released (Figure 1 and 2).

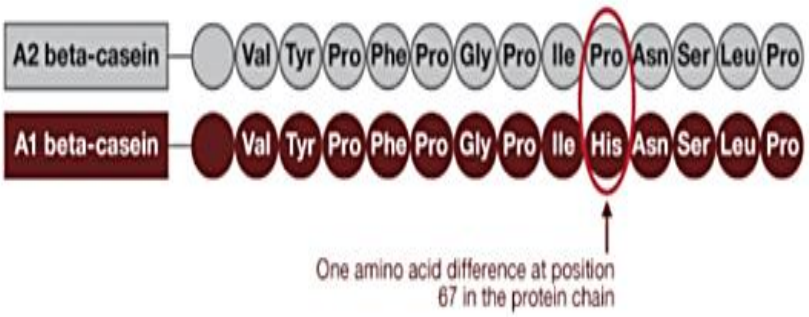

Figure 1. Protein chain showing amino acids in A1 and A2 β-casein.

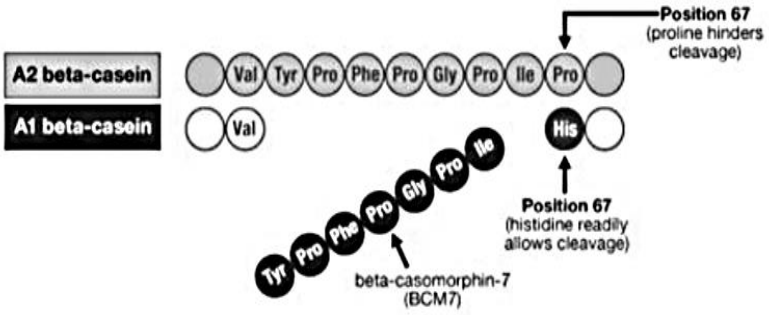

Figure 2. Release of BCM-7 from A1 beta-casein during digestion.

1.1.1.2 Genetics behind A1 and A2 Milk

The gene responsible for the production of A1 and A2 milk is present on chromosome 6. From ancient times cows producing A2 have been regarded as safe and nutritious. Around 5,000 years back beta-casein gene was mutated, and the 67th amino acid was changed from proline (A2 allele) to histidine (A1 allele). A cow has only two copies of the beta-casein gene. Hence, possibly having A2A2 homozygous genotype or A1A2 heterozygous genotype or A1A1 homozygous genotype. The alleles do not have a dominant-recessive relationship and both the alleles are co-dominant. Thus, an A1A2 cow will produce both A1 and A2 beta-casein alleles in equal proportion. An A2A2 genotype cow will only have A2 beta-casein, and an A1A1 cow will produce A1 beta-casein. A cow of A2A2 genotype will transmit the A2 allele to her progeny while an A1A1 cow will pass on the A1 allele, and for the A1A2 cow, there is an equal chance of transferring either allele. Breeding for A2A2 cows can be done by using semen from bulls of the A2A2 genotype (Behera et al., 2018) (Table 3).

Table 3. Breeding designs and genotype offspring (Behera et al., 2018).

Bull Genotype	Cow Genotype	Offspring Genotype
A2/A2	A2/A2	A2/A2
A1/A2	A2/A2	A2/A2 (50%)
A2/A2	A1/A2	A2/A2 (50%)
A1/A1	A1/A1	A1/A1

(Woodford, 2007; snowvillecreamery.com; www.zoetis.com; Indian Dairymen, 2017)

To increase milk production, the perpetual use of the European breeds in selective breeding improves reproduction gradually. A1 alleles have propagated through the breeding program. Some surveys have shown the frequency of getting A1/A2 is area-specific rather than breed-specific. For example, the A1 gene frequency in Holstein Friesen of North America and North Europe cow is very high (above 90%) but in German Holstein Frisian A2 gene frequency is very high (around 97%). In other countries, the frequency of A1 in Holstein Friesian is 40 –65%. Guernsey breed from the USA or Europe has high A2 frequency in cows and breeding bulls even more than 98 %, almost equal to Indian species. A2 gene frequency is usually higher in Jersey (60-80%) globally. Genotyping bulls for A1 and A2 alleles is a suitable method for lowering the risk of the A1 allele in human health and increasing milk and protein yield (Behera et al., 2018).

Jerseys, Guernsey, Asian, and African cow breeds produce A2 milk, while Holstein and Ayrshire cattle breed predominantly produce A1 milk (Figure 3). The proportion of A1 beta-casein is higher in the black and white species than yellow and brown breeds, such as Pezzata Rossa and Bruna breeds. A1 beta-casein is absent in the milk of pure Asian and African cattle (Bentivoglio et al., 2020). Additionally, the original A2 milk produced by species typically the more "traditional," such as Pezzata Rossa and Bruna cows, can be raised in marginal areas and have not been as significantly impacted by over-breeding.

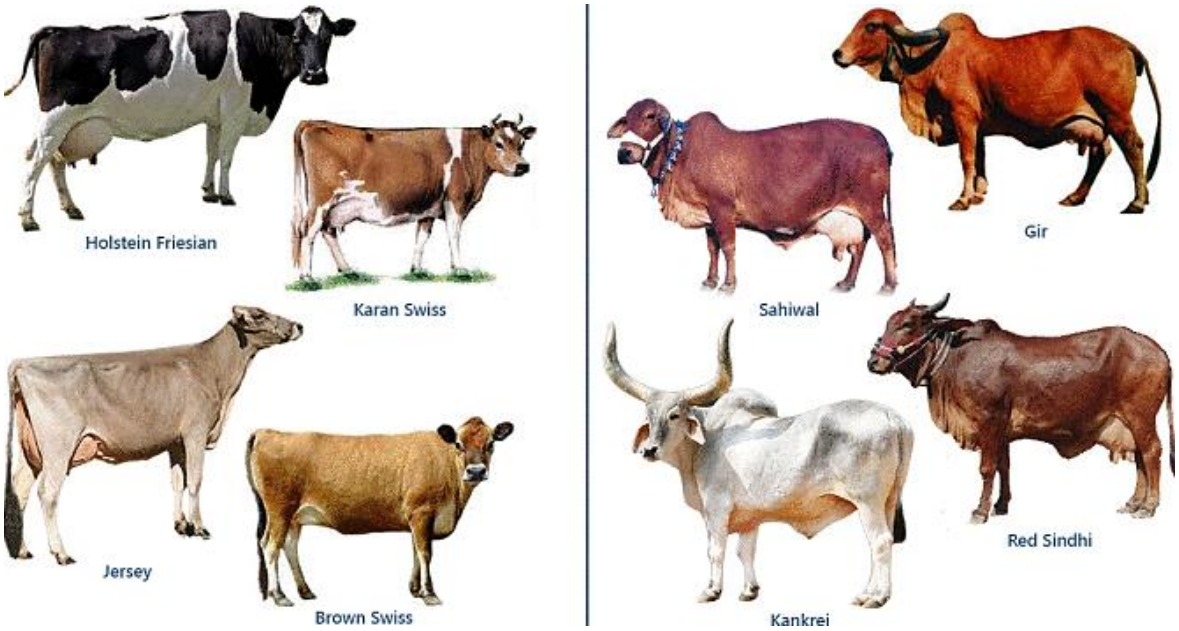

Figure 3. Cows producing A1 milk do not have a flap of skin beneath their neck and lack a hump, i.e., their back is straight throughout the body. In contrast, the A2 milk-producing cows have a skin flap in the neck region. The hump is also prominent, and its back is curved, especially in the tail region.

In 2009, the European Food Safety Authority (EFSA) reviewed their scientific literature and found that it is not that the possible bioactive peptides in milk containing both the A1 and A2 proteins have an adverse effect on health. The purity of A2 milk is typically related to the breeds considered more authentic, such as Pezzata Rossa and Bruna cows, raised in the outskirts and have not been affected by over-breeding.

1.1.2 Effects on human health of consumption of A1 and A2 milk

The controversy regarding A1 and A2 milk is related to the relative digestion of β-casein. When proteins digestion occurs, bioactive peptides are released. A small chain of amino acids is a peptide. BCM-7 is a seven amino acid peptide much more commonly released when A1-milk is digested than when A2-milk is digested. BCM-7 has opioid-like properties and is supposed to affect up to 25% of the human population (Hegde, 2019).

BCM-7 may be associated with type-1-diabetes, heart disease, infant death, autism, and digestive problems is suggested by few research groups (Brooke-Taylor et al., 2017). It is believed that BCM-7 may affect the digestive system. It's still unclear how much BCM-7 is

absorbed intact into the blood. Studies have shown that BCM-7 is not present in the blood of a healthy person who consumed cow's milk, but a few evaluations indicate that BCM-7 may be present in infants (Truswell, 2005).

1.1.2.1 Type-1- diabetes

Type-1-diabetes is a type of diabetes mellitus caused by insulin deficiency, also known as insulin-dependent diabetes, due to the destruction of insulin-producing β-cells in the islets of Langerhans of the pancreas by autoimmune processes. The contribution of cow's milk containing the A1-milk variant to the development of type-1-diabetes has been controversial for decade (Chia et al., 2018). Cow's milk which is the first food introduced to an infant. Children who have specific human leukocyte antigen genotype (HLA-DR) paired with a greater dietary intake of cow's milk protein may be at an increased risk of developing islet autoimmunity and progression to Type-1-diabetes (Lamb et al., 2015). Researchers reported that lower consumption of A1-milk might be related to the lower incidence of type-1-diabetes in Iceland than in Scandinavia. But it was found that there are more chances of developing a syndrome incidence in young childhood than in adulthood.

Similarly, it was found that the A1-milk consumption correlates strongly with the type-1-diabetes incidence in 0-14-year-olds (McLachlan, 2001). One study clearly showed a higher incidence rate in Finland and Sweden (highest A1-milk consumption/per capita) and low rates found in Venezuela and Japan (lowest A1-milk consumption/per capita) (Laugesen & Elliott, 2003)(Figure 4). In lactose-intolerant individuals, lactose malabsorption and digestive comfort with lactose-containing milk were improved with A2-milk exclusively.

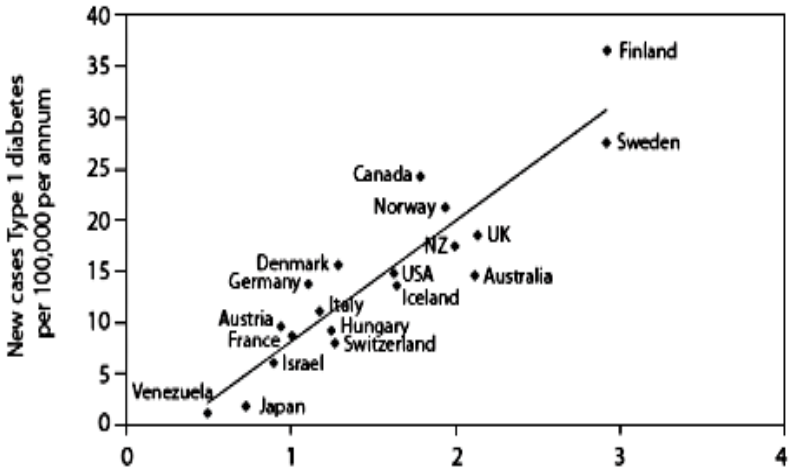

Figure 4. Type 1 diabetes death rates due to A1 beta-casein consumption.

1.1.2.2 Heart disease

According to earlier research, the consumption of the milk protein A1-milk (excluding milk protein in cheese) and ischemic heart disease mortality is closely correlated in West Germany, Toulouse in France, and Belfast in Northern Ireland (Figure 5). The value was a R2 = 0.86 (McLachlan, 2001). The same results were obtained for 20 countries showing that A1 β-casein per capita supply in milk and cream was greatly and positively correlated

with ischaemic heart disease (Venn et al., 2006). On the contrary, the studies in fifteen asymptomatic participants (six male and nine female) at high risk of developing cardiovascular disease have shown that the supplementation with A1-milk has no cardiovascular health disadvantage overconsumption of A2-milk (Chin-Dusting et al., 2006). Similarly, the studies by Venn et al. (2006) showed no evidence that dairy products containing beta-casein A1 or A2 exerted differential effects on plasma cholesterol concentration in humans.

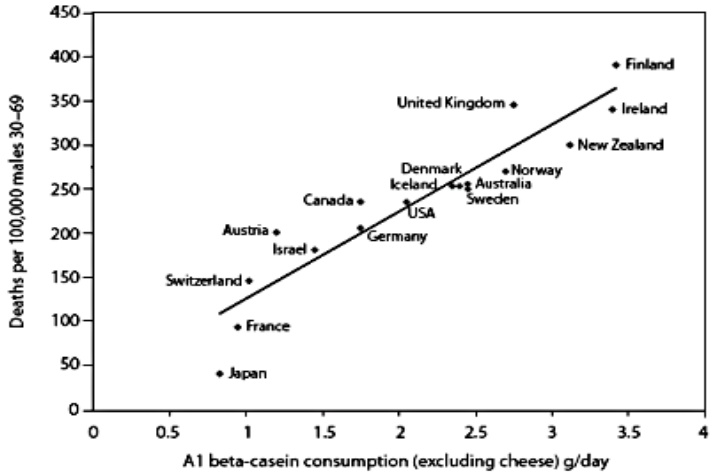

Figure 5. Ischaemic Heart Disease Death Rates (Source: MacLachlan CN (16))

1.1.2.3 Gastrointestinal disease

There are several studies on the impact of A1- or A2-milk on digestive tract health (Ho et al., 2014)(He et al., 2017). However, the increasing consumption of dairy products is associated with an increase in the risk of gastrointestinal function. Ho et al. (2014) found that A1-milk led to significantly higher stool consistency values (Bristol Stool Scale) than the A2-milk. Furthermore, the authors found a significant positive association between abdominal pain and stool consistency on the A1-milk diet (r=0.52) but not the A2-milk diet (r=-0.13). Jianqin et al. (2016) reported that milk consumption containing both types (A1-milk and A2-milk) was associated with significantly greater post-dairy digestive discomfort symptoms, higher concentrations of inflammation-related biomarkers and BCM-7, longer gastrointestinal transit times, and lower levels of short-chain fatty acids (Jianqin et al., 2016).

On the other hand, consumption of milk containing only A2-milk did not aggravate post-dairy digestive discomfort symptoms. Further studies in Germany have shown that after applying A2-milk in 10 people who cannot tolerate A1-milk, they have not had gastrointestinal problems. He et al. (2017) investigated that milk containing A2-milk attenuated acute gastrointestinal symptoms of milk intolerance, while conventional milk containing A1-milk reduced lactase activity and increased gastrointestinal symptoms. Animal studies have shown that the effect of A1- versus A2-milk on gastrointestinal was directly influenced (Haq et al., 2014)(Barnett et al., 2014). Similarly, it has been reported that casein and its derivatives, particularly BCM-7, exert various effects on gastrointestinal function in animals, including reducing the frequency and amplitude of intestinal

contractions. Interestingly, Barnett et al. (2014) also shown in rats that A1-milk feeding relative to A2-milk feeding significantly increased the colonic activity of the inflammatory marker myeloperoxidase by 65%, an effect also negated by the opioid blocker naloxone. In general, it has been shown that the consumption of milk containing A1-milk would lead to systemic inflammation and gastrointestinal motility through the release of BCM-7 (Ho et al., 2014)(Jianqin et al., 2016).

1.1.2.4 Autism

Autism, also called autism spectrum disorder, is a group of neurodevelopmental conditions characterized by social deficit and repetitive behaviours. It is believed that peptides like BCM-7 might play a role in the development of autism. However, the studies on this relationship are not always displayed(Hunter et al., 2003; Reichelt & Knivsberg, 2003). Breastfeeding is advantageous during the first month of life than artificial feeding for an infant's development and support the hypothesis for deterioration of bovine casomorphin elimination as a risk factor for delay in psychomotor development other diseases such as autism (Kost et al., 2009) . On the other hand, some authors have suggested that food peptides might determine toxic effects at the central nervous system level by interacting with neurotransmitters. Some worse neurological symptoms have been reported in autistic patients after the consumption of milk and wheat (Lucarelli et al., 1995). That means that there was a close relationship between food allergy and infantile autism. In conclusion, A1-milk has no significant effect on autism as compared to A2-milk.

1.1.2.5 Sudden death of infants

Sudden infant death syndrome is the most common cause of death in infants under 12 months old and its pathogenesis is complex and multifactorial. Casein-derived peptides as BCM-7 have been suggested to play a role in sudden infant death syndrome. Sun et al. (2003) described in a study the possible relationship between BCM-7 and sudden infant death syndrome (Sun et al., 2003). On the other hand, the studies by Wasilewska et al. (2011) have shown that the sera of some infants after an apnoea event contained more BCM-7 than that of the healthy infants in the same age (Wasilewska et al., 2011). These results indicate that some children may be sensitive to A1-milk. But more research is needed to get concrete results before any firm conclusions can be reached.

1.1.3 Consumer interest and market of A2 milk

The European Union (EU) dairy sector is the second biggest agricultural sector in the EU, representing more than 12% of total farm output. Over the past two decades, milk production in the EU has been steadily increasing. It increased from 152 million tons in 2000 to over 172 million tons in 2018 (an increase of almost 11%). The most likely explanation for this is that the quota system introduced in 1984 to increase milk production under control was abandoned at the end of March 2015. All 28 Member States produce milk. The leading producers of cow milk are Germany (19%), France (15%), the United Kingdom (9%), the Netherlands (8%), Poland (8%), Italy (8%), Spain (5%), and Ireland (5%), which together account for three-quarters of total EU production. The remaining Member States produce just a quarter of EU production (Bentivoglio et al., 2020).

The global a2 milk market was valued at $1,129.7 million in 2019, and is projected to reach $ 3,699.2 million by 2027, registering a compound annual growth rate of 15.8% from 2021 to 2027. In 2019, the liquid a2 milk segment accounted for the highest share in the market. Region-wise, the Asia Pacific region was leading the A2 milk market and expected to hold steady dominance (Figure 6)

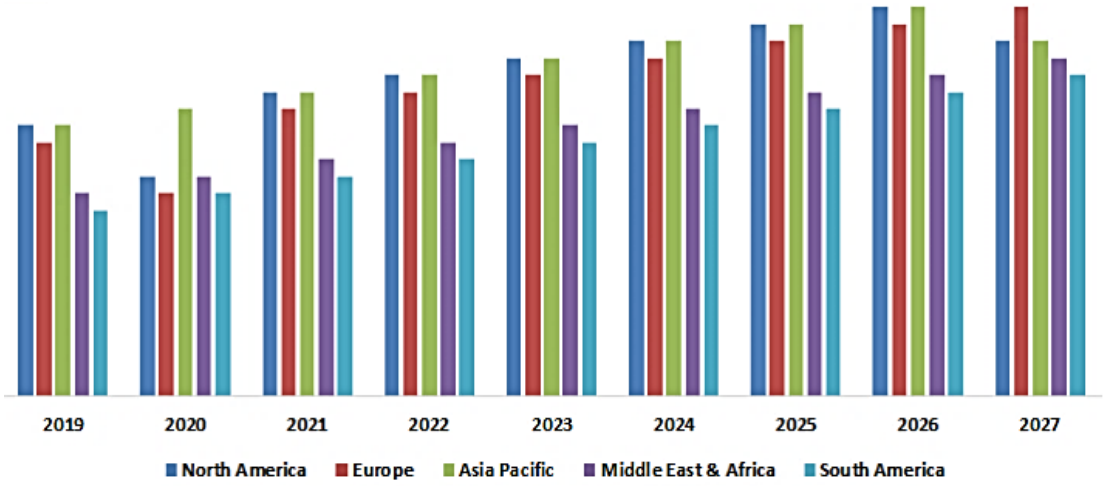

Figure 6. Global A2 Milk Market, By Region 2020-2027

In line with the negative trend of production of drinking milk, the EU per capita consumption also recorded a decrease annually. In particular, the volume of milk consumed dropped by 2% between 2013 and 2018. According to the EU agricultural outlook 2018–2030, European liquid milk consumption is expected to continue declining in the EU. Campaigns promoting lower dairy product intake because of the climate and environmental footprint of livestock products and an increase in lactose intolerance claims will negatively influence the consumption of dairy products. Consumer demand, in fact, drastically changed in recent years: consumers take more interest in aspects such as nutrition, health, and quality of the foods.

Consequently, with declines in per capita milk consumption and changes in consumer preferences, the dairy industry has to be creative and innovative in developing products to increase milk sales (Bigliardi & Galati, 2013; Madigan & Washburn, 2016). Nowadays, functional foods are one of the most dynamic and innovative categories in the food industry, with an estimated global value of over 40 billion US dollars and steady annual increases in sales (Oliveira et al., 2016). The markets of functional food are more expanding in the USA and Japan in comparison to the EU. Germany, France, United Kingdom, and the Netherlands represent the most important countries within the functional foods market. Meanwhile, in Italy, the overall category showed a negative performance, not only in 2019 (Bentivoglio et al., 2020)(Figure 8).

In the U.K., Australia, New Zealand, and recently, California, A2 milk has been introduced by the A2 Milk Company with a specific logo (Figure 7). The A2 Milk Company commercializes intellectual property relating to A1 protein-free milk sold under the A2 and A2 Milk brands and milk and related products like infant formula. This type of milk is sold as a functional dairy food due to its natural health benefits. A2 milk represents a relative newcomer to the ever-

expanding health food market. A2 milk is a variety of cows' milk that mostly lacks a form of β-casein proteins called A1, and instead, has mostly the A2 form. Nonetheless, farmers worldwide are incentivized to produce A2 milk to fulfil the increasing demand for a healthier alternative to conventional dairy.

Figure 7. A2 milk on the market shelves.

The growth can be due to the rising demand for dairy products and the product application in ghee, yogurt, milk powder, cheese, and butter in the regions such as India. Also, increasing consciousness about the health benefits obtainable by A2 milk products among the consumers in the Asia Pacific will further accelerate the A2 milk market growth trends in the region. The North American area is also rising rapidly in the A2 milk market in terms of increasing health concern and consciousness near the ill effects of A1 proteins and the high living standards of consumers. Major A2 milk-making companies are R&D products based on A2 milk to boost the growth of the A2 milk market in this region.

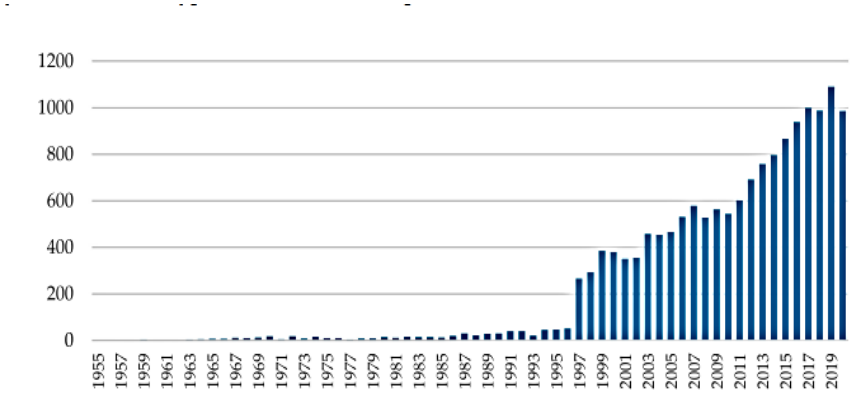

Figure 8. Documents by year from Scopus and Science Direct database, using the keywords "β-casein"(Bentivoglio et al., 2020).

This type of milk has also appeared in Italy without a specific logo and brand in recent years. Understanding the demand for this differentiated product constitutes a cornerstone for further analysis of consumer choices and price competition. One of the studies showed that Italian

consumers' intentions to purchase A2 milk were not influenced by sociodemographic variables but by the product's intrinsic (i.e., quality) and extrinsic attributes (i.e., method of production).

Research on A2 milk, Analysis of Italian Consumer Preferences (Bentivoglio et al., 2020) comprises four sections. The first section asked about sociodemographic characteristics (age, household income, gender, etc.). The second section comprised questions about milk consumption (purchase frequency, place of purchase, type of milk) and motives influencing milk purchase. Section three accounts for the knowledge and consumption of functional foods.

The results regarding Milk A2 consumption are contrasting. In total, 58% of the sample buy milk 1 or 2 times a week, 24% buy it 3 or more times a week, while 18% buy it less than once a week. Most of the milk (93%) is purchased in supermarkets/GDO. Based on the fat content, the most consumed type of milk is partially skimmed, representing 63% of the total, followed by whole milk, with 27%, and skimmed milk consumed by 10% of the sample. Meanwhile, based on other specific characteristics, the most consumed type of milk is long-life milk (UHT) with 42%, followed by lactose-free milk (21%) and fresh, high-quality milk (16%) (Table 4).

Table 4. Milk Purchasing Behavior (Bentivoglio et al., 2020).

Variables	Description	%	Variables	Description	%
Purchase frequency	<1 time	18%	Milk fat percentage	Whole	27%
	1 time	33%		Semi-skimmed	63%
	2 times	25%		Skimmed	10%
	3 times	12%	Type of milk	UHT milk	42%
	>3 times	12%		Lactose-free milk	21%
Place	Supermarket/Organized large scale distribution	93%		High quality milk	16%
	Retail	5%		Fresh milk	10%
	Local producers	2%		Microfiltered milk	3%
	Automatic dispenser	1%		Organic milk	3%
	Local market	0%		Raw milk	2%
				Mountain milk	1%
				Special milk	0%
				Flavored milk	0%

Moreover, the three attributes that consumers most selected were: the expiry date (18%), the origin (17%), and the price (14%). To understand better how the different attributes are significant, consumers were asked to rank, on a five-point Likert scale (from 1 = not important to 5 = very important), the importance of each attribute. The factors that result, by consumers, more important were the expiry date (4.02), the origin (3.80), and the organoleptic characteristics (3.72) (Figure 9).

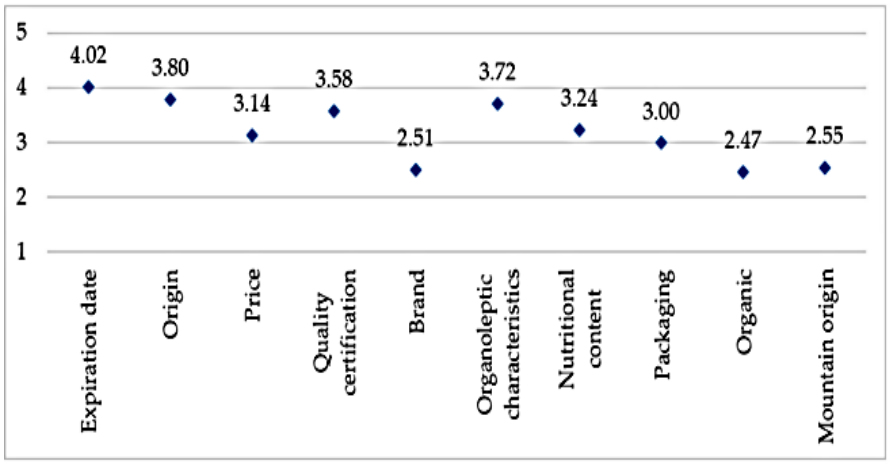

Figure 9. Factors influencing the purchase of milk (Likert Scale) (Bentivoglio et al., 2020)

1.2 Cheese

Cheese is considered among the most ancient processed food. It is believed that cheese-making dates back to 10,000 BC when sheep and goats were domesticated in the Middle East to produce milk. In warm climates, milk has a short life because of spoilage-causing microorganisms. Therefore, sour milk is separated into curd and whey, the solid curd providing an edible and healthy food. Curdling of milk is due to the fermentation of milk sugars and the breakdown of curd and provides solid curd and drinkable whey. The curds would be removed, drained, and lightly salted to provide a tasty and nourishing high protein food. The Romans increased this crude cheese-making to an early semblance of technology and spread it to various European regions. The primary reason for purposely processing the milk into cheese is preserving perishable food and converting it into a stable and storable product. It also expands the variety of food. Cheese produced in Europe, where the climate is more relaxed than in the Middle East, required less salting for preservation. Less salt and lower cheese acidity create an environment suitable for various beneficial microbes and molds, giving aged cheeses their pronounced surprising flavors (Walther et al., 2008).

Nowadays, cheese consumption is globally known throughout the world. However, the amount of cheese consumed varies from one country to another. Greece, France, Germany, Italy, and Switzerland have more than 20 kg per year per capita. In Ukraine, South Africa, Mexico, Japan, and China, cheese consumption is deficient at the other end of the scale. Nevertheless, in recent years, cheese consumption has increased in most selected countries worldwide (World & Situation, 2013)(Table 5).

Table 5. Per capita cheese consumption in various countries, 2001-2006 (Walther et al., 2008).

	2001	2002	2003	2004	2005	2006
			kg per capita			
Greece	26.6	27.5	28.7	28.7	28.9	28.9
France	25.8	25.0	24.8	24.5	24.5	23.9
Iceland	22.8	22.3	23.9	24.7	23.6	–
Germany	21.6	21.7	21.7	21.9	22.1	22.4
Italy	21.4	21.2	22.3	23.0	–	23.7
Austria	18.7	18.9	19.4	19.4	19.6	18.8
Switzerland	18.2	18.3	20.2	21.7	22.2	22.2
EU 25	17.5	17.6	17.8	18.0	18.3	18.4
Sweden	17.3	17.6	17.6	17.9	17.9	18.5
Finland	16.6	16.5	16.6	18.3	18.5	19.1
Norway	15.3	15.3	15.1	15.3	15.9	16.0
USA	15.1	15.3	15.3	15.7	15.7	16.0
Netherlands	14.7	14.7	14.7	14.7	14.7	20.4
Canada	14.1	13.9	14.0	14.3	14.4	–
Australia	11.6	12.3	12.4	11.7	11.9	–
Argentina	11.5	9.6	8.3	8.9	10.7	–
Poland	10.9	10.1	10.3	10.4	10.4	–
Ireland	10.3	10.3	10.5	10.5	10.5	10.5
Czech Republic	10.2	14.4	14.7	15.7	–	–
Portugal	10.0	10.1	10.2	10.3	–	–
UK	10.0	10.8	10.8	10.9	11.1	–
Hungary	8.7	8.9	8.8	9.0	10.0	–
Spain	8.7	9.1	9.5	9.5	9.6	–
New Zealand	7.3	7.1	7.1	7.1	7.1	–
Slovakia	6.3	9.1	9.3	8.2	9.3	–
Russia	5.3	5.5	5.6	5.7	6.2	–
Japan	1.9	1.8	1.9	2.0	–	–
Mexico	1.9	2.0	1.9	2.1	2.1	2.1
South Africa	1.0	1.0	0.8	0.6	–	–
Korea (Republic)	–	–	1.2	1.3	1.4	–

Today the main reason for cheese consumption is not the prevention of hunger but the supply of essential and essential nutrients, its various uses in the kitchen, and its enjoyment. Technological progress has led to the production of many types of cheese, which is different in texture and flavor from one another. Nowadays, a shift is observable from the optimum in product quality to the optimum for the consumer. So, research is no longer based on the production of high-quality cheese but more and more on the commercialization of cheese as a functional food. Technology is needed for gentle processing to retain or even accumulate desired nutrients and to remove unwanted compounds. The reason for the rapidly increasing market of functional foods are people who are health concerned.

1.2.1 Composition of Cheese

The various cheese types can be classified based on the milk used (goat, sheep, cow, buffalo), their production (rennet, sour milk cheese, ultrafiltration), consistency (extra-hard, hard, semi-hard, semi-soft, soft, fresh cheese), fat content (double cream, cream, total fat, three-quarters fat, quarter fat cheese, half fat cheese), fermentation type (lactic acid, butyric acid, lactic+propionic acid), surface (hard, soft, with smear, molds) and interior(eyes, molds). Additionally, the different flavors and some bioactive components are mainly created during the different ripening stages due to the breakdown of lactose, protein, and fat by fermentation, proteolysis, and lipolysis. Thus, the varieties of cheese on the market are enormous, which is also reflected in the variability in the composition of the different types of cheese. In most countries with high consumption of milk products, the central part is produced from cow's milk. So, we have concentrated on the composition of cow's milk. They mainly consist of fat, protein, water, and vitamins, minerals, and trace elements. Lactose is rarely present (Table 6).

Table 6. The average composition of fresh, soft, semi-hard, hard, and extra-hard cheese (Walther et al., 2008).

	Water	Protein	Fat	Lactose	Minerals + Vitamins
			$g \cdot kg^{-1}$		
Fresh cheese	700	110	80	30	80
Soft cheese	520	200	220	0	60
Semi-hard cheese	400	250	270	0	80
Hard cheese	350	270	310	0	70
Extra-hard cheese	300	290	330	0	80

Research today is concentrated more and more on the influence of nutrition on human health. Calcium, which is present in large quantities in cheese, has positively affected. Besides calcium, other constituents with potentially positive effects on health are found, e.g., bioactive peptides, which also decrease hypertension. In humans, the possible anti-carcinogenic effects of specific lipids (CLA, sphingolipids) have not yet been investigated, but animal studies suggest a particular potential (Walther et al., 2008).

1.2.2 Cheese Production

Virtually all cheese is made by coagulating milk protein casein to trap milk solids and milk fat into a curd matrix. This curd matrix is then consolidated to express the liquid fraction, cheese whey. Cheese whey contains those milk solids that are not held in the curd mass, particularly most of the sugar (lactose) and several soluble proteins. The processes involved are pasteurization, acidification, coagulation, cooking, salting, dehydration or syneresis, molding (or shaping), pressing, packaging, and maturation or storage (Moatsou, 2019) (Figure 10).

Pasteurization of the milk kills nearly all the microorganisms present, including the harmful pathogenic bacteria that cause diseases, such as tuberculosis and leptospirosis, and other undesirable organisms, such yeasts, and coliforms, that may alter the cheese characteristics by producing carbon dioxide and undesirable proteolysis.

Acidification of the milk is essential for the proper release of whey from the cheese curd and to control the growth of many undesirable bacteria. It is usually accomplished by the addition of lactic acid bacteria that convert lactose to lactic acid. Most varieties of cheese cannot be made without the addition of a "starter," which is a culture of carefully selected lactic acid-producing bacteria. The large volumes of starter required for cheesemaking are made in unique bulk starter fermentation pots. The milk is heat-treated to destroy unwanted bacteria, spores and then cooled to about 22°C, a temperature suitable for starter growth. The frozen starter is mixed in, and fermentation continues for about 6 to 16 hours. The amount of starter required varies for the different cheese varieties but, for Cheddar, this is generally between 1.25-2.0% of the cheese milk (Nasr, 2021).

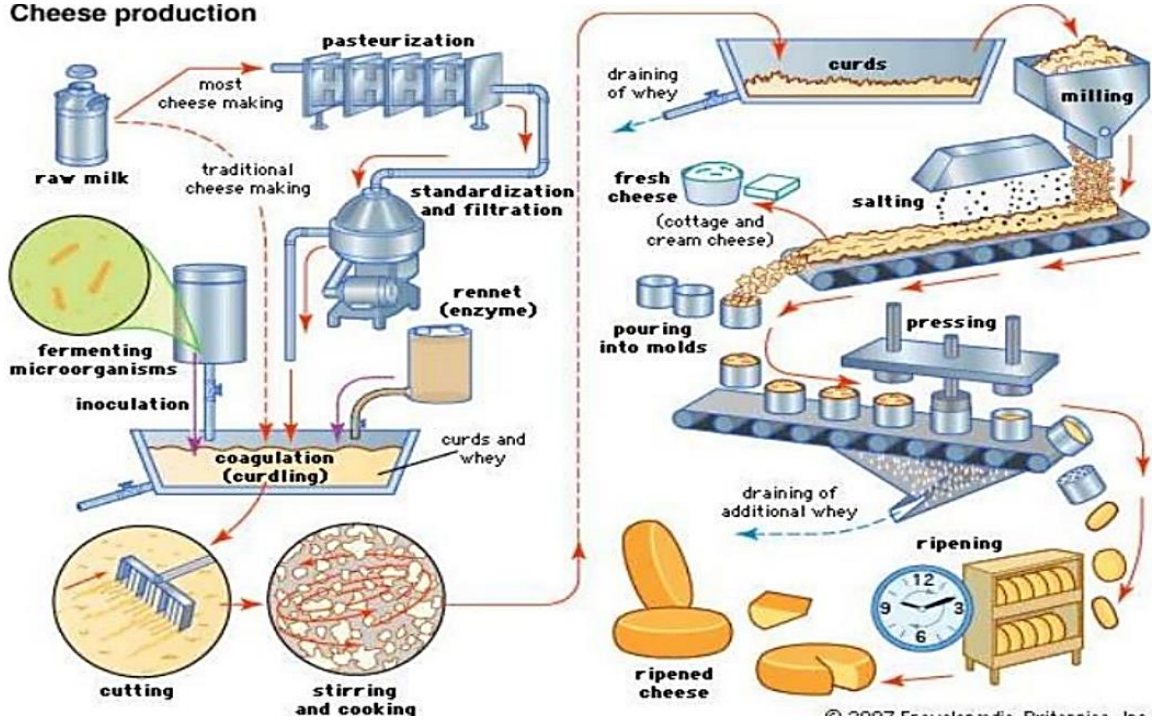

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Figure 10. Cheese Production.

Coagulation of the casein fraction of the milk to form a gel can be achieved by lowering the milk pH and the addition of "rennet," a mixture containing a specific proteolytic enzyme. The most commonly used rennet has the enzyme chymosin, either as an extract of calf abomasum or a recombinant product. Other types of rennet are derived from different animal sources, microorganisms, or plants.

The four main groups caseins in milk are the αs1-,αs2-, β- and κ-casein These phosphoproteins are held together by microclusters of calcium and phosphate. They exist in milk as micelles of about 100 nm in diameter containing hundreds of molecules of each type of casein. The more hydrophobic regions of these phosphoproteins are believed to be located inside the micelle with the more hydrophilic regions of κ-casein on the outside. The negatively charged carboxy-terminal of the κ-casein molecules is thought to protrude 'hair-like from the micelle and repel other casein micelles (charge stabilization). In addition to this, the hair-like macropeptide portions of κ-casein are unable to interpenetrate (steric stabilization). These two mechanisms are thought to enable the micelles to stay in the solution as colloidal particles. The addition of rennet (includes any of a range of acid proteinases) leads to the partial proteolysis of κ-casein. The release of the hydrophilic carboxy-terminal peptide (glycomacropeptide) destabilizes the micelles, which become less negatively charged and more hydrophobic. These micelles then aggregate (in the presence of calcium and at a temperature above 15°C) to form a coagulum. A rennet coagulum consists of a continuous matrix of strands of casein micelles, which incorporate fat globules, water, minerals, and lactose, and microorganisms are entrapped (Coker, Christina; Honoré, Craig; Johnston, Keith; Creamer, 1997) (Figure 11).

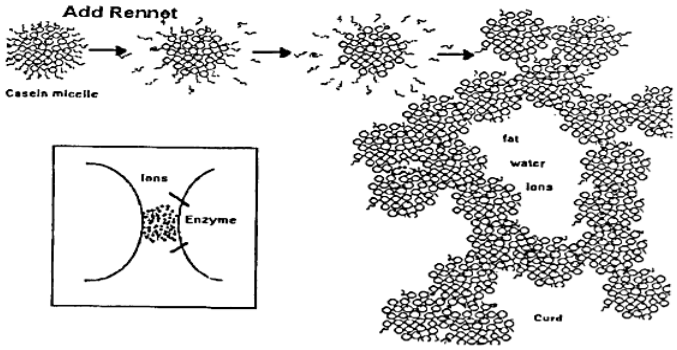


Figure 11. The action of rennet on the casein micelle. The enzyme in rennet cleaves the κ-casein releasing a large peptide. The surface of the micelle changes from being hydrophilic and negatively charged to hydrophobic and neutral. As a consequence, the micelles aggregate to trap fat globules and microorganisms in the developing curd.

(Coker, Christina; Honoré, Craig; Johnston, Keith; Creamer, 1997)

Syneresis, or shrinking, of the coagulum is primarily the result of continuing rennet action. It causes loss of whey and is accelerated by cutting, stirring, cooking, salting, or pressing the curd and the increasing amount of acid produced by the starter, which gradually increases during cheesemaking. As a result, the cheese curd contracts, and moisture is continuously expelled during the cooking stages.

Salt is added to cheese as a preservative and because it affects the texture and flavor of the final cheese by controlling microbial growth and enzyme activity. The salt can be added either directly to the curd after the whey is run off and before molding or pressing into shape, or by immersing the shaped cheese block in a salt brine for several days the following manufacture. In dry salt cheese, the curd/whey separation is done by screens and then cheddaring on the Alfomatic belt system for at least 2 hours, so the curd loses more water and clumps together. The curd is then milled, and 1.6-2% salt is added. Then block forming is done. In contrast, in brine salt cheese, the whey removal and block formation are done in Casomatic tower. Cheese molds are prepared and pressed. After demolding, the salting is done in a brine salt tank for 24-48 hours.

Cheese ripening is basically about breaking down carbohydrates (sugars and acids), protein, and lipids, releasing flavor compounds, and modifying cheese texture. Ripening varies from nil for fresh cheese to 5 years for some hard ripened cheese (Figure 12).

Figure 12. Cheese Ripening.

1.2.4 Ripened cheese

Cheese is a biologically and biochemically dynamic product, in which a series of sequential changes take place during the cheese-making process. Some technological procedures such as heat treatment, homogenization, pressure application, and milk coagulation can affect the structure of the milk constituents and promote the development or the release of bioactive compounds (Kumar et al., 2000).

Cheese ripening is a complex, dynamic system. In this process, the diversity of proteolytic enzymes naturally present in milk and the residual coagulants and the enzymatic metabolism of LAB play an essential role (Gagnaire et al., 2001)in the final cheese. During ripening, peptides are being constantly released by the action of plasmin and enzymes from LAB; some of these peptides are subsequently hydrolyzed, whereas others accumulate during storage. Bioactive peptides trapped or inactivated in the protein matrix can be released by enzymatic hydrolysis (e.g., pepsin, trypsin, and chymotrypsin) (Fitzgerald & Murray, 2006)(Figure 13).

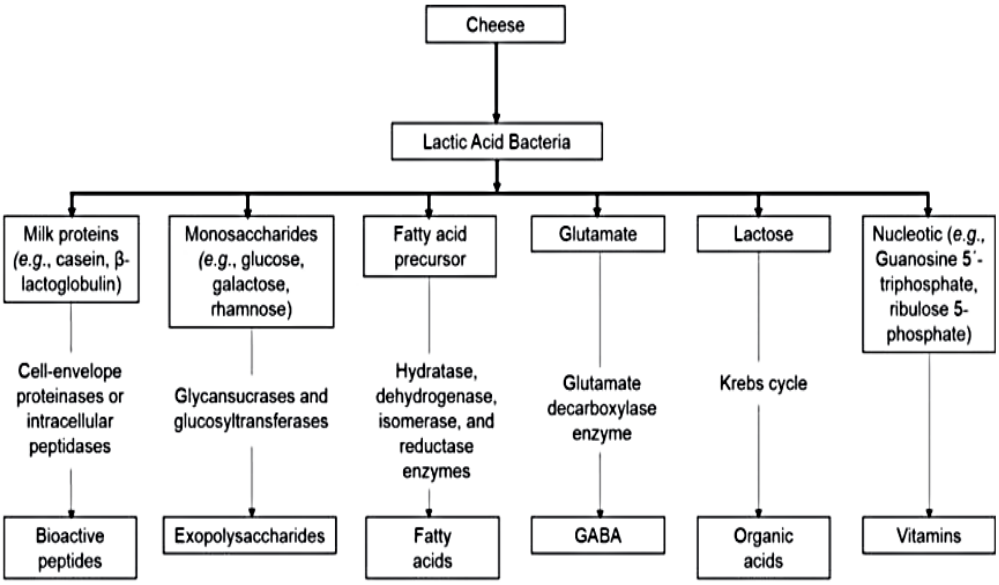


Figure 13. The role of lactic acid bacteria during the fermentation or ripening process of cheese. Lactic acid bacteria have been documented as precursors of bioactive compounds, and their action releases peptides, exopolysaccharides, fatty acids, γ-aminobutyric acid (GABA), organic acids, and vitamins. Different mechanisms and factors are involved during the release of bioactive compounds, including enzymes, pH conditions, ripening time, and temperature (Santiago-López et al., 2018).

In the complex microbial niche of cheese, Lactic acid bacteria (LAB) yeast, and some molds are present. These microbes play an essential role in developing the sensory characteristics of cheese and in the technological aspects of cheese production (Irlinger & Mounier, 2009). During the cheese-making process, LAB can be added as a starter culture, contributing to the coagulation. On the other hand, at least one group of nonstarter LAB (NSLAB; e.g., lactobacilli, pediococci, enterococci, and Leuconostoc) can also be present naturally in cheese. The primary source of NSLAB is raw milk, although other cheese ingredients or equipment can also be a source and enhance their concentration in the final product (Santiago-López et al., 2018). The NSLAB grows at a meager rate during the first weeks of ripening but eventually dominates the cheese microbiota after the death phase of the starter culture (Belletti et al., 2009). Both groups of LAB are essential for developing the biochemical characteristics of fresh cheese and for cheese ripening. LAB releases bioactive peptides, EPS, vitamins, CLA,

GABA, and oligosaccharides (Reyes-gavilán et al., 2015). During the ripening process, In cheese ripening, the proteolytic and peptidolytic pathways are called phase 1 and 2, respectively, and the conversions of amino acids, fatty acids, and so on leading to the actual cheese flavors, are known as the third phase of ripening (Smit et al., 2000)(Figure 14).

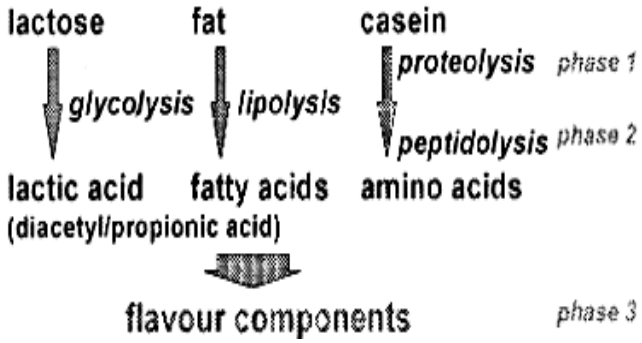


Figure 14. Flavor formation in Cheese (Smit et al., 2000).

1.2.5 Caciotta cheese

The general term Caciotta includes various soft cheeses produced in different Italian regions, especially in central Italy. It refers to small or average-sized cheeses that weigh 0.8–2 kg of cylindrical shape with less height, about 4–8 cm, and diameter of 8–10 cm may reach upto 16 cm (Figure 15). It is made with cow's milk, ewe's milk, or both, ripened from one to several weeks. The quality of this type of cheese reflects its production area and local tradition. Moreover, interactions between pedoclimatic characteristics, autochthonous genetic variations, and anthropic components create an environment so specific that it would be so difficult or impossible to reproduce it at some other place (Turchi et al., 2011) In order to preserve this product, i.e., their unique taste it is prepared in small diaries and family plants by using raw or thermised cow milk.

Figure 15. (left) Caciotta cheese samples in the lab (right) Caciotta cheese in the market.

Caciotta have a flat surface and a straight or slightly convex rim (4 cm). The rind is thin, uneven, and yellowish-white. From the inside, it is whitish and characterized by small eye-like spots and a semi-soft texture. Different diaries use different methods, and thus a wide variety of cheeses can be seen in the market. It greatly depends upon the quality of raw milk. This situation is usually related to improper milking management and causes microbial origin defects, owing to the proliferation of spoilage microorganisms during ripening (early gas-

blowing). The early gas defect is characterized by an incomplete ripening of the cheese and heterogeneous plastic mass. The occurring holes are irregular in shape; sometimes, many tiny holes may arise, unfavorably affecting the aspect of the cheese when cut. In addition, the organoleptic characteristics of these cheeses are altered, with an unpleasant taste and spongy. To reduce cheese defects, many farmers apply milk thermisation. This treatment, which is essential for lowering spoilage bacteria, particularly coliforms, causes the loss of lactic acid species necessary for fermentation and cheese ripening, even if its mildness can allow some mesophilic lactic acid to survive bacteria (LAB). The selection of LAB, to be used to produce cheese with thermised milk, allows the restoration of the wild microflora to maintain the unique character and taste of the cheese. Optimizing the wild microbial biodiversity plays a vital role in the characterization and improvement of dairy products on an artisan and industrial scale. Moreover, the activity of the starter is crucial for the control of coliforms by decreasing the pH and the amount of lactose in the curd.

1.3 Fatty acids

The principal lipids of milk are triacylglycerides, representing up to 98% of the total lipids (Christie, 1983). Triacylglycerides have molecular weights ranging from 470 to 890 Da, corresponding to 24–54 acyl carbons (Boudreau & Arul, 1993). Triacylglycerides are esters of glycerol composed of a glycerol backbone with three fatty acids attached. It is well established that milk fat is essential for developing the correct flavor in cheese during ripening. Lipolysis in cheese is due to lipolytic enzymes, which are hydrolases that cleave the ester linkage between a fatty acid and the glycerol core of the triacylglyceride, producing FFA and mono- and diacylglycerides (Deeth & Touch, 2000). Lipolytic enzymes may be classified as esterases or lipases. FFA are released upon lipolysis and contribute directly to cheese flavor, especially short- and inter-mediate-chain FFA (Bills & Day, 1964). The proportions of free C6:0to C18:3in Cheddar cheese appear to be similar to those in milk fat. However, free butanoic acid(C4:0) occurs at a greater relative concentration in cheese than in milk fat (Bills & Day, 1964), suggesting its selective release by lipases present in cheese or its synthesis by the cheese microflora.

In cheese, FFA is released due to lipolysis, especially short- and medium-chain fatty acids directly contribute to cheese flavor. FFA also act as precursor molecules for a series of catabolic reactions leading to the production of flavor and aroma compounds, such as methyl ketones, lactones, esters, alkanes, and secondary alcohol (Tobin, n.d.)

Lipids are an essential component of human nutrition, important in many physiological and pathophysiological processes and show great structural diversity (Schulze et al., 2018). Biological functions of fatty acids as one class of lipids depend on the chemical structure. According to their structural characteristics, fatty acids can be grouped into short- (SCFAs), middle- (MCFAs, 8–10 carbon atoms) and long-chain fatty acids (LCFAs, ≥ 14 carbon atoms) or in relation to the degree of saturation in saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids. Especially, the latter group is thought to have beneficial health effects; PUFAs showing a positive impact on cardiometabolic health in terms of lower incidence of coronary heart disease, lower cholesterol levels and better insulin sensitivity (Zárate et al., 2017). PUFAs also exhibit an antihypertensive and antimicrobial effect.

Fatty acids are the main constituents of oil and fats whose applications are overwhelming. They possess a simple structure consisting of a long hydrocarbon chain and one or more carboxylic groups. It is composed of carboxylic acid “head group” and a long hydrocarbon “tail” (Figure

16). The broadest definition includes all chain lengths, but most natural fatty acids are C4 to C22, with C18 most common.

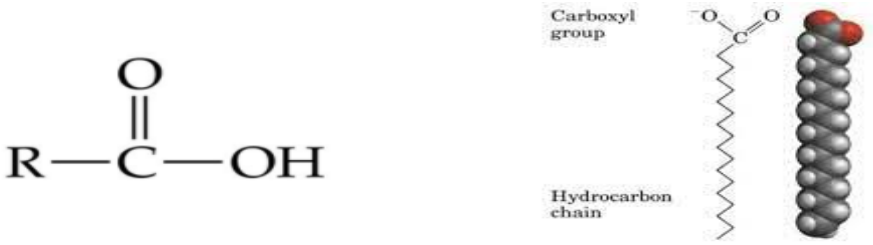


Figure 16. Structure of Fatty Acid.

Fatty acids are of two types: saturated fatty acids and unsaturated fatty acids. Saturated Fatty acids (SFA) do not contain double bonds (Figure 17a). The body can synthesize this type of fat, and its primary dietary source is food from animal sources, such as full-fat dairy products, red meat, and poultry (Rustan & Drevon, 2005). Moreover, there are numerous types of SFA according to the length of their chain (contains 4–16 carbon atoms). In comparison, Unsaturated Fatty Acids have one or more double bonds with a terminal carboxylic acid (Figure 17b).

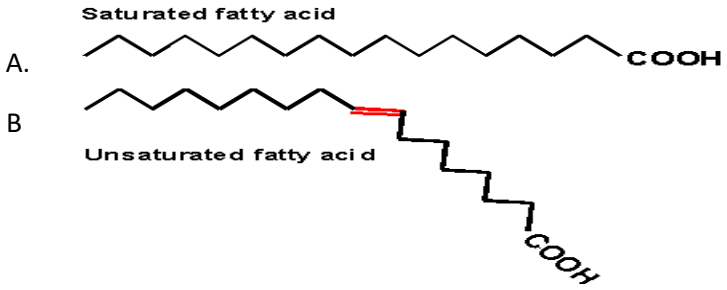
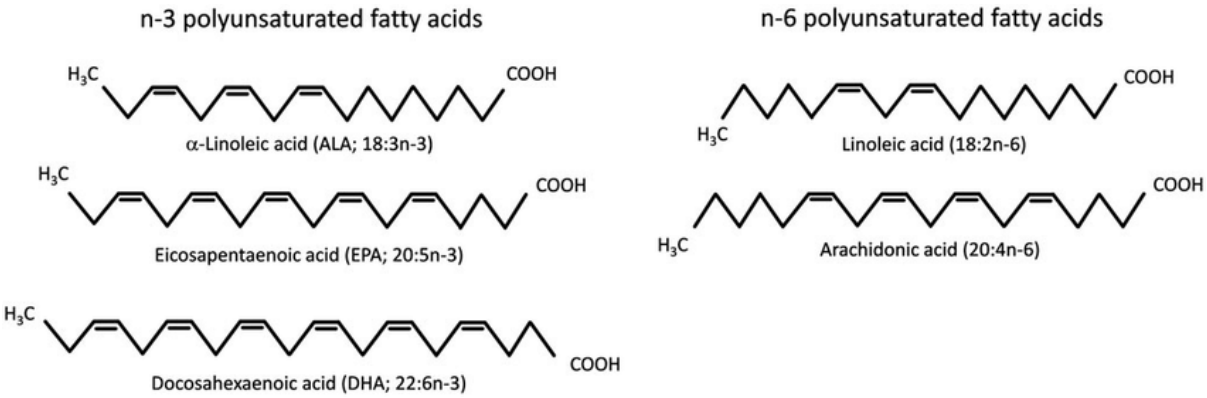
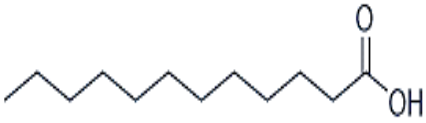


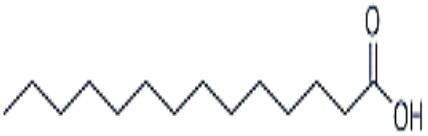
Figure 17. (a) Saturated Fatty acid (b) Unsaturated Fatty acid.

These fatty acids are subdivided into two groups depending on the number of double bonds. A single, double bond fatty acid is termed Monounsaturated Fatty Acid (MUFA), and those with more than one double bond are termed Polyunsaturated Fatty Acid (PUFA) (Figure 18).

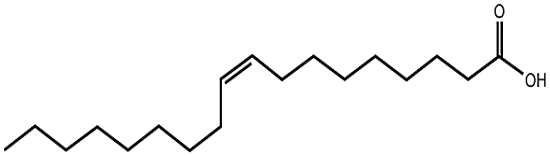




Lauric acid C12:0

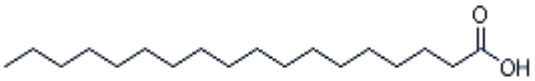


Myristic acid C14:0



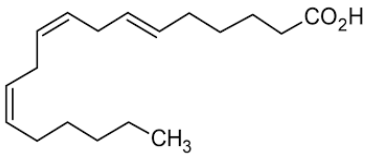
Oleic acid C18:1ω-9

Palmitic acid C16:0



Stearic acid C18:0

Linoleic acid C18:2ω-6



Linolenic acid C18:3ω-3

Figure 18. Saturated, Monounsaturated and Polyunsaturated Fatty Acids.

1.3.1 Fatty Acid Methyl Esters (FAMEs)

Lipids are one of the major components of milk solids. Fatty acid (FA) composition of milk fat is closely related to the nutritive quality of milk and physicochemical properties of dairy as animal products (Chen et al., 2004). Fatty acid composition of milk fat is influenced by various factors such genotypes, diets and physiological status of cows (Shingfield et al., 2005). As a result, analysis of fatty acids composition is of great importance in lipid-related research and for dairy-based industries. Fatty acid methyl esters (FAMEs) analysis is an important tool for characterizing fats and oils and determining the total fat content in foods. Fats can be extracted from a matrix using a nonpolar solvent and saponified to produce salts of the free fatty acids. After derivatizing the free acids to form methyl esters, the mixture can readily be analyzed by gas chromatography (GC) due to the volatility and thermal stability of the FAMEs. Generally,

Fatty acids are quantified as to their methyl esters by gas chromatography (GC) following a two-step sample preparation procedure, i.e., lipid extraction and FA methylation (Liu et al., 2020).

The primary reasons to analyze fatty acids as fatty acid methyl esters include their free, underivatized form. Free atty acids and bounded fatty acids (triglycerides) may be challenging to analyze because these highly polar compounds tend to form hydrogen bonds, leading to adsorption issues. Reducing their polarity may make them more amenable for analysis. Additionally, the polar carboxyl functional groups must first be neutralized to distinguish between the very slight differences exhibited by unsaturated fatty acids. This allows column chemistry to perform separations by boiling point elution and by the degree of unsaturation, position of unsaturation, and even the cis vs. trans configuration of unsaturation (Restek, 2018).

2. Aim of the thesis

This thesis aims to evaluate the impact of the genetic biodiversity of dairy cattle with the A2A2 (a2) genotype of beta-casein against the other genotypes (a1) on the fatty methyl esters profile of milk and cheese (fresh or during ripening).

The framework of this thesis is included in the project **I-Milka 2- PSR MARCHE 2014.** The project aims to protect the genetic biodiversity of dairy cattle with A2A2 genotype, determining the impact of milk production with beta-type A2 on consumer health, cheese processing efficiency, and quality dairy products. The project includes the analysis of the qualitative and technological parameters of A2 milk and its derived products and the evaluation of possible beneficial effects on human health of beta-casein A2 through studies on human adipose cells in culture.

3. Materials and methods

3.1 Experimental design

Initially, the bovine capital of Angolo di Paradiso company (Amendola, Italy) was screened to identify the beta-casein gene genotype of all cows and thus identify homozygous A2A2 subjects. Two groups of cows were generated, producing two different kinds of milk: milk from cows with genotype A2A2 vs. milk from cows with other genotypes. Samples of milk (A2A2 vs. other genotypes) were collected along two years of monitoring (2020-2021), creating two pools of milk from different subjects but always separated in the function of the genotype. From each pool of milk, cheese (Caciotta) was produced to evaluate the impact of the bovine beta-casein genotype on cheese quality. The cheeses were analyzed fresh but also ripened (in cellar at 10-15°C) at different periods (maturation from 15 days to 14 months). The list of samples is reported in Table 7.

Table 7. Experimental design of milk and cheese. Milk a2 represents the milk from cows with genotype A2A2 of beta-casein while milk a1 from the cow with other genotypes. 26 samplings of each type of milk (58 samples in total) were performed at different periods from 16 January 2020 till 1 July 2021. The respective cheeses (26 samplings) were collected fresh and ripened (18 samplings) till 14 months of maturation. Only the high-lightened samples were analyzed as the number of samples was very extended.

	Fresh milk		Fresh cheese		Ripened cheese		
Date of sampling	Milk a1	Milk a2	Cheese a1	Cheese a2	Cheese a1	Cheese a2	Months
16-Jan-20	1 A1	1 A2	1 A1	1 A2			
30-Jan-20	2 A1	2 A2	2 A1	2 A2			
2-Feb-20	3 A1	3 A2	3 A1	3 A2	3 A1	3 A2	13.9
13-Feb-20	4 A1	4 A2	4 A1	4 A2	4 A1	4 A2	13.5
27-Feb-20	5 A1	5 A2	5 A1	5 A2	5 A1	5 A2	13.1
16-Jun-20	6 A1	6 A2	6 A1	6 A2	6 A1	6 A2	9.4
2-Jul-20	7 A1	7 A2	7 A1	7 A2	7 A1	7 A2	8.9
16-Jul-20	8 A1	8 A2	8 A1	8 A2	8 A1	8 A2	8.4
27-Aug-20	9 A1	9 A2	9 A1	9 A2	9 A1	9 A2	7
10-Sep-20	10 A1	10 A2	10 A1	10 A2	10 A1	10 A2	6.5
24-Sep-20	11 A1	11 A2	11 A1	11 A2	11 A1	11 A2	6.1
7-Oct-20	12 A1	12 A2	12 A1	12 A2	12 A1	12 A2	5.6
22-Oct-20	13 A1	13 A2	13 A1	13 A2	13 A1	13 A2	5.1
5-Nov-20	14 A1	14 A2	14 A1	14 A2	14 A1	14 A2	4.7
19-Nov-20	15 A1	15 A2	15 A1	15 A2	15 A1	15 A2	4.2
3-Dec-20	16 A1	16 A2	16 A1	16 A2	16 A1	16 A2	3.7
28-Jan-21	17 A1	17 A2	17 A1	17 A2	17 A1	17 A2	1.9
11-Feb-21	18 A1	18 A2	18 A1	18 A2	18 A1	18 A2	1.4
25-Feb-21	19 A1	19 A2	19 A1	19 A2	19 A1	19 A2	0.9
11-Mar-21	20 A1	20 A2	20 A1	20 A2	20 A1	20 A2	0.5
25-Mar-21	21 A1	21 A2	21 A1	21 A2	21 A1	21 A2	0.5
22-Apr-21	22 A1	22 A2	22 A1	22 A2			
6-May-21	23 A1	23 A2	23 A1	23 A2			
20-May-21	24 A1	24 A2	24 A1	24 A2			
17-Jun-21	25 A1	25 A2	25 A1	25 A2			
1-Jul-21	26 A1	26 A2	26 A1	26 A2			

3.2 Fat extraction from milk

10.0 ml of milk was mixed with 10 ml ethanol and 1 ml H2S04 (2.5 mol/L). Extraction was carried out with 15 mL diethyl ether/heptane (1:1, v/v) in a 50 mL screw-capped centrifuge tube. After centrifugation at 2500 rpm for two minutes at room temperature, the upper solvent layer was transferred to a 100 mL conical flask containing 1g anhydrous sodium sulphate to adsorb residual water. The extraction procedure was repeated twice with 10 mL diethyl ether/heptane (1: 1, v/v), as reported by De Jong & Badings (1990). The collected fractions were pooled together and taken to dryness with rotavapor at 35°C, and the extracted fat was kept for later fatty acids methyl ester analysis.

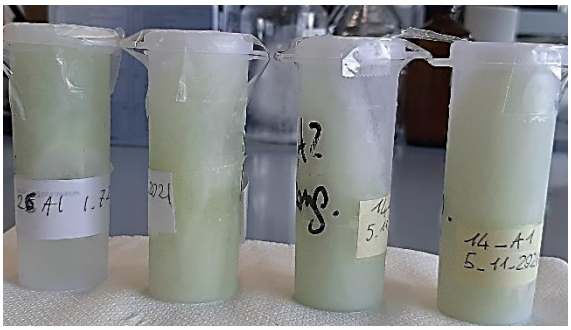


Figure 19. A1 and A2 Milk samples.

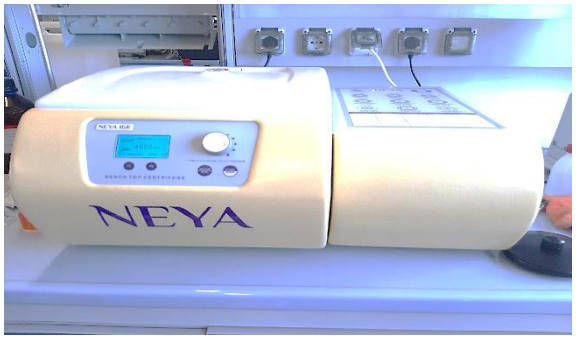


Figure 20. Centrifuge.

3.3 Fat extraction from cheese

After grinding 1.0 g of cheese (**Figure 21**) with 3 g anhydrous sodium sulphate, 0.3 ml H2S04 (2.5 mol/L) was added to perform protein precipitation. This mixture was extracted three times with 3 mL diethyl ether/heptane (1:1, v/v). Each time the solution was clarified by short centrifugation as for milk. The collected fractions were pooled together and taken to dryness with rotavapor (Figure 22) at 35°C, and the extracted fat was kept for later fatty acids methyl ester analysis.

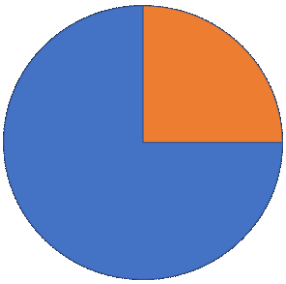


Figure 21. Example of a section of cheese for the grinding.

Figure 22. Rotavapor.

3.4 Fatty acid methyl esters (FAME) analysis

Fatty acid methyl esters (FAME) were obtained from total lipids through transmethylation by BF_3-MeOH reagent (Medina et al., 1992). Briefly, 20 mg of fat were added of *n*-hexane (0.5 mL), BF_3-MeOH solution (0.5 mL) and vortexed. After 15 min at 100 °C, the reaction was stopped with distilled water (0.5 mL), and the mixture was centrifuged (4500 rpm, 3 min). The organic phase was analyzed by capillary gas chromatography, as reported by Balzano, Pacetti, Lucci, Fiorini, and Frega (2017).

The qualitative analysis of FAMEs (weight% of total fatty acids) was performed by means of gas chromatography using Thermo Scientific TRACE 1300 apparatus (Massachusetts, USA) (Figure 23) equipped with a flame ionization detector set at 270°C (FID) and an RT-2560 fused silica capillary column (100 m 0.25 mm i.d., film thickness 0.2 µm; Restek, USA). The carrier was helium at a flow rate of 1.6 mL/min. The oven temperature program was: 6 min at 115°C, raised to 240°C at a rate of 10°C/min, then held for 20 min. The injector temperature was 250°C. The sample was injected into a split injection port at a split ratio of 6.

Figure 23. Gas Chromatography Instrument (GC-FID).

3.5 Statistical Analysis

Data are reported as mean values ± standard deviation (SD) of three replicates. Data were analyzed with the principal component analysis (PCA) using R software version 3.5.0.

4. Results and Discussion

4.1 Fatty acid methyl esters profile of milk a1 and a2

The fatty acid methyl esters profile was analyzed in 26 samplings of each type of milk (58 samples in total), collected at different periods from 16 January 2020 till 1 July 2021. Milk a2 represents the milk from cows with genotype A2A2 of beta-casein while milk a1 from the cow with other genotypes. The identified fatty acid methyl esters are reported as examples of GC-FID chromatograms in Figure 24 and Figure 25**. In both kinds of milk (a1 and a2), the most abundant compounds (in terms of relative percentage) were the palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) as saturated fatty acids. The most abundant unsaturated fatty acids in both samples were as follows oleic acid (C18:1), linoleic acid (C18:2 omega6), α-linolenic acid (C18:3 omega3).**

Our results of fatty acid methyl esters profile are in accordance with Van Nieuwenhove et al. (2009). The author explained that palmitic (C16:0), stearic (C18:0), and myristic (C14:0) acids were the most abundant fatty acids. The monounsaturated fatty acids were 26.6 and 27.4 g/100 g of fatty acids, respectively, with oleic acid (cis-9, C18:1) as the most abundant.

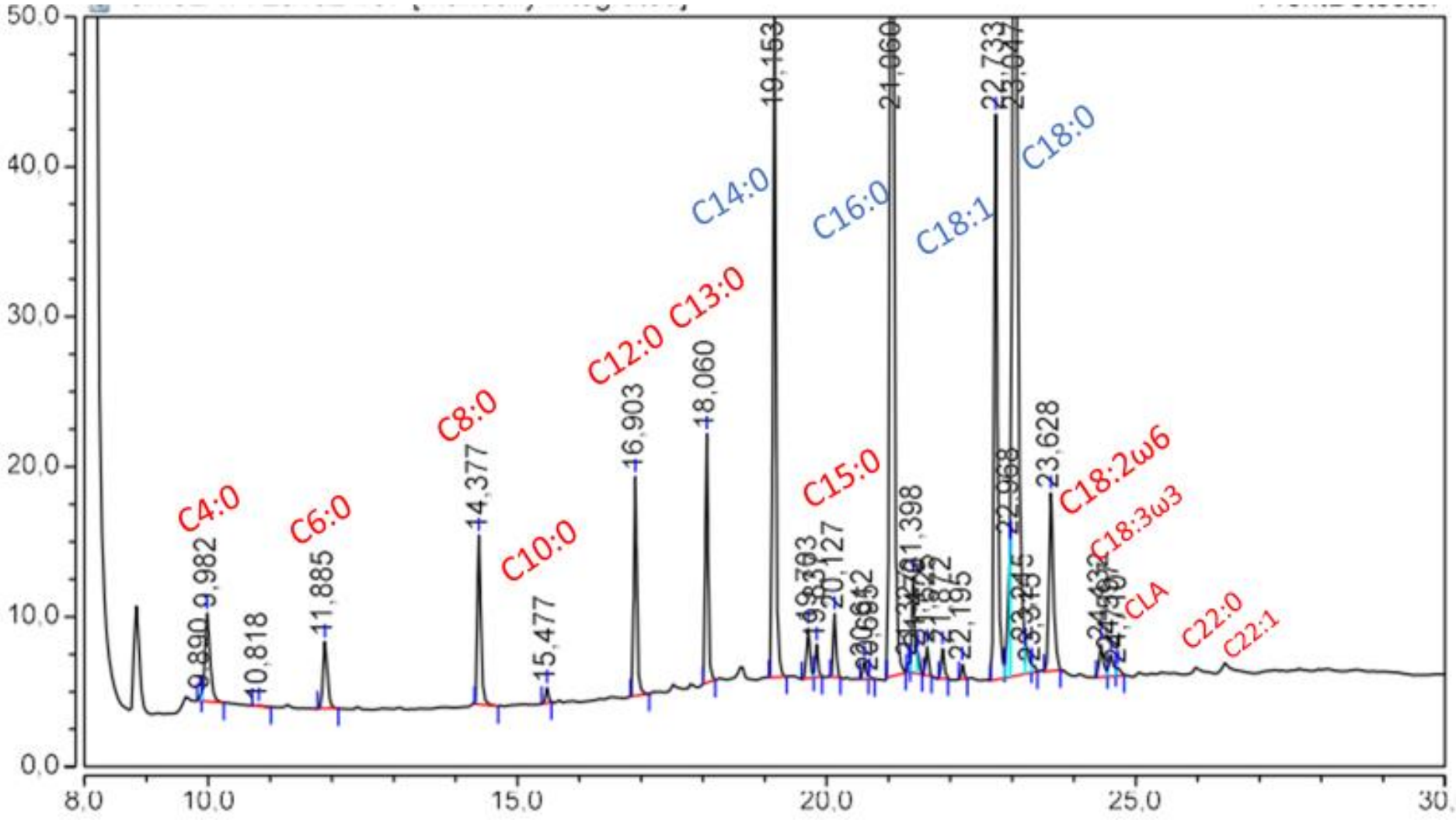


Figure 24. Example of GC-FID chromatogram of fatty acid profile in milk a1.

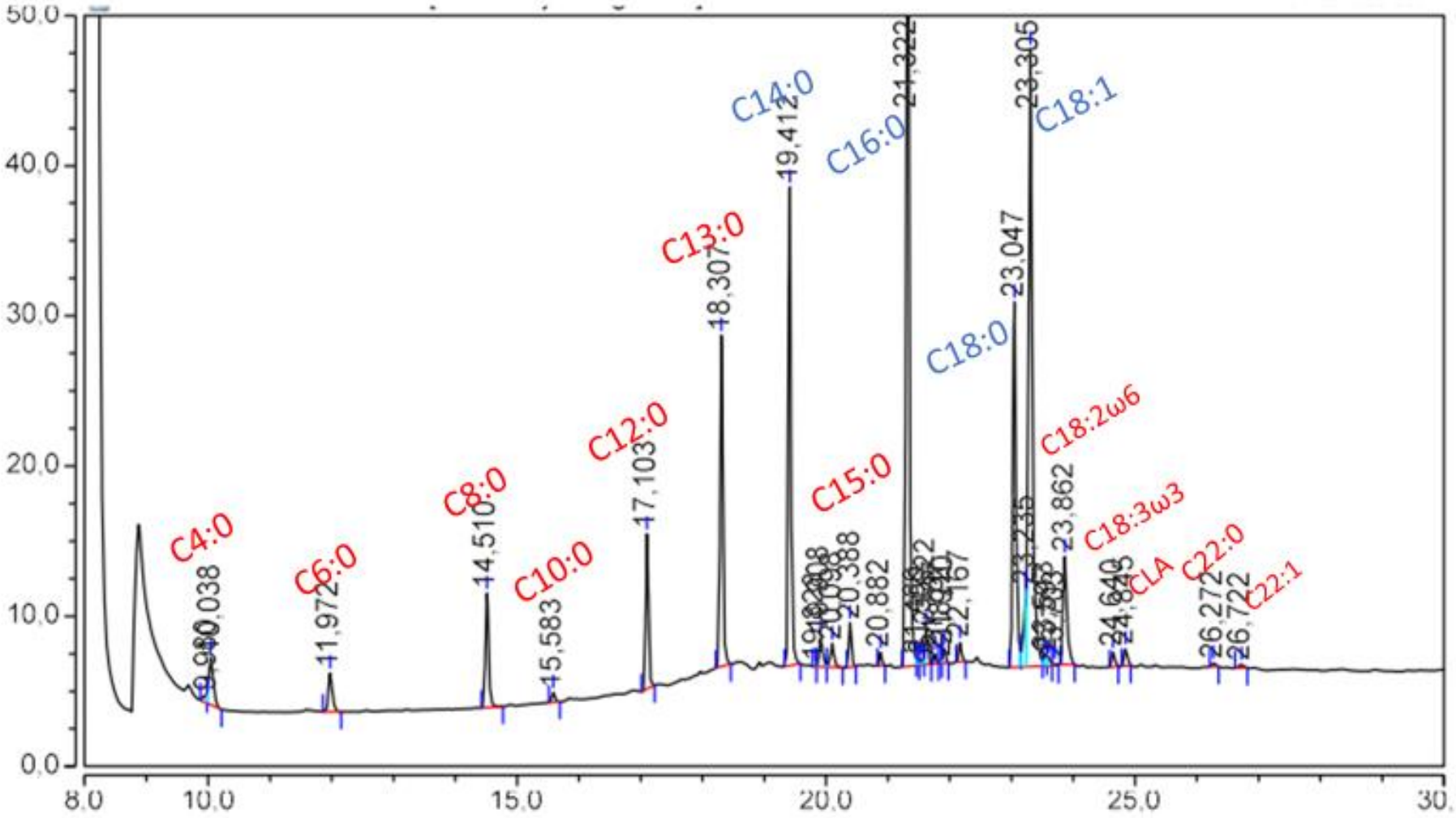


Figure 25. Example of GC-FID chromatogram of fatty acid profile in milk a2 farms nor between the seasonal periods (P>0.05).

4.1.1 SFA, MUFA, PUFA and trans fatty acids of milk a1 and a2

The fatty acid profile in terms of SFA (solid fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated acids), and trans (trans fatty acids) of milk a1 and a2 are reported in Table 8. **SFA of a1 milk ranged from 58.6 to 72.5 %, while 55.2 to 69.9% for a2 milk. PUFA (omega 3 and omega 6 fatty acids) varied in a range of 4.3-7.0 and 4.2-7.2 for milk a1 and a2, respectively. Trans fatty acids were present in an average value of 3.5 and 3.4% for a1 milk and a2 milk, respectively.**

Our results in the fatty acid profile of cow milk agree with Mollica et al. (2021). The authors explained that the milk of ruminants is characterized by a high content of SFA (about 70% of total fatty acids), a low content of PUFA (less than 3%, including ω-3 fatty acids and CLA, conjugated linoleic acid), and trans-fatty acids (around 4%, including vaccenic acid or trans-11 C18:1).

Table 8. Fatty acid profile in terms of SFA (solid fatty acids), MUFA (monounsaturated fatty acids), PUFA (polyunsaturated acids), and trans (trans fatty acids) of milk a1 and a2 collected simultaneously in different periods, expressed as % of fatty acids. Data are reported as a mean value of two replicates ± standard deviation.

Sample	SFA a1	MUFA a1	PUFA a1	trans a1	sample	SFA a2	MUFA a2	PUFA a2	trans a2
1 A1	63.2 ± 0.4	26.6 ± 0.2	7.0 ± 0.1	3.3 ± 0.1	1 A2	62.5 ± 1.1	27.6 ± 1.4	6.8 ± 0.4	3.1 ± 0.1
3 A1	60.0 ± 0.3	32.2 ± 0.2	5.8 ± 0.2	2.1 ± 0.3	3 A2	63.7 ± 6.9	27.9 ± 5.2	5.9 ± 1.6	2.5 ± 0.1
4 A1	60.4 ± 0.3	29.8 ± 0.2	5.7 ± 0.2	4.1 ± 0.2	4 A2	55.2 ± 0.1	33.6 ± 0.8	7.2 ± 0.1	4.0 ± 0.7
5 A1	58.6 ± 0.6	32.3 ± 0.7	5.5 ± 0.2	3.7 ± 0.0	5 A2	66.4 ± 0.2	25.0 ± 0.0	5.2 ± 0.3	3.4 ± 0.1
6 A1	66.8 ± 0.6	24.7 ± 0.4	5.4 ± 0.1	3.1 ± 0.1	6 A2	67.9 ± 0.9	23.9 ± 0.8	4.7 ± 0.3	3.5 ± 0.1
7 A1	67.1 ± 0.1	25.3 ± 0.1	4.3 ± 0.0	3.4 ± 0.2	7 A2	65.1 ± 0.9	26.2 ± 0.8	5.5 ± 0.1	3.2 ± 0.0
8 A1	64.4 ± 0.5	25.4 ± 0.1	5.6 ± 0.2	4.6 ± 0.4	8 A2	64.2 ± 0.6	26.4 ± 1.0	5.4 ± 0.4	4.0 ± 0.9
9 A1	62.3 ± 0.1	27.9 ± 0.1	5.2 ± 0.1	4.6 ± 0.1	9 A2	68.0 ± 0.9	24.4 ± 0.5	4.7 ± 0.3	2.9 ± 0.0
11 A1	61.5 ± 0.1	29.1 ± 0.1	5.4 ± 0.0	4.1 ± 0.0	11 A2	66.0 ± 0.2	26.7 ± 0.0	4.3 ± 0.0	3.0 ± 0.2
12 A1	62.8 ± 0.2	27.8 ± 0.0	5.8 ± 0.1	3.7 ± 0.3	12 A2	62.3 ± 0.2	28.6 ± 0.1	5.2 ± 0.0	3.9 ± 0.1
13 A1	60.1 ± 0.1	30.6 ± 0.1	5.4 ± 0.1	3.9 ± 0.0	13 A2	63.5 ± 2.1	27.7 ± 1.3	5.1 ± 0.3	3.7 ± 0.5
15 A1	61.0 ± 0.2	30.4 ± 0.1	5.0 ± 0.0	3.6 ± 0.3	15 A2	66.3 ± 0.5	25.9 ± 0.9	4.2 ± 0.3	3.6 ± 1.1
16 A1	69.1 ± 0.7	23.3 ± 0.6	5.1 ± 0.0	2.6 ± 0.1	16 A2	68.8 ± 0.4	23.2 ± 0.3	5.0 ± 0.1	3.0 ± 0.2
18 A1	63.6 ± 0.5	27.0 ± 0.4	5.7 ± 0.2	3.7 ± 0.1	18 A2	66.8 ± 0.0	24.4 ± 0.2	5.3 ± 0.1	3.5 ± 0.3
20 A1	64.9 ± 0.7	25.3 ± 1.0	5.3 ± 0.1	4.6 ± 0.1	20 A2	65.6 ± 0.1	25.1 ± 0.0	5.3 ± 0.1	4.0 ± 0.0
21 A1	72.5 ± 0.6	20.0 ± 0.6	4.3 ± 0.0	3.2 ± 0.1	21 A2	69.9 ± 0.9	20.3 ± 0.7	5.8 ± 0.2	4.0 ± 0.0
22 A1	65.5 ± 0.7	26.5 ± 0.2	5.3 ± 0.2	2.7 ± 0.3	22 A2	69.9 ± 0.5	22.0 ± 0.3	5.4 ± 0.0	2.7 ± 0.1
23 A1	65.8 ± 0.5	25.3 ± 0.5	5.6 ± 0.0	3.2 ± 0.0	23 A2	66.5 ± 0.2	25.4 ± 0.1	5.0 ± 0.1	3.1 ± 0.2
24 A1	63.9 ± 0.1	26.4 ± 0.3	6.1 ± 0.3	3.5 ± 0.6	24 A2	65.6 ± 1.2	25.7 ± 0.5	5.4 ± 0.4	3.4 ± 0.3

4.1.2 Principal component analysis of milk a1 and a2

To better evaluate the influence of genetic variability of cows in terms of beta-casein on milk fatty acids, we performed the PCA, Principal Component Analysis using 33 variables (fatty acids identified and quantified in milk samples) as reported in Figure 26. Our sampling plan explained 38.14% of the total variance, with Principal component 1 accounting for 22.97% of the variance and Principal Component 2 of 15.17%. The samples are plotted on the Score Plot (left) in the function of the Loading Plot (right) which reports the variables (loading) used to explained the variance, having more or less of variance as reported but the length of the arrow (longer=higher impact on variance). The samples are scattered on the 4 quadrants of the plot

with some outliers. In the Score plot the samples were colored in red for a2 milk and in black for a1 milk samples. We can see an attempt of separation of a1 and a2 samples in the quadrants, but this separation is not well defined, meaning that **the fatty acid profile did not varied between a1 and a2 milks as the total variance explained with the Principal component analysis was of 38.14%.**

The variance of data could be due also to the sampling as the milks samples were collected from January 2020 till July 2021, and it is well know that animal feeding affects the fatty acid profile. The animal feeding this study was standardized across the samplings, but little variation of the feeding could explain variability of our results. Frelich et al. (2012) reported that the seasonal change between the pasture-based and silage-based diets was found to be a principal cause of the changes in the milk fat profile. The fresh herbage intake resulted in the increase in long-chain fatty acids (stearic and unsaturated FAs) and the decrease in the short- and medium-chain FAs including the hypercholesterolemic lauric, myristic and palmitic acids. The seasonal increase in unsaturated FAs against the saturated FAs and omega-3 against omega-6 polyunsaturated FA indicated that the milk yielded in summer was more beneficial to consumers' health than that yielded in winter.The concentration of oleic acid – the major MUFA of a cow milk – was higher in the pasture period than in the winter period. The substitution of saturated fatty acids with oleic acid is desirable because it reduces the risk of a coronary heart disease. The mean CLA concentration in milk was more than twice as high in the pasture period than during the winter period.

Perna et al. (2016)showed that casein haplotype significantly affected the fatty acid composition of milk. In this study, despite significant differences in some individual SFA, they found no significant effect for total SFA content. In BB-A2A2-BB and BB-A1A1-AA the first, second and third place represent different allelic combinations of loci αS1-, β-, and κ-CN. In BB-A2A2-BB and BB-A1A1-AA milk have lower C4:0 content compared with milks of other haplotypes. Several studies have highlighted the role of C4:0 fatty acid on human health as C4:0 regulates fluid transport, protects colonocytes from oxidative stress, and modulates cell proliferation and differentiation; it is also important for its alleged antitumor activity. The BB-A2A2-AB, BB-A2A2-BB, and BB-A2A1-AA milks showed the highest contents of saturated short-chain fatty acids C6:0, C8:0, and C10:0). These fatty acids are particularly digestible, have a low tendency for adipose formation, and (together with C18:0) do not affect serum cholesterol levels.

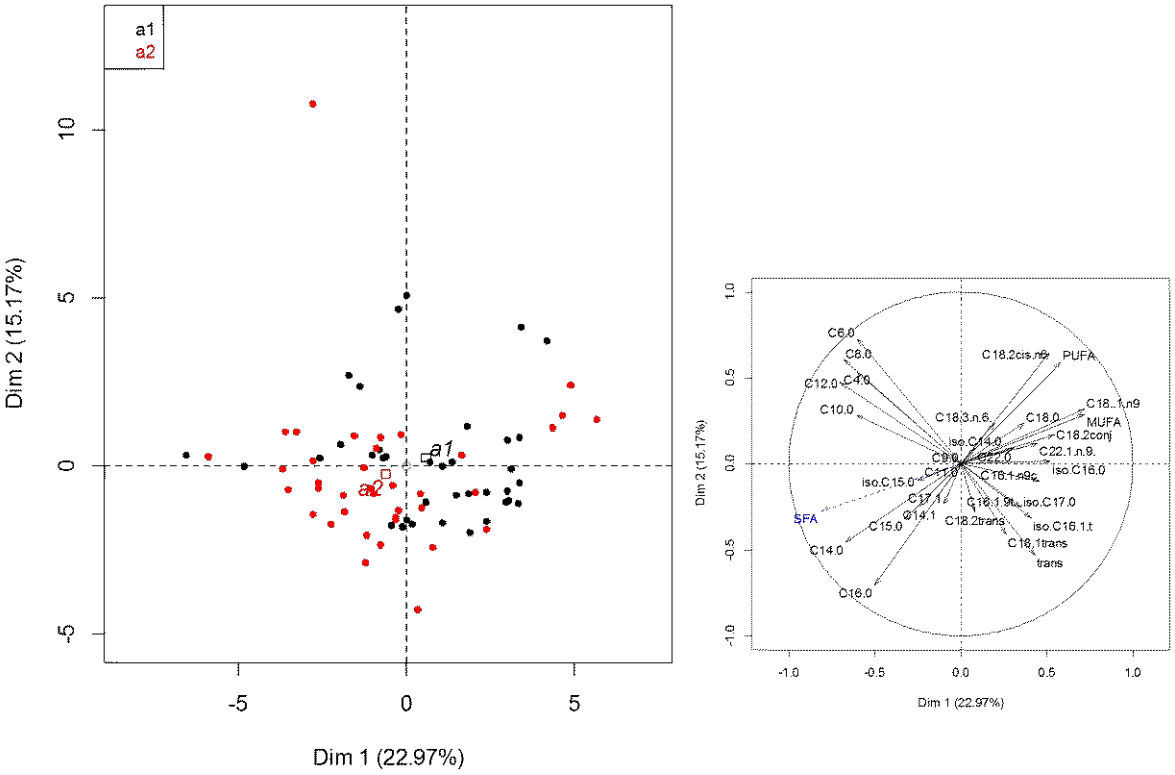


Figure 26. Principal component analysis (PCA) of milk a1 and a2 collected in 2020 and 2021. 19 sampling were performed with 38 analysis conducted in two replicates. Score plot (left) and loading plot (right). 33 variables (fatty acids identified) are included in the PCA.

4.2 Fatty acids methyl esters profile of cheeses a1 and a2

The fatty acid methyl esters profile was analyzed in 7 fresh cheese samples and 8 ripened cheese samples (15 samples in total) collected in 2020 and 2021. The identified fatty acid methyl esters are reported as examples of GC-FID chromatogram in Figure 28 and Figure 29. **In cheeses prepared from milk a1 and a2, the most abundant compounds (in terms of relative percentage) were the palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) as saturated fatty acids. Lauric acid (C12:0), capric acid (C10:0), caproic acid (C6:0), linoleic acid (C18:2 *cis-9,12*) and caprylic acid (C8:0) were also detectable while the others occurred only in traces**.

Our results of fatty acid methyl esters profile in Cheese is almost similar with the Eisenstecken et al.(2021). He reported that the most abundant fatty acids found in cheese-extracted fat were palmitic acid (C16:0), oleic acid (C18:1 *cis-9*), mystiric acid (C14:0) and stearic acid (C18:0), accounting for about 79% of the total fatty acids.

In comparison, to ripened cheese, freshly manufactured Caciotta cheese, the level of butyric acid (C4:0) and caproic acid (C6:0) is low whereas the level of long-chain fatty acids were considerably higher (Figure 27).

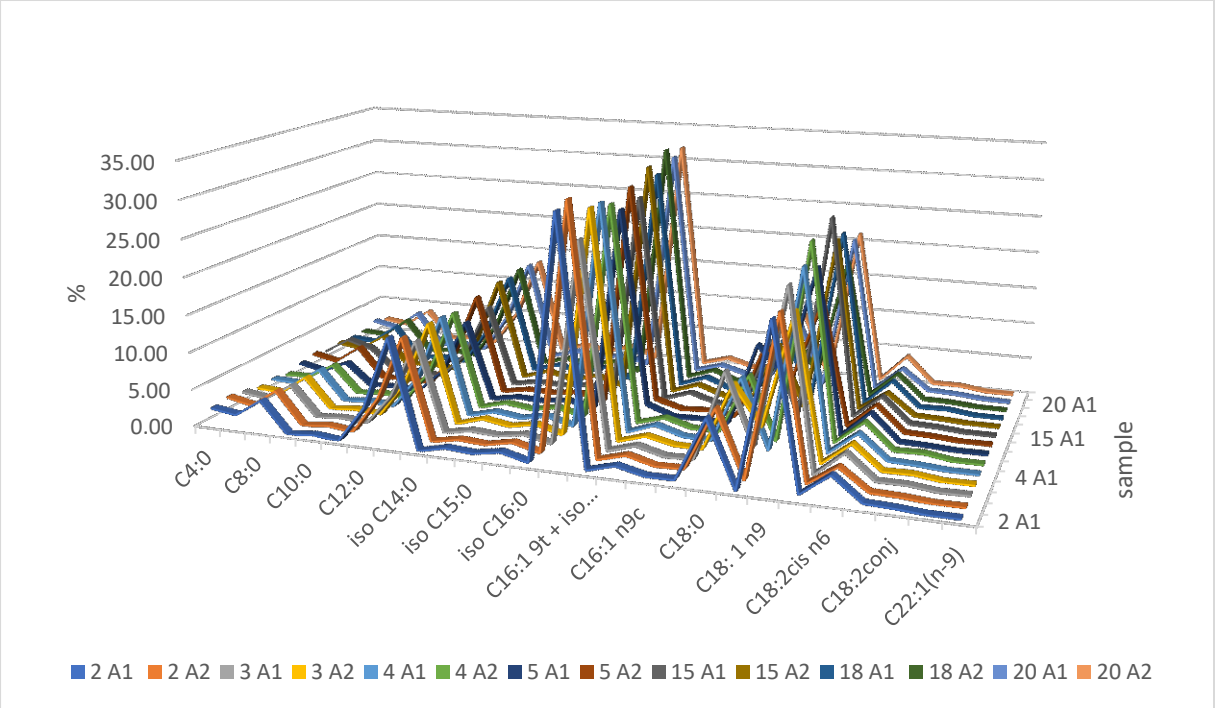


Figure 27. Fatty acids profile of fresh cheeses (15 days of ripening) produced with milk a1 and milk a2. Data are reported as % of fatty acids of one replicate.

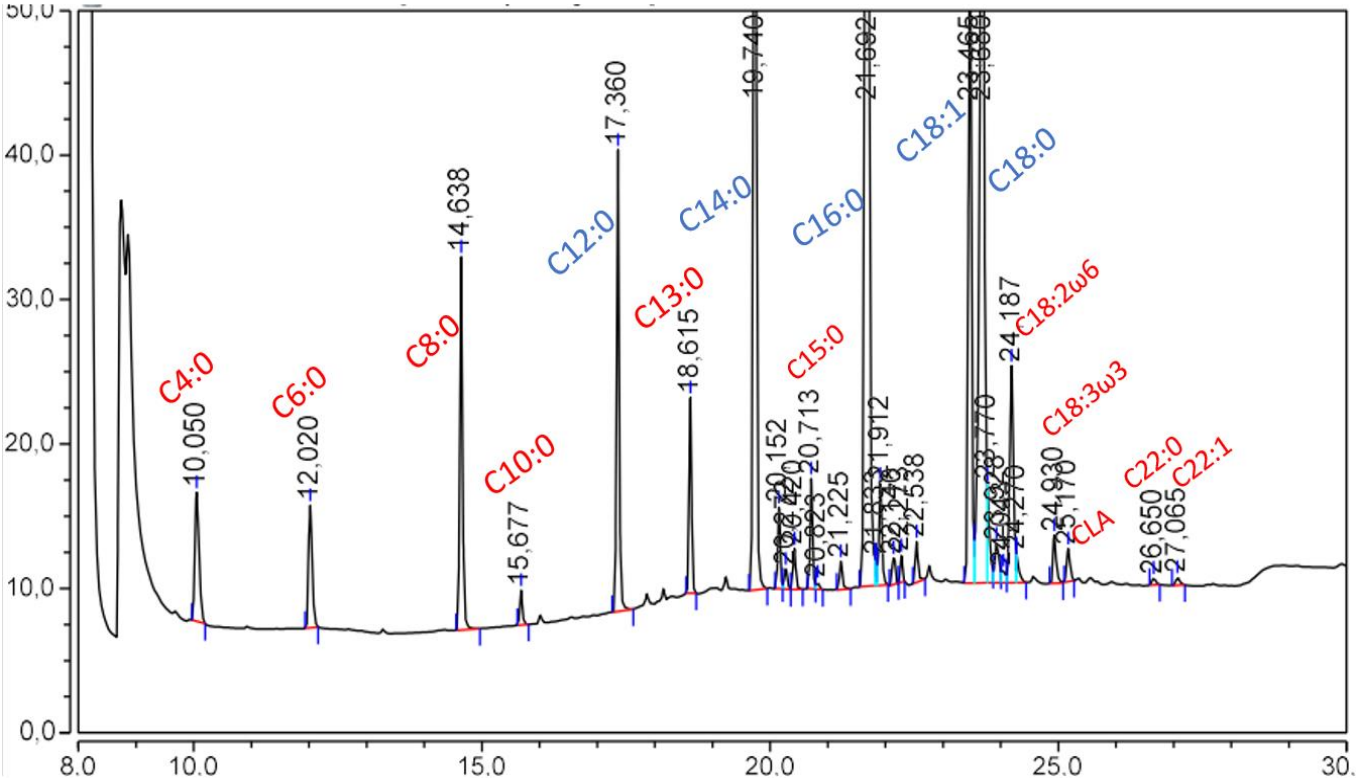


Figure 28. Example of GC-FID chromatogram of fatty acid profile in cheese a1.

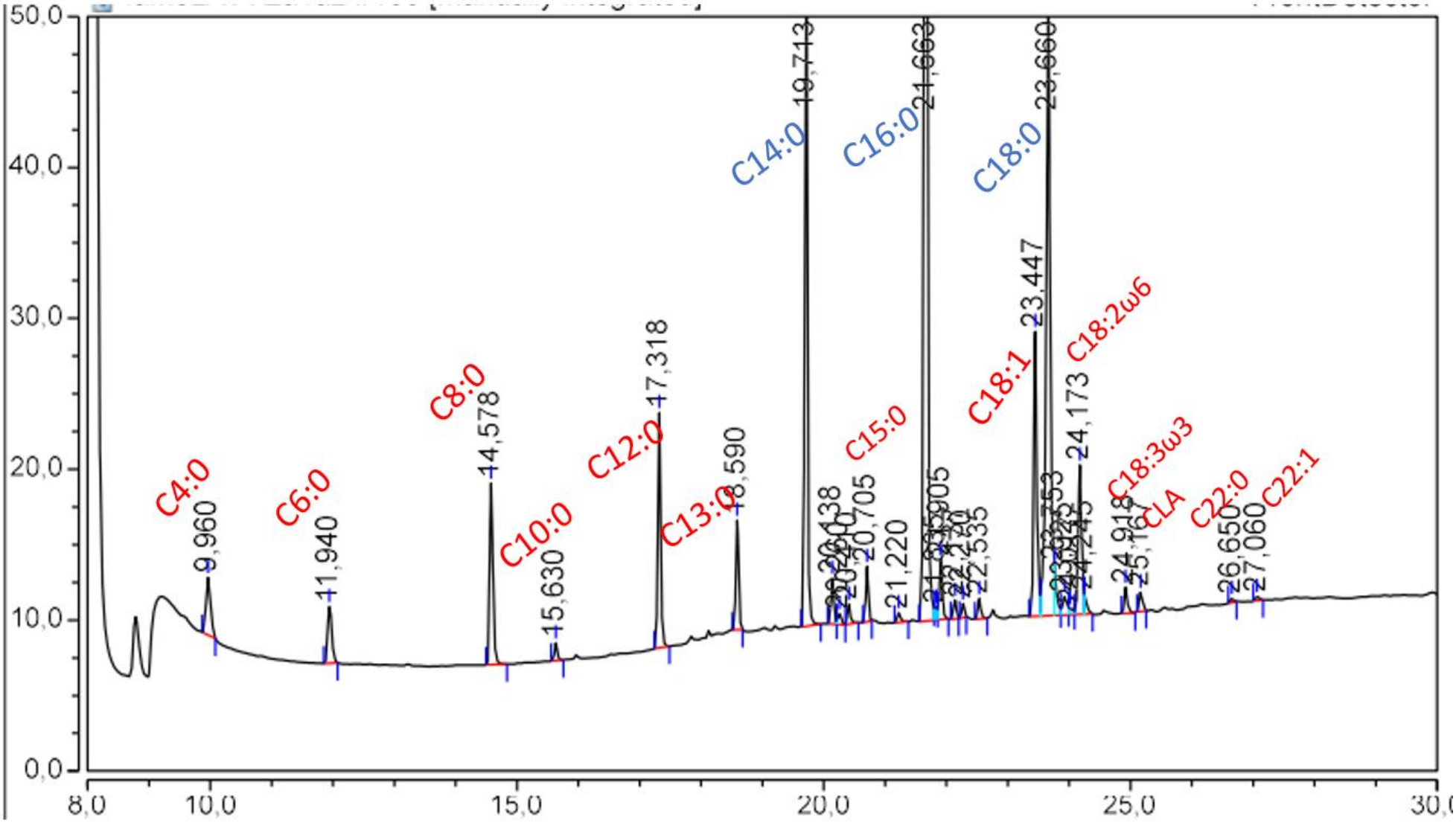


Figure 29. Example of GC-FID chromatogram of fatty acid profile in cheese a2

4.2.1 Influence of ripening

The evaluation of influence of ripening on milk fatty acid profile is done by the PCA, Principal Component Analysis using 33 variables (fatty acids identified and quantified in ripened cheese samples) as reported in Figure 30. **Our sampling plan explained 46.17% of total variance, with the Principal component 1 accounting for 26.34% of variance and the Principal Component 2 of 20.17%.** The samples are plotted on the Score Plot (left) in function of the Loading Plot (right) which reports the variables (loading) used to explained the variance, having more or less of variance as reported but the length of the arrow (longer=higher impact on variance). **The samples are scattered on the 4 quadrants of the plot. The fresh cheese are characterized with Saturated fatty acids (SFA), butyric acid (C4:0), caproic acid (C6:0) and caprylic acid (C8:0) whereas the cheese which 8.4 months ripened are characterized with Polyunsaturated fatty acids (PUFA) and long chain fatty acids. In addition, the 5.6 month ripened cheese are characterized by the monounsaturated fatty acid (MUFA), decanoic acid (C10:0) and oleic acid (C18:1n9).**

Woo & Lindsay (1984)mentioned that the concentrations of carbons 4 and 6 free fatty acids decreased, whereas those of carbons 14 to 18 increased during latter stages of aging.

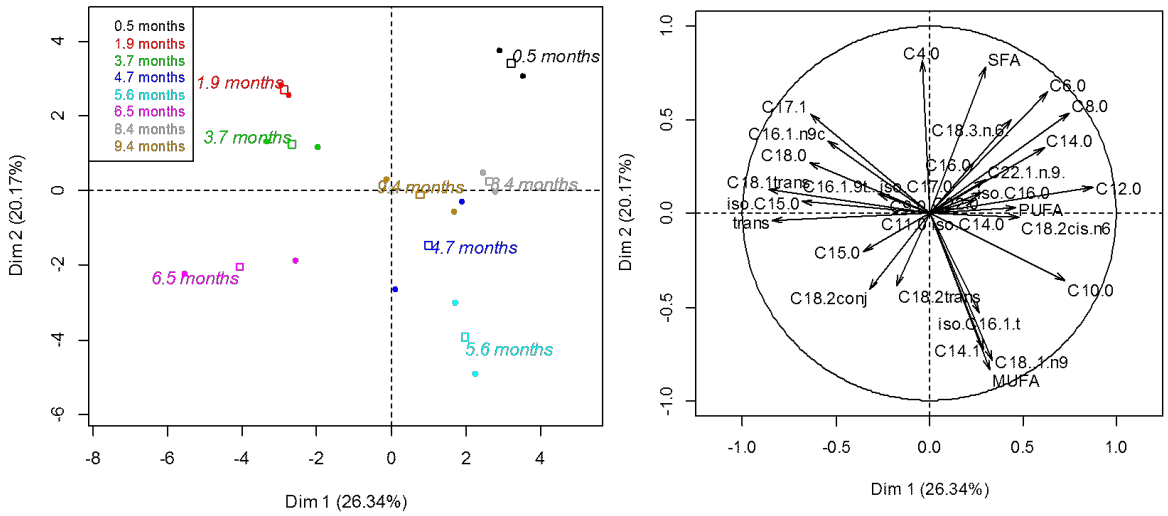


Figure 30. Principal component analysis (PCA) of ripened cheeses a1 and a2 collected in 2020 and 2021. 8 samplings were performed with 16 analysis conducted in one replicate. Score plot (left) and loading plot (right). 33 variables (fatty acids identified) are included in the PCA. In the score plot is reported with different colors the supplementary factor (age of ripening).

4.2.2 Influence of genetic variation a1 and a2

The assessment of influence of genetic variation in cheese prepared from milk A1 and A2 in terms of beta-casein is done by Principal Component analysis using 33 variables (fatty acids identified and quantified in milk samples) as reported in Figure 31. **Our sampling plan explained 46.17% of total variance, with the Principal component 1 accounting for 26.34% of variance and the Principal Component 2 of 20.17%.** The samples are plotted on the Score Plot (left) in function of the Loading Plot (right) which reports the variables (loading) used to explained the variance, having more or less of variance as reported but the length of the arrow (longer=higher impact on variance). The samples are scattered on the 4 quadrants of the plot. In the Score plot the samples were colored in red for a2 milk and in black for a1 milk samples. The samples are scattered on the four quadrants of plot. This may be possible due to the ripening condition of cheese as different samples are at the different stage of ripening and ripened cheese whether prepared from A1 or A2 doesn't relate with Beta-casein.

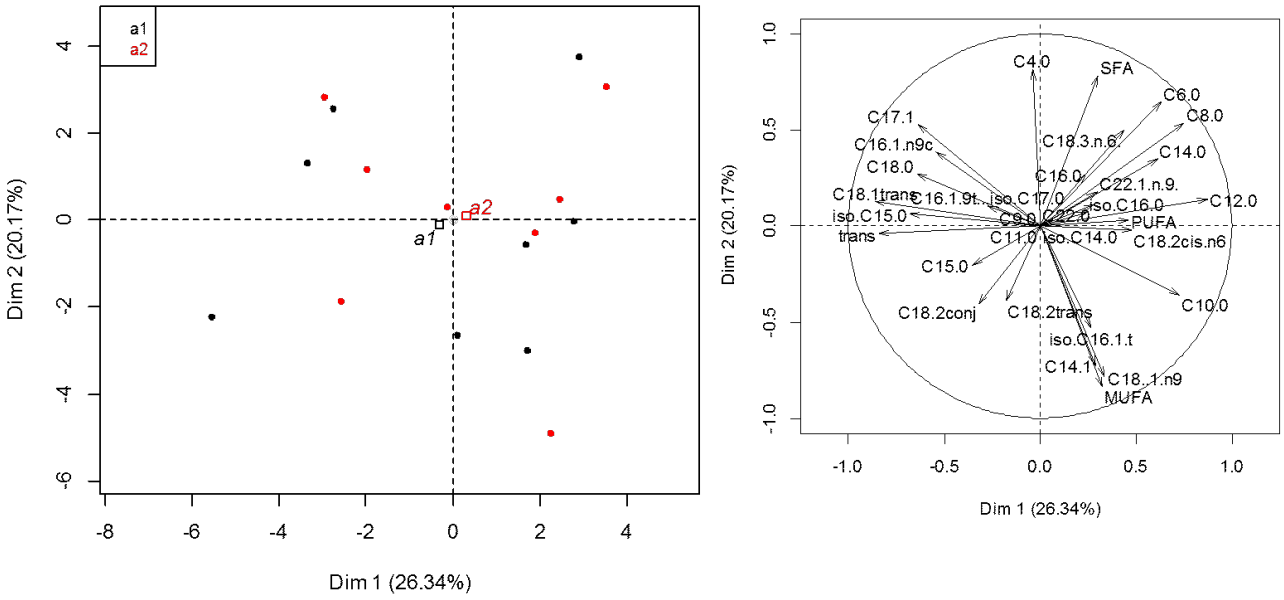


Figure 31. Principal component analysis (PCA) of ripened cheeses a1 and a2 collected in 2020 and 2021. 8 samplings were performed with 16 analysis conducted in one replicate. Score plot (left) and loading plot (right). 33 variables (fatty acids identified) are included in the PCA. In the score plot is reported the supplementary factor (genetic variation of beta-casein), in red and black.

5. Conclusion

The market of A2 milk is growing globally. A2 milk could be beneficial in alleviating symptoms of gastrointestinal distress in a certain demographic of individuals. Nonetheless, the mechanisms for these interactions and criteria to identify consumers that would benefit from A2 milk are still poorly understood. Therefore, evidence to support the promotion of A2 milk is currently insufficient.

This thesis work was focused on the fatty acid profile of milk A1 and A2, fresh and ripened cheese. The results are quite interesting as it shows the fatty acid profile of both milk and the cheese prepared from these milk are similar. In particular, **saturated fatty acids of a1 milk ranged from 58.6 to 72.5 %, while 55.2 to 69.9% for a2 milk whereas PUFA (omega 3 and omega 6 fatty acids) varied in a range of 4.3-7.0 and 4.2-7.2 for milk a1 and a2, respectively. Trans fatty acids were present in an average value of 3.5 and 3.4% for a1 milk and a2 milk, respectively. In cheeses prepared from milk a1 and a2, the most abundant compounds (in terms of relative percentage) were the palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) as saturated fatty acids. Lauric acid (C12:0), capric acid (C10:0), caproic acid (C6:0), linoleic acid (C18:2 *cis-9,12*) and caprylic acid (C8:0) were also detectable while the others occurred only in traces**.

The Project I-Milk a2- PSR MARCHE 2014 is analysing also other parameters to better understand the difference between A1 and A2 milk.

6. References

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