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Bovine Lactation and Reproduction Physiology: A Survey on Some Fundamental Features In Autochthonous Dairy Breeds Reared in Northern Italy

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to my family…

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CHAPTER 1

Foreword

1. Foreword

1.1. Demographic pressure, food needs, depletion of water, vegetal and animal biodiversity resources.

The United Nations is alerting worldwide population: our planet will undergo a drastic change in less than 40 years. Human population will increase to reach 9 billion inhabitants; consequently, cereal annual production should increase to be about 3 billion tons compared to current 2.1tons. According to a Food and Agriculture Organization's report FAO, "FAO 2050", human birthrate is expected to prevail in developing countries. Thus, food resources, such as wheat, corn, barley, and others, will be used to ensure people survival in these countries, which will reduce availability of silage and concentrates addressed to cattle breeding. This redistribution of primary resources will force bovine species to a diet based on forage biomass with low energy and water consumption, associated with the use of less productive areas such as foothills, forests and pastures, thus implementing availability of cereals for human consumption. Noticeably, climate changes will persist and modify any environment where plants grow. In this context, farmers and breeders will be faced with new challenges. Maintaining genetic biodiversity in plants including some wild peculiarities may help to solve some of current agricultural problems and protecting such biodiversity appear therefore crucial to increase a sustainable and efficient use of land. According to 'Europe 2020' protocol established by United Nations, greenhouse gas emissions must be reduced by 20%, energy efficiency increased by 20% with a reduction in consumption, and energy from renewable sources pushed up by 20%. Therefore, it is expected that in certain areas long-term and perennial crops will be implemented; energy consumption for sowing, harvesting, and drying products for livestock consumption will be reduced; availability of mature manure to improve soil fertilization, reducing easily washable nitrogen, will be increased. In the coming decades, an actually thorny scenario will affect future generations: processes such as steady population and consumption growth will cause a reduction in the response capacity of ecosystems, and a consequent decrease in food resources, water and energy. The agricultural system is strictly implied in the abovementioned issues, and plays its role as a multifaceted character. In fact, agriculture can give answers to the drawing demand for food while consuming huge quantities of water and fuels on the planet to achieve its goals. To date, care for biodiversity is pivotal to improve and increase a sustainable and efficient use of land.

1.2. Dairy cattle breeding: European and Italian scenarios

In parallel with vegetal selection aimed to maximize production, even farm animal biodiversity has been damaged to select animals with rapid food conversion into milk and meat. This mono-aptitude selective criterion has caused a decline in several sectors, the main ones being reproductive performances and quality of products (Schennink et al., 2007), problems easily noticed in the dairy cattle scenario. In fact, mainly in Friesian breeds, reproductive performances have decreased worldwide, with negative consequences to cows robustness and longevity by increasing stress, udder health disturbances and locomotion disorders (Roxström et al., 2001a). Holstein, Brown Swiss and Jersey, subjected to a mono-aptitude selection in the last 40 years, almost aimed to a quantitative milk production, reach, from a physiological point of view, a very critical situation: they have missed good reproductive efficiency characters, (e.g. calving interval and conception rate (Sørensen et al., 2007), excellent longevity in farm, resistance to stress and diseases (metabolic syndrome, ketosis, mastitis and foot diseases)(Roxström et al., 2001a; Roxström et al., 2001b; Carlén, 2004)), whereas they have dramatically increased "energy and financial voracity" (diet based on starch and protein meals, great health and structural investments due to several high recurring diseases (Ingvartsen et al., 2003; Collard et al., 2000; Carlén et al., 2004).

Italian dairy breeding has followed the European trend, by selecting high yielding Holstein cows, influencing some important milk quality parameters, such as milk fat, protein and somatic cell count, all involved in cheese making processes. Recently, Italian researchers have demonstrated that milk with a somatic cell content greater than 400,000 cells/ml evidences a scarce aptitude to rennet coagulation and, in general, it does not seem to be suitable for cheese production, with particular reference to Grana cheese production (Sandri et al., 2010). Italy is the most important cheese producer and exporter in Europe, with its about 460,000 tons of products and almost 3 billion Euros (data from www.clal.it, www.ismea.it) derived from PDO (Protected Designation Origin) and PGI (Protected Geographical Indication). The most representative Italian products are certainly Parmigiano Reggiano and Grana Padano, which have recently increased by 9.8% their export trend to Germany, the United States, France, Switzerland and the United Kingdom. Therefore, both for the Italian breeding system and for the whole country it is very important to try to solve current problems related to health and welfare of their dairy cows, in order to maintain a unique cheese production and exportation.

An innovative idea lies in finding solutions to the current dairy breeding scenario by orienting our sight on the existing Italian bovine heritage, and diversifying from the current thought that has driven genetic selection of dairy cosmopolitan breeds in the last 40 years. Using very rustic and frugal cattle with double (milk and meat) or threefold (milk, meat and work) aptitude, such as Italian autochthonous cows, showing a good food conversion into milk and meat, would be highly advantageous, compared with the more cosmopolitan and selected Holstein, Brown Swiss and Jersey, unable to maintain high milk yield and reproduction standards, reared with a protein-deficient diet mainly based on forage and cereals.

1.3. Autochthonous dairy cattle of northern Italy

1.3.1. Breed: origin, diffusion, traits, aptitude – Cabannina (Bigi and Zanon, 2008)

Figure 1.1 - Cabannina cow.

These cows are native to the province of Genoa and are reared in Liguria region and in Pavia province in Lombardy; currently, about 220 heads are enrolled in the population register. They have a dark brown coat with light-colored lines and reddish shades. Their head is small, short, light; their muzzle is black and widely white-bordered. Their reduced size (withers height of 1.22 cm for females and 134 cm for males, with a maximum adult live weight of 4.5 quintals), short and powerful limbs, and very hard claws, make these animals excellent to grazing, the only animals able to effectively use plant resources in the high slopes of the

Ligurian Apennines. The Cabannina Breeding, based on local grazing for a large part of the year, determines the specific characteristics of flavor and authenticity of the final products, milk and cheese. For this reason, it can be said that Cabannina is the testimony for the province of Genoa to the indissoluble link between land and its products, and that it perfectly expresses the adaptation process of the characteristics of both breed and environment, where it has evolved and maintained over time. Milk production from this cow breed is kept on 20 to 30 quintals/year and its high longevity (it is common to find in farm cows aged over 12 years) confirms their excellent adaptation to their territory. Their cheese, entirely derived from raw milk, is called "U Cabanin" (The Cabanin). It was established in 2007 thanks to the intervention of the Breeders Association and the Chamber of Commerce of Genoa. From 2010, U Cabanin is one of Slow Food Presidia and according to Carlo Petrini, Slow Food Association founder and President, Cabannina cow breed represents a correct mix of pleasure for food and responsibility, sustainability and harmony with nature.

1.3.2. Breed: origin, diffusion, traits, aptitude - Varzese-Ottonese-Tortonese (Bigi and Zanon, 2008)

Figure 1.2 - Varzese cow

As clearly shown by its long compound name, this breed'sarea of origin includes the northern Italian Apennines and 4 neighbouring regions: Lombardy, Emilia Romagna, Liguria and Piedmont. Currently there are about 240 heads enrolled in the population register, atomized in few farms in Pavia, Piacenza, Alessandria and Milano provinces. The cows in this breed show a uniform reddish-blond coat, more or less intense, with limited lighter shades around their muzzles, eyes, bellies, inner thighs and distal limbs. Their medium size (withers height of 135 cm for females and 145 cm for males with a maximum adult live weight of 5.5 quintals), and their distinct characteristics of rusticity, frugality, fertility and longevity (this breed can easily reach 10 births and the goal of one calf/year) make these animals be used as first choice in marginal areas like mountain, wood and foothill grazes. Born to be primarily used for work - in fact, bulls were famous for their strength, endurance, docility and for their resistant hoofs), and even cows were employed for rapid and light draft - this breed was then appreciated for the production of meat and milk. used to make excellent cheese today including some important cheese named "Nisso", “Robiola”, “Montebore” and "Molana", all produced in Oltrepo regions, areas lying south of the River Po. The Varzese breed has been recently required by farms where tourists can be lodged and/or consume local products. In addition, it can be found in teaching farms and, as it recalls traditional aspects of the ancient rural world, it plays a role in folklore events.

CHAPTER 2

Objectives

2. Objectives

2.1. Comparative Study Among Breeds.

To understand to what extent the regulatory mechanisms of production and reproduction of high yielding dairy cows can be altered, comparative physiological studies within Italian dairy breeds experiencing no genetic improvement were necessary. This investigation aimed to increase scientific information about these animals in the perspective of developing a more efficient and sustainable dairy breeding. Data now available, provided by ongoing research at our laboratory, are actually encouraging. In our research, two endangered local bovine breeds of Northern Italy, Varzese and Cabannina, were considered and compared with Friesian cows in relation to some aspects characterizing lactation and reproduction.

CHAPTER 3

MUFA and PUFA content in milk of Varzese and Cabannina cows, two local bovine breeds of Northern Italy

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3. MUFA and PUFA content in milk of Varzese and Cabannina cows, two local bovine breeds of Northern Italy

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3.1. Abstract

In the last 40 years, the first goal pursued by dairy cattle community has been to increase individual production from a quantitative point of view, by high yielding dairy cows selection (BLAP). This mono-aptitude criterion has caused a decline in reproductive performances worldwide (eg increase in calving interval). The comparative physiological study of autochthonous cattle to value "Ancient Autochthonous Biodiversity" opens up interesting prospects for livestock to comply the FAO 2050 target, as these animals are guarantors of a good milk production, excellent and typical cheese making, peculiar characteristics of rusticity, frugality, longevity and resistance to multiple diseases. Therefore they appear to be good candidates for traditional food production. The study of autochthonous breeds lactation and reproduction physiology could be strategic to improve knowledge and develop a more rational dairy livestock in view of

reduced availability of cereals and plant proteins for livestock in the next 40 years.

From a productive point of view the most variable component was analyzed. The importance of fat composition of milk in human and animal nutrition has in the last years come rapidly to the fore. Several nutritionist Associations recommend the intake of higher levels of unsaturated fatty acids (*cis*-MUFA and PUFA) in milk despite of saturated fatty acid levels: increases in *cis*-MUFA and PUFA in milk fat can be reached by enhancing the activation of the enzyme Δ9-desaturase or by selecting subject with high desaturating ability. In this research, two endangered local bovine breeds of Northern Italy, Varzese and Cabannina, were considered for the *cis*-MUFA and PUFA content and compared with the *cis*-MUFA and PUFA levels in Friesian cows bred in the same conditions. The milk collections were performed from the 40^{th} to the 180^{th} day of lactation. Milk fat was analyzed by gas chromatography and desaturase indices for C14, C16 and C18 fatty acids were determined. Varzese and Cabannina milk fat contains significantly overall higher levels of some *cis*-MUFA and PUFA with respect to Friesian, and higher desaturase indices, mainly C16 desaturase and the total desaturase indices. The results rise the importance of the recovery and the reevaluation of local breeds, not only for the biodiversity *per se*, but also in order to bring out peculiar characteristics of niche milk.

3.2. Introduction

Fatty acid composition is an important feature in milk, since it influences its physical, organoleptic and nutritional properties (Chilliard et al., 2000). Recent studies showed that the composition is determined by several factors, as diet (Banks et al., 1983; Grummer, 1990; Perfield et al., 2006; Perfield et al., 2007), stage of lactation and season (Coulon, 1994), genetics (Schennink et al., 2007).

Breed has a relevant role in milk composition and especially in fatty acid profile (Soyeurt et al., 2006). In a recent report, Malacarne et al. (2001) have demonstrated interesting differences between some Italian bovine breeds, such as Italian Friesian, Italian Brown, Reggiana and Modenese, reared in the Parmigiano Reggiano cheese production area. The most important differences were observed for palmitic acid (C16:0) and oleic acid (C18:1), more abundant and less present in milk from Italian Friesian, respectively.

Milk fat is characterized by high amounts of saturated fatty acids (SFA), especially myristic acid (C14:0) and palmitic acid (C16:0), and by a low amount of mono- and poly-unsaturated fatty acids (Soyeurt et al., 2006).

Milk is the major contributor of SFA to human diet and this fact had led to the widespread conviction that milk and dairy products have negative influences on human health. A recent meta-analysis conducted by Elwood et al. (2010) has demonstrated a reduction in risk for all causes of death (ischaemic heart disease, stroke and incident diabetes) in the subjects with the highest consumption of dairy products as regards those with the lowest intake.

There is a large heterogeneity of SFA and all of them have different effects on human health. In the case of SFA, myristic acid has negative effect on cardiovascular diseases (hypercholesterolemia), whereas stearic acid does not seem to have the same effect (Bonamone and Grundy, 1988; Hu et al., 1999; Dabadie, 2002).

In order to understand lifestyle-related diseases, such as obesity, hyperlipidemia, arteriosclerosis, diabetes mellitus and hypertension, researchers have oriented their attention to diet and in particular to dietary lipids (Vessby, 2003). The most studied milk components are ω-6 and ω-3 poli-unsaturated fatty acids (PUFA), conjugated linoleic acids (CLA) and SFA, but to date also mono-unsaturated fatty acids (MUFA) should be considered to confer important nutritional quality to milk. Not only MUFA profile but also Δ9-desaturase activity could be interesting since it is the key enzyme to convert SFA in MUFA and to control conjugated linoleic acids concentration. Some researchers (Schennink et al., 2007; Soyeurt et al., 2007) have estimated a moderate heritability of this enzyme and the concentration of MUFA to have another chance to improve unsaturated fatty acids (UFA) in milk composition. In mammary gland, Δ9-desaturase catalyzes the insertion of a double bond between carbon atoms 9 and 10 of fatty acid (Pereira et al., 2003).

In the mammary gland, fatty acids originating from the blood or from de novo fatty acid synthesis can be desaturated and the degree of unsaturation is often calculated by a so-called "desaturase index": the concentration of the unsaturated product proportional to the sum of the unsaturated product and the saturated substrate (e.g. for C=14 + C14:1/C14:1+C14:0) (Schennink et al., 2008). The degree of unsaturation is an optimal indirect index of Δ9-desaturase activity in the mammary gland.

Italy is a peninsula and its characteristic is important to guarantee a big variety of environment, such as alpine, countryside, seaside. In Northern Italy are reared a number of bovine breeds, cosmopolitan and autochthonous (Food and Agriculture Organization (FAO), 2000). In this study it was considered Cabannina breed: a cattle population, about 220 heads, which maintained important "climbing" features to exploit the steep pastures available in the region where it lives, Liguria. In this region there is Aveto Valley, that Ernest

Hemingway called ‘the most beautiful valley in the world’ in his journal and where this bovine breed originated.

The second breed studied in this research is the Varzese, autochthonous breed reared in Lombardia, in the southern region of Po river, in Northern Italy. The breed counts about 240 heads, the mean milk production is about 4550-5550 kg milk/lactation, with 4.0% fat and 3.5% protein content. These breeds are tolerant to roughage and show a high coarse-feed efficiency producing high quality milk and very tasty meat.

These local breeds were chosen because they are an important animal Italian biodiversity heritage. Moreover, the international association “Slow Food” has recently oriented its interest towards these two populations recognizing their dairy products under the name “U’ Cabanin and Montebore”, respectively from Cabannina and Varzese milk.

The aim of this project was to compare milk fatty acid composition and Δ9-desaturase activity in different cows breeds, in order to differentiate milk from autochthonous dairy cattle than the more cosmopolite, provide useful data on the quality of autochthonous bovine milk that currently do not exist on the national scientific environment.

3.3. Materials and methods

3.3.1. Animals

A total number of 13 lactating cows were enrolled. Two local breeds were taken into account (Varzese, n=4 and Cabannina, n=4) and compared with a cosmopolite breed (Friesian, n=5). All animals were raised in the same center located in Northern Italy and fed with the same diet, reported in Table 3.1.

Table 3.1 - Mean daily composition of the diet in the three groups of cows

Feed Name	Amount As Fed
Corn silage	15 kg/die
Alfa alfa hay	8 kg/die
Corn meal	4 kg/die
Rye grass	2 kg/die
Sunflower seeds	1.2 kg/die
Beet Pulp	1 kg/die
Soybean	0.6 kg/die
Linseed meal	0.3 kg/die
Minerals and vitamins complex	0.2 kg/die
Total	32.3 kg/die

The reduced sample size in the three selected groups arises from the difficulties encountered during the selection of the subjects for the research: in Northern Italy, mainly in the Apennine region including Lombardy, Piedmont, Liguria and Emilia-Romagna, the local breeds rearing is atomized and somewhat scattered. Therefore, it is almost impossible to find farms with the contemporary presence of three/four breeds. If any, the subjects are very limited, inhomogeneous for physiological status, and moreover, the lactating cows are often in a different lactation stage. Milk samples (10 mL) from the whole udder were collected during the morning milking and from the lactometer. Samples were forwarded to the laboratory analysis at 4°C and analyzed within 2 hours. The animals were in early and mid lactation ranging from 40 to 180 days and the intervals of collection were of 20 days. The starting point of milk collection was chosen in order to assure the full physiological uterine involution in all heads.

3.3.2. Milk fat globule granulometry

Distributions of fat globules diameters were performed using a granulometer laser scatter, according to the method proposed by Lopez, 2005. Size distributions were characterized by volume weighted diameter of the globules (d43, in microns) and by specific surface area (SSA, in cm2 * ml-1). Data obtained were subjected to analysis of covariance (ANCOVA, GLM procedure, SPSS ver. 17.0 per Windows); breed was taken into account as a fixed factor, and day of lactation as a covariate. The differences between breeds were evaluated by LSD (least significant difference) for multiple comparisons. To assess the evolution of globular diameter (expressed as d43) in relation to the days of lactation the following relationship was applied:

$$D43 = (A+B*(\text{days of lactation}))/(1+C*(\text{days of lactation}))$$

where A, B, C are constants.

3.3.3. Milk fat content

The milk fat determination was performed by a UV Spectrophotometric method, according to Forcato et al. (2005). 60 μL of raw milk was added to 3 mL of absolute ethanol in vials and stored for 1 h at −20°C. This procedure allows the precipitation of proteins and hydrophobic peptides that could interfere with UV measurement. After thawing, samples were centrifuged at 13.000 rpm for 15 minutes and the supernatant transferred to micro cuvettes to be measured at 208 nm by a Shimadzu UV-visible recording Spectrophotometer.

3.3.4. Fatty acid extraction, derivatization and desaturase indices

Individual milk samples were stored at -20°C until analysis. Lipid extraction was performed according to a modified Bligh & Dyer method (Manirakiza et al., 2001) with chloroform/methanol mixtures, chloroform and micro-filtered distilled water. The samples (7.25 mL) were thus placed in centrifuge at 2000 rpm for 10 minutes at room temperature (20°C) in order to separate the solution into three phases. The deepest phase (solution of chloroform containing the lipids) was drawn and placed in a glass tube in order to be dried under a slight nitrogen flow.

The solution previously dried was derivatized according to Molto-Puigmartì et al. (2007), using solutions of NaOCH3/MeOH 0.5M and BF3. After complete derivatization, 0.5 μL of solution containing lipids was finally injected and analyzed by gas chromatograph Trace GC 2000 (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with a TR-CN100 column (100 m, 0.25 mm i.d. x 0.20 μm film thickness) and a 5 m precolumn (i.d. 0.25 mm) deactivated (TeknoKroma, Barcelona, Spain).

The oven temperature program was: from 70°C held 3 min, then from 70 to 240 °C at 2.5 °C min-1, held for 10 min. Carrier gas was nitrogen at 0.5 mL min-1. Fatty acids were identified using external standards (Standard containing 37 fatty acids, FAME Mix 37, Supelco, USA) and two CLA standards (Matreya, USA) and quantified using 19:0 (nonadienoic acid) as internal standard. Peak areas were corrected according to the theoretical relative FID response correction factors (TRFs) published by Ackman (2002). The results are presented as g/100 g fatty acids (% by weight).

The percentage of the single contribute of each fatty acid was calculated on the total of the area under the known peaks. The percentages of myristoleic acid (C14:1-cis-9), pentadecenoic acid (C15:1-cis-10), palmitoleic acid (C16:1-cis-9), heptadecenoic acid (C17:1-cis-10), oleic acid (C18:1-cis-9), eicosaenoic acid (C20:1-cis-11), erucic acid (C22:1-cis-13), were taken into account.

Desaturase indices (Δ) were calculated according to Schennink et al. (2008) and the total desaturase index (TDI) was calculated according to Mele et al. (2007) on C14, C16 and C18 fatty acids; briefly, the individual Δ was calculated as $Cx:1/(Cx+Cx:1)*100$, where x is the number of carbons of fatty acid; The total desaturase index was calculated as $(C14:1+C16:1+C18:1/(C14+C14:1+C16+C16:1+C18+C18:1)*100$.

3.3.5. *Statistical analysis*

Raw data were resumed as mean ± standard deviation. In order to compare the results between breeds, the data were analyzed by an analysis of covariance model (ANCOVA), taking into account the breed as fixed factor and the time as covariate. The differences between breeds were assayed through the corrected t-test for multiple comparisons. All data were analyzed by the software JMP® ver. 7.0.2 (SAS Institute Inc.) for Windows platform.

3.4. Results

The mean production of the three groups of cows during the observation period was 14.24±4.36 kg, 24.17±6.84 kg, and 11.84±4.59 kg for the Cabannina, Holstein and Varzese, respectively. The mean productions were significantly different (ANOVA, $p<0.001$), and the three groups were all significantly different when compared pairwise (Tukey test, $p<0.05$). The three breeds showed an overall fat percentage of 4.05±1.14% (Cabannina, n=36), 3.53±1.01% (Holstein, n=39) and 4.27±0.87 (Varzese, n=30). By adjusting for the time trend, a significant difference ($p<0.05$) is evidenced between Holstein cows and the other two breeds; no differences are highlighted between Cabannina and Varzese milk fat content. The overall time trend for fat was significantly positive ($p<0.05$).

The univariate descriptive statistics for the variables considered are resumed in Table 3.2. The results report also the ANCOVA significances for breed and time. At a first glance, great differences between groups can be appreciated: in particular, the Varzese breed shows higher percentages of C15:1*c*, C17:1*c*, C18:1*c*, ΣMUFA, and higher levels in Δ14, Δ16, Δ18 and ΣΔ with respect to the other breeds; Cabannina milk evidenced significantly higher levels in C14:1*c*, Δ14, Δ16 and Δ18. Holstein cows maintain the lowest percentages for all the MUFAs determined. Positive significant temporal trends ($p<0.05$) were observed for C14:1*c*, C16:1*c* and for Δ14, Δ16, Δ18 and ΣΔ. Significant negative trends appeared in C15:1*c*, C17:1*c*, C20:1*c* and C22:1*c*. A significant trend for the other variables was not evidenced.

Table 3.2 - Univariate descriptive statistics and ANCOVA results for *cis*-MUFA profiles and desaturase indices in different dairy cow breed

	Breed			ANCOVA		
Fatty acid (%)	**Cabannina**	**Friesian**	**Varzese**	**Breed**	**Day of lactation**	**Time trend**
C14:1-*cis*	1.14±0.25[a]	0.96±0.32[b]	1.03±0.40[ab]	*	***	↑
C15:1-*cis*	0.08±0.16[c]	0.13±0.18[b]	0.17±0.21[a]	***	***	↓
C16:1-*cis*	1.93±0.75	1.80±0.73	2.13±0.80	n.s.	***	↑
C17:1-*cis*	0.15±0.20[b]	0.18±0.17[b]	0.27±0.23[a]	**	***	↓
C18:1-*cis*	19.11±5.45[b]	16.83±4.03[c]	22.49±4.08[a]	***	n.s.	=
C20:1-*cis*	0.06±0.16	0.07±0.12	0.03±0.10	n.s.	**	↓
C22:1-*cis*	0.01±0.03	0.02±0.03	0.01±0.02	n.s.	*	↓
∑*cis*-MUFA	22.40±5.35[b]	19.91±4.11[c]	25.31±5.27[a]	***	n.s.	=
Desaturase						
Δ14	6.95±1.44[a]	6.06±2.08[b]	6.77±2.63[ab]	0.07	***	↑
Δ16	5.15±1.80[a]	4.20±1.72[b]	5.71±2.21[a]	*	*	↑
Δ18	67.32±3.70[ab]	65.06±5.14[b]	69.85±5.90[a]	**	*	↑
ΣΔ	26.58±4.18[b]	23.00±4.41[c]	29.98±4.05[a]	***	n.s.	=
C18:0	9.20±2.57	9.08±2.17	9.78±2.70	n.s.	***	↓
C18:1-*cis*/C18:0	2.03±0.45	1.92±0.49	2.47±0.59	***	n.s.	↑
C18:3n3	0.43±0.33[a]	0.25±0.37[c]	0.37±0.48[b]	n.s.	***	↑
C18 3n6	0.001±0.005	0.008±0.04	0.06±0.3	n.s.	n.s.	
C20:2	0.01±0.01	0.01±0.02	0.01±0.02	n.s.	n.s.	
C20:3n3	0.01±0.03	0.01±0.02	0.04±0.08	n.s.	n.s.	
C20:3n6	0.04±0.05	0.02±0.04	0.08±0.24	n.s.	n.s.	
CLA 9-11	0.34±0.11[a]	0.18±0.17[b]	0.31±0.29[b]	***	***	↑

cis-MUFA = *cis*-monounsaturated fatty acids

[a-c]Different superscripts indicate a p<0.05 difference (correct t-test for multiple comparisons).

*=p<0.05; **=p<0.01; ***=p<0.001.

Δ = Desaturase Index; ΣΔ = Total Desaturase Indices.

n.s. = not significant.

↑ = increasing time trend.

↓ = decreasing time trend.

The fatty acid, the desaturase indices and the milk fat percentage patterns during the observation period are reported in Figures 3.1, 3.2 and 3.3.

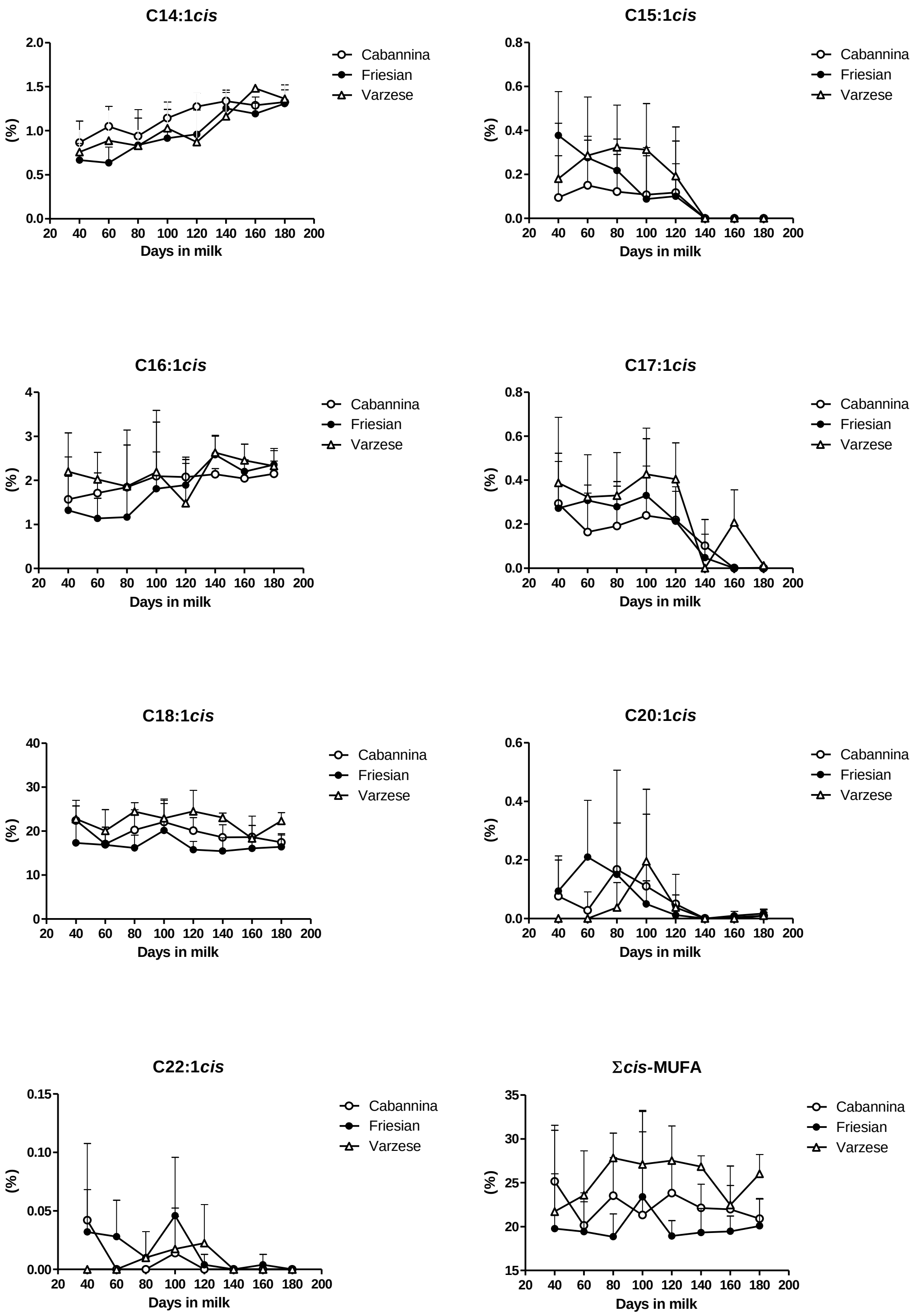

Figure 3.1 - *cis*-MUFA patterns in the three breeds during lactation.

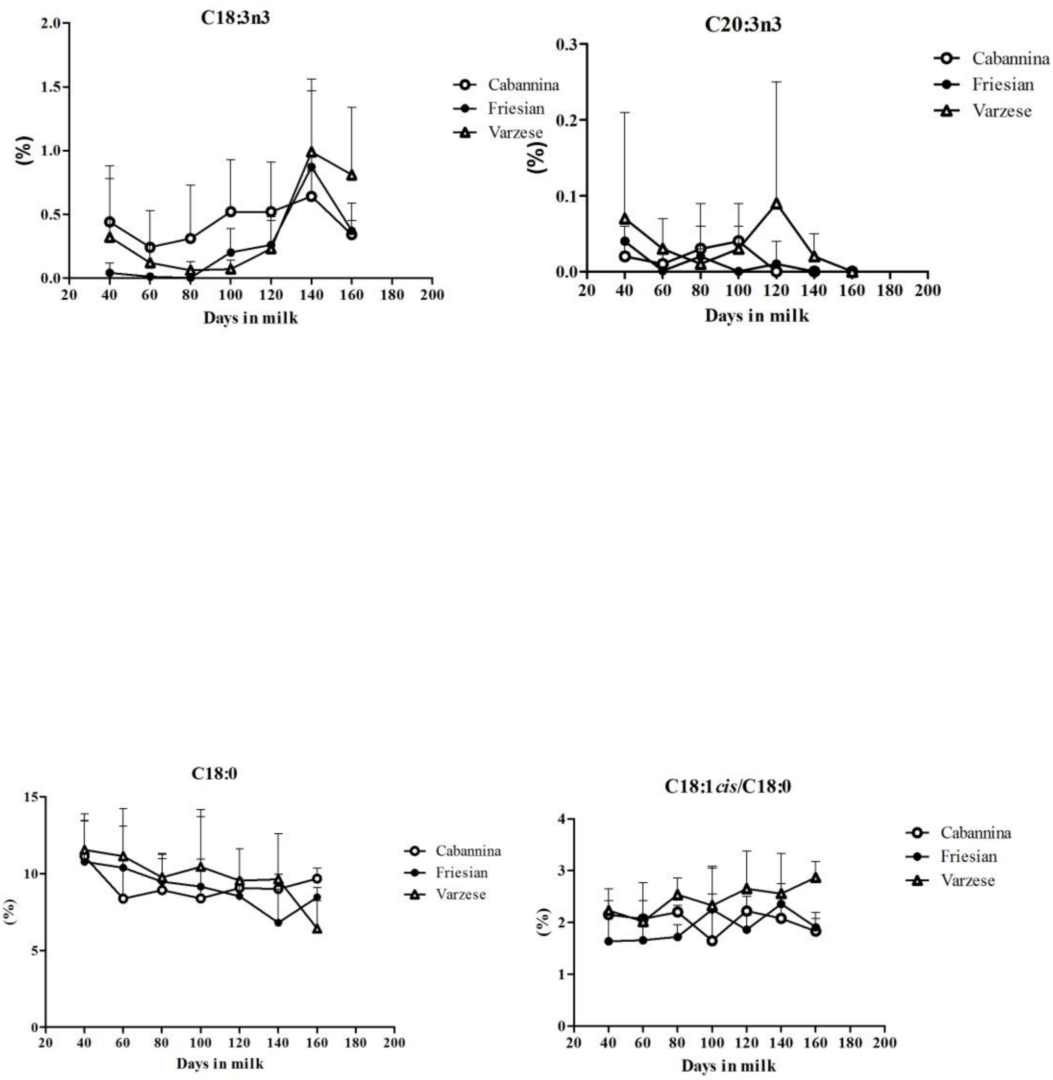

Figure 3.2 – PUFA, C:18 and C18:1-*cis*/C18:0 patterns in the three breeds during lactation.

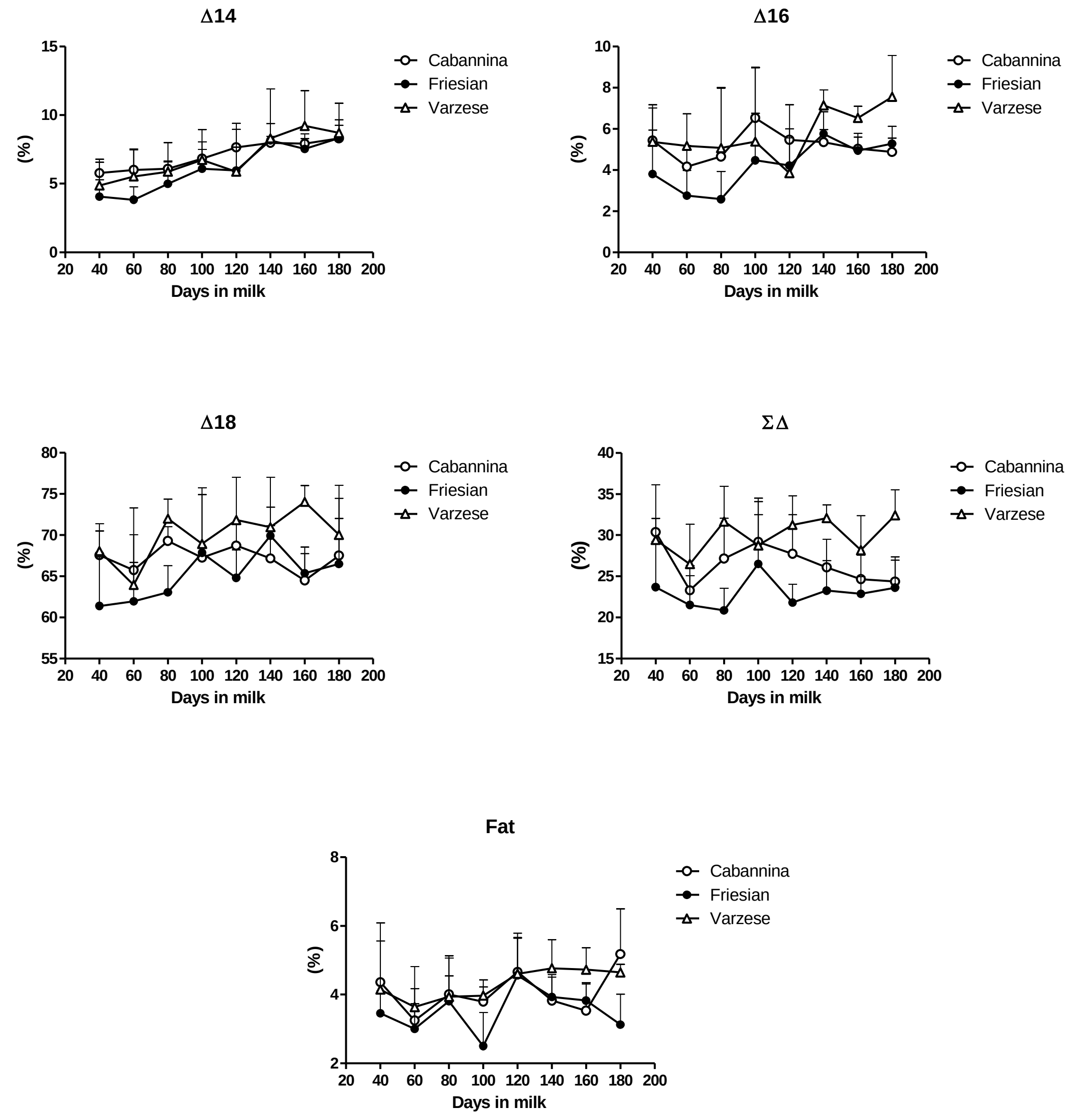

Figure 3.3 – Desaturase Indices and fat content in the three breeds during lactation.
Δ = Desaturase Index; ∑Δ = Total Desaturase Indices;

The analysis of fatty acid content in milk of the three cow breeds taken into account (Cabannina, Friesian, Varzese), as reported in table 3.2, showed statistically significant differences for C18:3n3 and CLA 9-11contents; the first showed differences only for time factor and the second both for the time factor and breed factor. Moreover, Cabannina cows showed, for these fatty acids, a higher and persistent content over time than the other two breeds observed. For all other PUFAs analyzed there were no notable differences from a statistical point of view both for time factor and breed factor.

3.5. Discussion and conclusions

The present work put in evidence some important between-breed variations in the composition of fatty acids in local and cosmopolite cows, adjusted for the stage of lactation. The content in *cis*-MUFA found in the present research is quite similar to the data reported in the recent literature (Table 3.3) with some exceptions: in particular, the C14:1-*cis* level was higher in all analytical groups, mainly in Cabannina and Varzese cows, with respect to reported values (Table 3.3). The same conclusions can be drawn for c16:1-*cis*, whereas C18:1-*cis* the three breeds show slight lesser levels with respect to the reported data, with the exception of the results of Heck et al. (2009). Desaturase indices overlap with those reported by other authors (Table 3.2), with slightly reduced Δ14 levels.

The enhancement of the *cis*-MUFA in milk is desirable for the human consumption, as reported by Givens (2008): higher intakes of *cis*-MUFA and a reduction in short fatty acids reduce the plasma insulin levels, the total plasma cholesterol and the LDL-cholesterol concentrations, therefore reducing the risks of coronary heart disease (CHD). The higher intake of *cis*-MUFA is therefore well-considered in type-2 diabetes, as reported by Ros (2003), that underlines as *cis*-MUFAs are an alternative to low-fat diets in diabetes management. Moreover, Lauszus et al. (2001) indicate that the intake of MUFAs may prevent the blood pressure rising in gestational diabetes mellitus, without influences on lipid and lipoprotein concentrations; if such mechanism is insulin-mediated, is unclear. The lowering in plasma insulin levels and a following reduction in white fat deposition was observed by Liao et al. (2010) in the hamster when a MUFA-rich diet with high polyunsaturated/saturated fatty acid ratio is administered.

In a survey conducted in 11 EU member states, the intake of *cis*-MUFA from dairy products ranges from 8.3 to 28.6% of the total intake of *cis*-MUFA (Givens, 2008), confirming the huge contribute of milk and dairy in the *cis*-MUFA intake. The report of Givens suggests that the modification and modeling of fatty acid content in milk by replacing short fatty acids enhancing the *cis*-MUFA content may reduce the risk of CHD in the population, although the agricultural policies would be deeply changed. The positive effects of *cis*-MUFA can exert also on the udder health: during mastitis, for example, an enhancement in lipase activity can be appreciated, with increase in free fatty acids, mainly short chain fatty acids (Randolph and Erwin, 1974); several fatty acids are endowed of a good antibiotic power, that can be expressed via inhibition of enzyme/fatty acid synthesis/nutrient uptake, cell lysis, metabolites leakage, disruption of electron transport chain, interference with oxidative phosphorylation and lipid peroxidation (Desbois and Smith, 2010). Some of the most relevant antibiotic effects of MUFAs are reported in Table 4.

By enhancing the activity of stearoyl CoA desaturase (SCD), the nutritional value of milk would be positively ameliorated, but a simple upregulation of its activity seems to be limited, as reported in a comprehensive milk lipid synthesis model (Shorten et al., 2004). In the present research, the local breeds show either higher levels in *cis*-MUFAs or in desaturase indices: these features could be linked to genetics, as evidenced by Schennink et al. (2008), by complex interaction in gene/allele expressions.

Taking into account the amount of milk produced by the three different breeds there were substantial differences: in fact, in the two autochthonous breeds, showing considerably lower productions than the Friesian ones, it has been

detected more abundant quantities of PUFA, as it can be deduced from results previously described.
It is important to note that all animals taken into account were reared in the same farm, fed with the same diet and so substantial differences of fat content and fatty acids profile showed among breeds could probably due to different genetic component of each animal.
Another important characteristic taken into account was oleic acid content in relation to stearic acid content. Varzese cows showed more abundant content as described in table 3.2 and in figure 3.2. Cabannina cows were in a middle position, whereas Friesian cows showed lower levels during all days in milk. In all three breeds ratio C18:1-cis/C:18 trend increases with the progression of the lactation. Recent studies (Schwartz et al., 2008; Dipasquale et al., 2010) had demonstrated how oleic acid introduced by diet is the most important substrate to produce a lipid amide by enterocytes involved in the induction of satiety. This compound is oleoyethanolamide (OEA)and, as reported by Dipasquale et al. (2010), has several characteristics of “satiety factor”: it inhibits feeding by prolongin the interval to next meal; its synthesis is regulated by nutrient availability and its levels undergo circadian fluctuations. Fu in 2005 had shown the effects of OEA on lipid metabolism, stimulating lipolysis in white adipose tissue and liver, as well as reducing blood cholosterol and triglyceride levels. As reported by Schwartz et al. (2008) OEA, by its specific pathways of activation, could be considered as a key physiological signal that liks dietary fat ingestion to across-meal satiety and nutritional and pharmacological strategies aimed at magnifying this lipid-sensing mechanism might be useful in treatment of obesity and other eating disorders.

In conclusion, the recovery of endangered niche breeds can undoubtedly give a boost to local products and to the conservation of livestock biodiversity; the

FAO sustains the livestock biodiversity as a "safety net for the future", mainly in developing countries, as reported in a recent FAO document (FAO, 2010). These principles can be extended also to developed countries, aiming to better exploit the local resources and preserve relic breeds from an impending extinction, with the loss of a priceless legacy.

The research undertaken and the results suggest that it is interesting to assume that from breed biodiversity derives a "milk biodiversity". This feature will show in the forthcoming years peculiar nutritional and nutraceutical aspects in these different milks and dairy products derived.

Table 3.3 - Fatty acid contents in bovine milk from recent literature

Fatty acid (%)	n	Mean	Dispersion†	Min	Max	Reference	Breed
C14:1-*cis*	-	0.8	0.4	0.4	1.3	Mansson, 2008	Swedish
	10401	1.03	0.22	-	-	Soyeurt et al., 2008	Pool of 7 breeds
	229	1.023	0.257	-	-	Moioli et al., 2007	Jersey, Piedmontese and Valdostana breeds pooled
	2408	0.79	0.27	0.15	2.09	Garnsworthy et al., 2010	Holstein
	18	0.91	0.13	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown Swiss pooled
	52	1.23	-	1.13	1.34	Heck et al., 2009	Dutch
C16:1-*cis*	-	1.0	0.0	0.9	1.8	Mansson, 2008	Swedish
	10401	1.88	0.40	-	-	Soyeurt et al., 2008	Pool of 7 breeds

	229	1.484	0.374	-	-	Moioli et al., 2007	Jersey, Piedmontese and Valdostana breeds pooled
	2408	1.69	0.46	0.39	3.49	Garnsworthy et al., 2010	Holstein
	18	1.50	0.15	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown Swiss pooled
	52	1.64	-	1.55	1.72	Heck et al., 2009	Dutch
C17:1-*cis*	-	0.1	0.0	<0.1	0.3	Mansson, 2008	Swedish
	229	0.317	0.134	-	-	Moioli et al., 2007	Jersey, Piedmontese and Valdostana breeds pooled
	18	0.32	0.07	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown Swiss pooled
	52	0.22	-	0.21	0.24	Heck et al., 2009	Dutch
C18:1-*cis*	-	22.8	1.0	19.7	24.7	Mansson, 2008	Swedish
	10401	22.31	4.48	-	-	Soyeurt et al., 2008	Pool of 7 breeds
	229	23.016	4.757	-	-	Moioli et al., 2007	Jersey, Piedmontese and Valdostana breeds pooled
	2408	27.96	4.61	15.29	47.36	Garnsworthy et al.,, 2010	Holstein
	18	23.65*	1.85*	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown

							Swiss pooled
	52	17.60	-	16.06	19.18	Heck et al., 2009	Dutch
C20:1-*cis*	18	0.04	0.01	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown Swiss pooled
C22:1-*cis*	18	0.01	0.02	-	-	Gaspardo et al., 2010	Friesian, Simmental and Brown Swiss pooled
Δ14	1933	10.5	7-14.6 (25-75th percentile)	-	-	Schennink et al., (2008)	Holstein
	2408	8.11	1.99	2.90	16.53	Garnsworthy et al., 2010	Holstein
	52	9.8	-	9.3	10.14	Heck et al., 2009	Dutch
Δ16	1933	4.2	2.9-6.3 (25-75th percentile)	-	-	Schennink et al., (2008)	Holstein
	52	5.0	-	4.7	5.4	Heck et al., 2009	Dutch
	2408	6.66	1.85	1.68	14.07	Garnsworthy et al., 2010	Holstein
Δ18	1933	67.6	59.5-75.3 (25-75th percentile)	-	-	Schennink et al., (2008)	Holstein
	2408	73.98	3.68	60.13	85.52	Garnsworthy et al., 2010	Holstein
	52	63.2	-	62.5	64.7	Heck et al., 2009	Dutch

Table 3.4. Biological antibiotic effects of the fatty acids taken into account in the paper.

Fatty acid	Effect
C14:1-*cis*	Antibacterial (Gram +) – antifungal – *Candida albicans* germination inhibitor*
C16:1-*cis*	Antibacterial (Gram + /-) – antifungal - antiviral
C18:1-*cis*	Antialgal – antibacterial (Gram+/-) – antiprotozoan – antiviral – haemolytic (sheep RBC) – toxic to brine shrimp larvae and mosquito larvae
C20:1-*cis*	Antialgal – antibacterial (Gram +)
C22:1-*cis*	Antialgal

All data are after Desbois and Smith (2010), except * (Clément et al., 2007).

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CHAPTER 4

Hydroxyproline monitoring during the post partum period in autochthonous bovine breeds to characterize the profile of uterine involution

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4. Hydroxyproline monitoring during the post partum period in autochthonous bovine breeds to characterize the profile of uterine involution

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4.1 Abstract

In the last 40 years, the first goal pursued by dairy cattle community has been to increase individual production from a quantitative point of view, by high yielding dairy cows selection (BLAP). This mono-aptitude criterion has caused a decline in reproductive performances worldwide (eg increase in calving interval). The comparative physiological study of autochthonous cattle to value "Ancient Autochthonous Biodiversity" opens up interesting prospects for livestock to comply the FAO 2050 target, as these animals are guarantors of a good milk production, excellent and typical cheese making, peculiar characteristics of rusticity, frugality, longevity and resistance to multiple diseases. Therefore they appear to be good candidates for traditional food production. The study of autochthonous breeds lactation and reproduction physiology could be strategic to improve knowledge and develop a more rational dairy livestock in view of

reduced availability of cereals and plant proteins for livestock in the next 40 years.

From a reproductive point of view serum hydroxyproline (HYPRO) trend was considered for the uterine involution monitoring. Postpartum period is a physiological process between birth and complete uterine involution, essential to bring the female genital apparatus back to favorable conditions for optimal embryonic development and implantation, resumption of ovarian cyclicity and future reproductive efficiency; the latter is a parameter that unequivocally determines the career of a cow in the herd.

In this research, the same cows involved for milk analysis, were subjected to blood collection and hydroxyproline determination. Blood samples were performed the day of birth (day 0), 1,2, 3, 4, 5, 10, 15, 20, 25, 30. The two autochthonous breeds showed lower starting points and average concentrations than the Friesian cows. Only the Cabannina cows showed a peak in day 4 statistically higher than the value at day 0. All breeds showed at day 40 after birth a completely uterine involution and so it seems obvious that HYPRO patterns are characteristic of each breed. It remains to clarify the origin of HYPRO district that could not come solely from the uterine matrix, given the ubiquitous nature of collagen. These results can be considered as a starting point to try to clarify the mechanisms of uterine involution process, the intrinsic and extrinsic factors of the animals and any phenomena that would affect reproductive performance of cows.

4.2 Introduction

Fertility is a complex parameter undoubtedly influenced by genetics, environment and management. These components exist and act in synergy and simultaneously, making it extremely difficult for any strategies and technologies applied to set up a stabilization of reproductive efficiency.

The post-partum period has a basic effect on the resumption of ovarian cyclicity and future reproductive efficiency, a parameter that unequivocally determines the career of a cow in the herd. By definition, the postpartum period is a physiological process between birth and complete uterine involution, essential to bring the female genital apparatus back to favorable conditions for optimal embryonic development and implantation (Badinand 1993; Kaidi et al., 1991b).

The length of involution is subject to seasonal variations and results in periods longer during winter and summer than in the other seasons (Bottarelli, 1989).

The physiological puerperium is characterized by a series of events that define the process of uterine involution during which the uterus returns to its pre-pregnancy size, the endometrium is regenerated, the reproductive tract removes bacteria and contaminants and ovarian cyclic activity is restored. The uterine involution is characterized by haematic cytological, bacteriological and biochemical changes of uterus and its contents (Badinand F., 1993). This process affects the future success of a new insemination and the inter-calving period assumes a considerable economic importance (Oltenacu et al., 1983, Villancourt, 1987).

The process of uterine involution seems to be influenced by many hormonal mechanisms, not yet fully clarified: these phenomena would involve sex steroids (estradiol and progesterone) and prostaglandin F2α (PGF2α) whose massive release has a duration of 2-3 weeks (Burton et all. , 1987) after birth.

Immediately after birth a cow uterus weighs about 10 kg and in a month it reaches a weight of 1 kg physiologically (Badinand 1993).

The uterus reduction in size during post partum is due to myometrial contraction phenomena that occur at intervals of 3-4 minutes and due to the size of myofibrils, which decrease from 700-750 μm at birth to 200 μm at day 3 post partum (Kaidi et al., 1991)

Uterine involution in cattle can be affected by many factors, such as dystocia, uterine prolapse, abortion and bacterial infections (93% of uteri in cattle can be infected by a large amount of bacteria until 15 days post-partum) (Elliot et al., 1968).

Marion et al. (1968) suggest that the delay in the uterine involution may be caused by a reduction of the uterine motility, whereas Ko et al. (1989) reported that retention of fetal membranes is not due to a reduction in contractility of the uterus in the early post partum period.

Metabolic disorders have a negative influence on uterine involution. Animals subjected to hypocalcaemia are characterized by a reduction of uterine contractions (Roberts, 1989; Al-Eknah and Noakes, 1989), with a decrease in the rate of involution of the uterus and the cervix (Fonseca et al., 1983). In hypocalcaemic cows uterine and cervical involution is longer than normocalcaemic ones, reaching pre pregnancy conditions average 6 days later (Kamgarpour et al., 1999).

The administration of PGF2α can reduce time of uterine involution (Lindell and Kindahl 1983), cows with a short interval between birth and complete uterine involution have a release of PGF2α longer in the post-partum period.

Multiparous cows require a longer time period to complete uterine involution than primiparous (Foote et al., 1960; Oltenacu et al., 1983, Marion et al., 1968; Schira and Martinet, 1982), in disagreement Tennant et al (1967) did not observe any difference between rates of uterine involution in primiparous and multiparous. Bastidas et al. (1984), however, have found a more rapid rate of uterine involution in multiparous than in primiparous.

The uterine involution occurs more rapidly in subjects who give birth in spring and summer compared to those who give birth in autumn and winter (Marion et al., 1968). The uterine involution rate is faster in rainy periods than in dry ones (Bastidas et al. 1984). Were not detected, however, significant variations between seasons and uterine involution (Johanns et al. 1967).

The feeding is the most important stimulus for the oxytocin release, and uterine involution in cattle feeding is faster than the non-feeding ones (El-Fouly et al. 1976; Riesen 1968). Okan and Fukuhara (1980) also conclude that early weaning delayed uterine involution, in contrast Moller (1970) does not detect any effect of feeding on the uterine involution process, in agreement with that observed by Oxenreider (1968).

Menge et al. (1962) have shown that the cow weight at calving is positively correlated with the time of uterine involution, while Agastia et al. (1976) did not detect any correlation between these parameters.

The uterine involution was longer in the heavier beef breeds than in the lighter dairy breeds (Sloss and Dufty 1980). Marion et al. (1968) report a range of 26-52 days for the uterine involution in dairy subjects and 37-56 days in the beef breeds.

The uterine involution can be monitored by observing the reduction in the size of the vulva, vagina, cervix and uterus (body and horns). These observations can be conducted using different methods:

• Transrectal palpation of reproductive tract after delivery to complete uterine involution (Marion et al., 1968, Moller 1970);

• removal of the cow reproductive at slaughter predetermining delivery intervals (Moller 1970);

• transrectal ultrasound of the reproductive tract (Mateus et al. 2002; Wehrend et al. 2003);
• cervical forceps (Wehrend et al. 2003);

Garcia and Larsson (1982) assessed the transrectal palpation of the cervix, uterus and ovaries. These authors assumed that the size, tone, symmetry and location of the uterus in the pelvic cavity were indicators of uterine involution. The uterine involution was considered complete when the uterus back in its normal location in pelvic cavity, when the uterine horns pre pregnancy returned to the size and when it did not occur a thickening of the uterine wall. Marion et al. (1968) applied transrectal palpation twice a week to monitor uterine involution, using the criteria outlined as a marker of complete uterine involution.

Moller (1970) used the trans-rectal palpation method by applying the "4-stages." During the "first stage" (1-8 days post partum), the vagina can be felt as a stretch of approximately 8 cm in width in the first 24 hours post-partum. The cervix is not discernible by day 3 and in day 4 and 5 it has enough tone to be distinguished from the uterus which is usually located at the front of the pelvic floor. The surface of the uterus is hard and corrugated and the uterine caruncles can be perceived only through the uterine wall when it is relaxed. In the "second stage" (8-10 days post partum), the whole uterus can be appreciated. The surface is smooth and soft with fluctuations in the horn post pregnancy. Caruncles are felt as spherical structures and the cervix, in young animals, is solid and placed in the pelvic cavity. During the "third stage" (10-18 days post partum), the uterus has an elastic and soft body, caruncles and the fluctuations are less pronounced, the cervix is solid and continues to reduce its size such as uterine post pregnant horn. During the "fourth stage" (18-25 days post partum) show greater firmness and the horn of the uterus after pregnancy returns to the same proportions of the non-pregnant one.

Gier and Marion (1968) found that the uterine involution continues up to 25-30 days in post-partum period. Marion et al. (1968) concluded that the uterine involution, monitored by transrectal palpation, lasts 40 days. Under normal conditions a uterus can be completely taken in a hand after 2-3 weeks and never before 10-12 days after birth (Badinand 1993).

Wehrend et al. (2003) have used as a method the ultrasound and the forceps placed in cervical duct. The distance between the two handles of the forceps is proportional to the opening of branches of the same. To check the level of intracervical extremity openness it can be used a meter that determines the distance between the handles of the forceps.

Uterine involution is characterized by a significant tissue remodeling: measuring cellular turnover markers, such as hydroxyproline (HYPRO), the most abundant amino acid present in uterine collagen, could provide useful information on involution progress.

The serum concentration of this protein increases gradually in late gestation and is related to the mechanism of placental separation and uterine involution (Kaidi et al., 1991a).

4.3 Materials and methods

4.3.1 Animals

A total number of 16 lactating cows were enrolled. All animals were multiparous, with eutocic partum, normal post-partum period and raised in the same center located in Valle Salimbene (Pavia) in Northern Italy. All animals were followed for the first 30 days after birth and were divided into 3 treatment groups based on breed, specifically:

- 4 Varzese cows (VAR group);
- 5 Cabannina cows (CAB group);
- Friesian cows (FRI group);

All cows did not undergo any gynecological clinic examination as manipulation (palpation and retraction of uterus in the pelvic cavity) could produce stimuli influencing a physiological uterine involution (Rosemberger, 1979). In this regard Hurtgen and Ganiam (1979), in a trial conducted on mares, showed that intracervical or intrauterine manipulation during the luteal phase of the estrous cycle, may directly or indirectly stimulate release of endogenous prostaglandins that trigger regression of the corpus luteum, followed by oestrus onset and ovulation (Hurtgen and Ganiam (1979). In our trial animals that showed persistent hyperthermia (40.5 °C) for over 48 hours, or post-partum syndromes requiring drug administration that could affect uterine involution (e.g., administration of boron gluconate drugs, steroidal and non steroidal anti-inflammatory drugs) were excluded.

4.3.2 Solutions preparation

Buffer solution:

In 200 mL of distilled water were dissolved 25 g of citric acid monohydrate, 6 mL of glacial acetic acid, 60 g of natrium trihydrate acetate and 17 g of natrium hydroxide (NaOH). Then volume was carried to 500 mL using distilled water. The solution pH was corrected 6, adding NaOH 5 mol/L or glacial acetic acid.

Chloramine T reagent:

0,70 g chloramine T powder (Sigma Chemicals) was dissolved in a mixture of 5 mL of water, 5 mL of n-propanol and 40 mL of a buffer prepared as above.

P-dimethylaminobenzaldehyde reagent (PDAB):
15.5 mL of n propanol and 6.5 mL of 70% perchloric acid have to be added to 3.75 g PDAB, drop by drop under vigorous stirring.

Standard solution of hydroxyproline:
A standard HYPRO solution was prepared by adding 100 μg of HYPRO per mL of a HCl solution 1 mmol/L.

4.3.3 Serum hydroxyproline determination

The whole serum was deproteinized with trichloroacetic acid (TCA) at 10% (w/v): 1000 μL of TCA and 200 μL of distilled water were added to 1000 μL of bovine serum, the mixture was processed to vortex for 30 seconds and then centrifuged at 1500 rpm for 15 minutes. The supernatant was drawn and immediately analyzed for free HYPRO concentration. The method is based on the HYPRO oxidation by the chloramine-T and on the formation of a red compound reacted with PDAB. 1200 μL of supernatant was taken from each deproteinized serum sample and 500 μL of reagent chloramine T was added to each one. Samples were incubated for 20 minutes at room temperature. After incubation 500 μL of PDAB reagent were added into each tube, then put in incubation for 15 minutes at 65 °C. At the end of incubation, samples were cooled to reach room temperature for 15 minutes. Then the mixture was transferred into cuvettes with an optical length of 10 mm; the optical densities were read at a wavelength of 550 nm against distilled water.
The optical densities of the samples were compared with a calibration curve obtained with HYPRO standard prepared as above mentioned with volumes of 10, 20, 40, 60, 80 and 100 μL of standards brought to 100 μL with distilled water, added with 600 μL of water and 500 μL of TCA. Each standard solution

was subsequently treated with chloramine T and PDAB reagent according to the same methodology used for serum samples. HYPRO concentrations in standards were 1, 2, 4, 6, 8 and 10 μg HYPRO/mL.

4.3.4 Statistical analysis

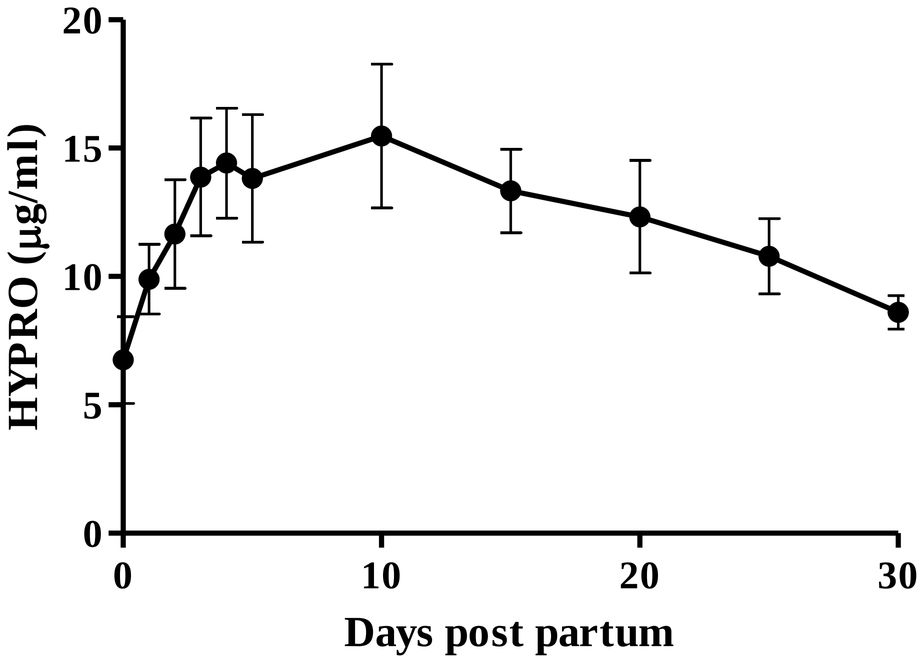
The data of serum HYPRO concentration during the observation time relatively to the 3 breeds, were summarized in mean values ± standard deviation. In order to evaluate the effects of breed and time on HYPRO concentrations, a model to analyze variance has been applied to (ANOVA) with two criteria of classification (Breed x Time).

4.4 Results

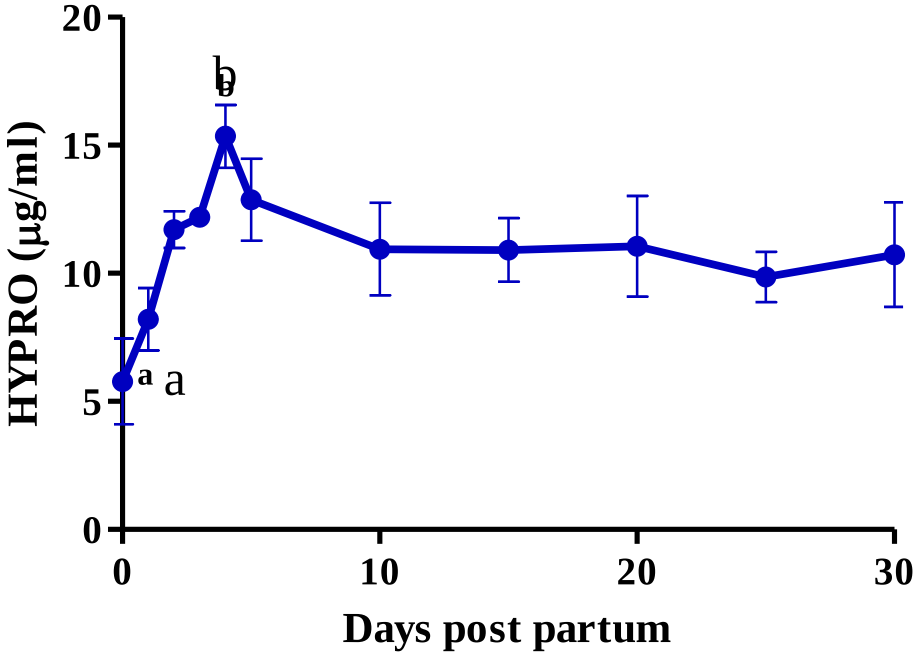
All summarized data about HYPRO levels in individual breeds during observational period are shown in graphs 4.1, 4.2, 4.3 and 4.4. Analyzing the trends in the 3 bovine breeds considered, we can see that at day 0 Cabannina and Varzese show the lowest values (~6-7 mg/ml) while Friesian shows an higher concentration (~10 mg/ml). From day 0, during the first few days postpartum, in all breeds values rise and around the fourth day post-partum a peak of HYPRO reaches up to 15 mg/ml. After this period all the graphs put in evidence a stabilization of the trends that lasts almost until the end of the determinations. The last observation period (26-30 days postpartum) is characterized by slight values elevation in Cabannina and Friesian, while Varzese is characterized by an opposite trend.

For the whole observational period, in each breed, was not observed any significant peak in relation to the time, except for Cabannina in which the

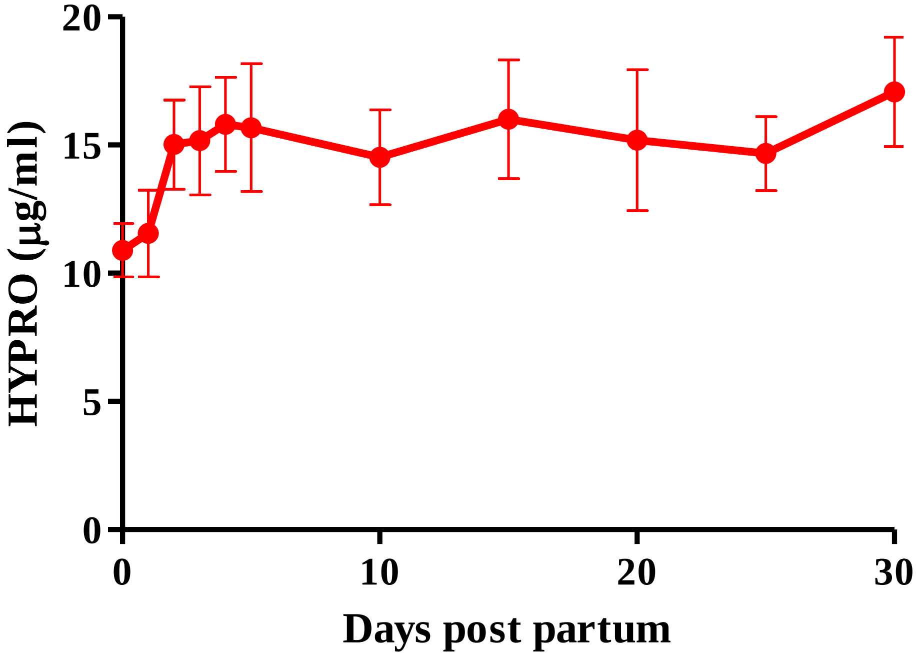
HYPRO value was significantly higher at 4th day postpartum than the value at day 0. It must be mentioned that Varzese breed showed a peak in relation to the time, at borderline significance (P = 0,09).



Graph 4.1 - HYPRO trend in Varzese Breed

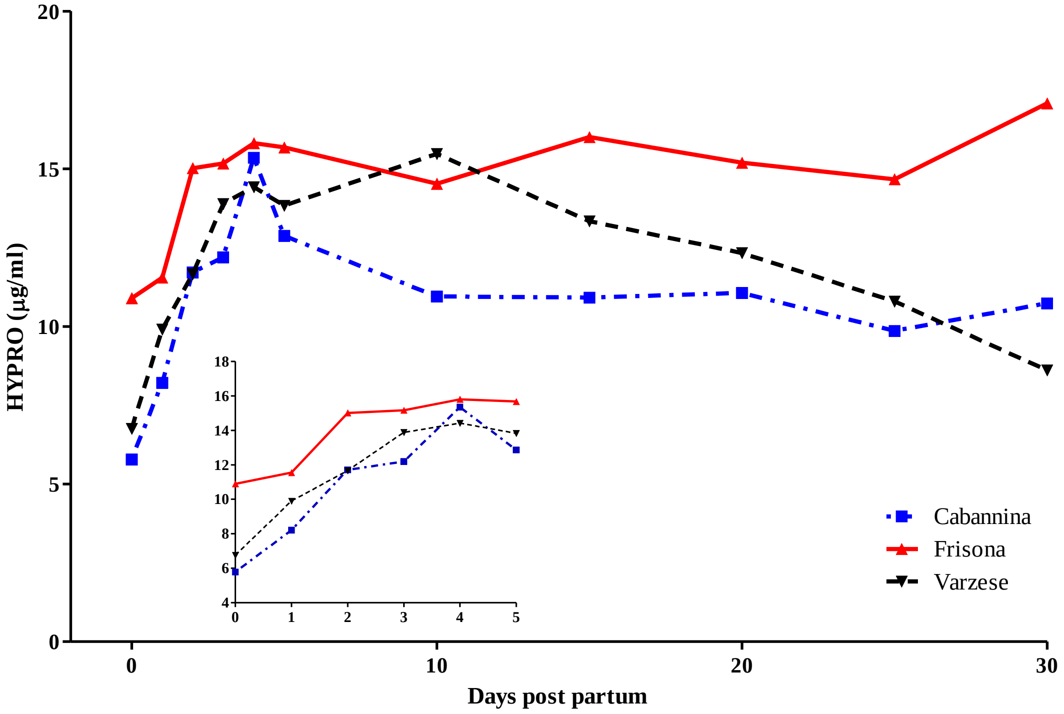


Graph 4.2 - HYPRO trend in Cabannina Breed.
a, b: significant difference with P<0.05



Graph 4.3 - HYPRO trend in Friesian Breed

Graph 4.4 shows the HYPRO concentration trend in the three bovine breeds observed; it is interesting to notice that the comparison between different breeds highlights the Friesian serum concentrations trend: the average value of this trend is significantly higher than those in the other breeds. The values are higher starting from day zero and remain higher for the whole observations time, tending to increase between 25 and 30 days postpartum.



Graph 4.4- all trends in the three observed breeds are shown; the detail of trend in the first five days post partum in all breeds is put in evidence.

The conclusions drawn from graph 4.4 are confirmed by two-way Anova that considers breed and time effects on the HYPRO concentrations. The ANOVA procedure has put in evidence a significance ($P < 0.01$) both in time and in breed factor. LSD test for multiple comparisons revealed significantly higher HYPRO values in Friesian than in the other breeds.

4.5 Discussion and conclusions

This work aimed to evaluate the HYPRO patterns in the first month post partum in three bovine breeds, both autochthonous and cosmopolitan. The HYPRO aminoacid trends don't have the same evolution, suggesting different metabolisms and regressive processes. In particular, in Friesian breed, we assist to a rapid increase in post-partum until day 5, followed by a plateau that persists for 25 days postpartum; after that the HYPRO concentration rises again to reach the zenith of this breed. The two-way ANOVA has shown that Friesian breed has a HYPRO pattern significantly different from the other breeds.

In the three examined breeds we recognize three different types of HYPRO profile. Cabannina has shown a peak (significant only for this breed) followed by a decrease and a plateau that lasts until the end of the observational period. Varzese breed does not reach a real HYPRO concentration peak, but has maximum at 10 days (at borderline significance probably due to the low sample size) followed by a decreasing trend in levels up to 30 days post partum. The Friesian HYPRO profile is characterized by high concentrations since the delivery day, with a rise on day 3 post partum and then a stabilization of values.

It must to be noticed that from a clinical point of view all animals had a complete uterine involution around day 40. And so it seems obvious that HYPRO patterns are characteristic of each breed. In particular, Friesian breed, which has undergone a genetic selective process oriented to maximize the production, shows a different collagenic metabolism.

It remains to clarify the origin of HYPRO district that could not come solely from the uterine matrix, given the ubiquitous nature of collagen. These results can be considered as a starting point to try to clarify the mechanisms of uterine involution process, the intrinsic and extrinsic factors of the animals and any phenomena that would affect reproductive performance of cows.

About reproduction physiology, the results obtained indicate that the reproductive physiology of Varzese and Cabannina is characterized by an early resumption of ovarian activity and by an early fecundation opportunity: in fact, the onset of first estrus can be observed 20 days after birth and the opportunity to impregnate can occur in the following cycle, i.e. approximately 40 from birth. That would allow farmers to achieve the goal of a calf/year, as the primary indicator of welfare, reproductive efficiency and good mammary function. According to unpublished data, obtained during trials, it could be said that autochthonous breeds have peculiar features to solve current problems of the scenario of high yielding dairy cows. As previously said, in the current system of cattle breeding, cows have dramatically increased "energy and financial voracity" (diet based on starch and protein meals, great health and structural investments due to several high recurring diseases (Ingvartsen et al., 2003; Collard et al., 2000; Carlén et al., 2004). In post partum period, energy needs required by high-yielding Holstein cows has increased by 25% compared to thirty years ago, despite the considerably limited growth in muscle mass (Agnew et al., 2003). All experts know about mobilization of various constituents from adipose tissue to support breast functions in producing milk (Veerkamp, 1998), but few know that the muscle is an important structure for reserves of aminoacids. In highly selected cows this phenomenon is much more marked than in cows genetically less selected (Pryce 2004). A cow's energy balance decreases even a couple of weeks before parturition, as a result of the animal's reduced ability of food ingestion. In the first weeks after birth, food ingestion cannot compensate the wide adipose tissue mobilization. Therefore, cows maintain this status of negative energy balance (NEB) for 5-7 weeks from birth (Grummer 2007). At the beginning of lactation, mobilization of adipose tissue and low blood glucose bioavailability are key events to induce metabolic syndromes (Ingvartsen et al., 2003), ketosis, liver diseases, paretic-spastic syndromes and foot diseases

(Collard et al., 2000). In autochthonous dairy farms ketosis and other metabolic syndromes are hardly ever present: in fact, these cows can keep up their double aptitude for maintaining a good milk production and creating a favorable muscle mass. A feature giving Cabannina and Varzese cows an interesting physiological ability to solve imbalances during NEB status through abundant energy reserves (consisting of subcutaneous and inframisial adipose tissue and muscle itself) immediately available to provide the animals with glucose and amino acids.

4.6 References

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CHAPTER 5

General Discussion

5. General discussion

5.1. Future and perspectives

Thanks to our studies directed to 2010 the "Year of Biodiversity" started about 5 years ago, we verified that the autochthonous dairy cattle, in our case Varzese and Cabannina, with their distinct characteristics of rusticity, longevity and frugality fully satisfy the future requirements identified by "2050 report" FAO. In this report it is expected that world population will reach nine billion people and birthrate will prevail in developing countries. Food resources (wheat, corn, barley, etc..) will be used to ensure people survival in these countries, thereby reducing the availability of silage and concentrates addressed to cattle breeding. This redistribution of primary resources will force bovine species to a diet based on forage biomass with low energy and water consumption, associated with the use of less productive areas, such as foothills, forests and pastures, designing the cereals availability to human consumption.

In this context it should not be overlooked that climate changes will continue to advance, modifying the environments in which plants grow. This fact will put farmers and breeders facing new challenges. According to the protocol called 'Europe 2020' we need to reduce greenhouse gas emissions by 20%, increase energy efficiency by 20% with a reduction in consumption and 20% increase energy from renewable sources. Therefore, it is expected that in certain areas will be implemented long-term and perennial crops, will be reduced energy consumption for sowing, harvesting, grain drying destined to livestock consumption and will increase the availability of mature manure to improve soil fertilization, reducing the nitrogen easily washable. Using cattle with double (milk and meat) or threefold (milk, meat and work) aptitude very rustic and frugal, such as Italian autochthonous cows, able of a good food conversion into milk and meat, will make a beneficial usage, compare with the most cosmopolitan and selected Holstein, Brown Swiss and Jersey unable to maintain high milk yield and reproduction standards, reared with a diet mainly based on forage and cereals and protein-deficient.

Holstein, Brown Swiss and Jersey, subjected to a mono-aptitude selection in the last 40 years, almost aimed to a quantitative milk production, reach, from the physiological point of view, a very critical situation: they have missed good reproductive efficiency characters (calving interval and conception rate), excellent longevity in farm, resistance to stress and diseases (metabolic syndrome, ketosis, mastitis and foot diseases), whereas they have dramatically increased the "energy and financial voracity "(diet based on starch and protein meals, great health and structural investments due to several high recurring diseases. The innovative idea is to find solutions to the current dairy breeding scenario, orienting our sight on an existing Italian bovine heritage, diversifying from the current thought that has driven genetic selection of dairy cosmopolitan breeds in the last 40 years.

The data at our disposal, provided by ongoing research at the laboratory are very encouraging.

Milk is the most complete food in nature; its nutritional value is mainly attributable to proteins and fats. Milk fat is present as a suspension of defined globules, with a biological membrane which gives to each globules an identity and a well-defined structural and functional behavior. The globules dimensions have been extensively described in mass milk of Holstein cows, both native and subjected to treatments such as homogenization and pasteurization. The fat globules interact with milk casein of curd and their size can influence the processes of lipolysis and ripening of cheese.

The results achieved by comparative evaluations of Varzese, Cabannina and Holstein breeds from the same farm and lactation period have highlighted relevant geometric and dimensional differences. The milk of Varzese and Cabannina cows has percentage of fat more abundant than Holstein ones and the size of fat globules are smaller, with a larger surface area available for interactions with proteins for the enzymatic digestion. These features give good conditions for dairy products creation with different intrinsic characteristics, not neglecting their better digestibility.

An important characteristic that influences physical, organoleptic and nutritional properties of milk is its fatty acid composition: in fact, it may change in relation to many exogenous factors (environment, nutrition, management, season, milking) and some endogenous (related to genetics such as breed, stage and number of lactation). The strategies used in recent years have been oriented to increase the unsaturated fatty acids levels such as oleic acid (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA), thanks to their beneficial effects on human health in the prevention of major diseases typical of the '"Western man", such as cardiovascular diseases. Recently, some authors have based their studies to the cis-monounsaturated fatty acids (cis-MUFA), demonstrating specific biological activities. Among these myristoleic acid (C14:1-cis), palmitoleic acid (C16:1-cis) oleic acid (C18:1-cis) have shown antibacterial, antifungal, antiprotozoal and antiviral activities(11.12). The milk quality is therefore not limited to the fat content in toto, but can be improved by the unsaturated fatty acids profile. These are produced in the mammary gland by Δ9-desaturase enzyme that catalyzes the insertion of a double bond between atoms carbon C9 and C10 and the evaluation of its activity can provide an additional parameter of milk quality. Therefore, a high cis-MUFA content, which was demonstrated in milk of autochthonous cows, destined to human diet, not all of the newborns, could be welcome thank to its important effects on human health (reduction in insulin, total cholesterol and cholesterol LDL plasma levels).

Another important component of human diet derived from animal is represented by meat. Even this, like milk, is the bearer of fat with saturated and unsaturated component, the first more abundant in adipose tissue while the second, a fundamental part of phospholipid membranes, more abundant in the muscle compartment. The fatty acid composition is influenced by the diet, but even more by the different mechanisms of digestion in different species: it is sufficient to consider that polyunsaturated fatty acids are more abundant in the adipose tissue of pigs whereas in intramuscular fat of ruminants. Fat is an important conditioning factor in some sensory aspects of meat, such as tenderness, fluid retention capacity, degree of oxidation and the aromas that result from cooking. Even in muscle fat the presence of fatty acid mono-and poly-unsaturated depends upon the desaturase enzyme and it is supposed that its activity is related to its genetic expression, therefore dependent on the breed. As noted by the data from milk studies, we expect that these fatty acids may be present in fat meat in different amounts. From fatty acids oxidation and in particular from linoleic acid (C18:2-n6) and linolenic acid (C18:3-n3) derives the volatile compound, responsible for the typical meat aromas. This component and other natural antioxidants, such as vitamins A and E, give the meat an intrinsic capacity to resist to oxidation, improve palatability, its time and storage stability. In muscle should not be forgotten connective component , whose major exponent is collagen. In latter, the amino acids more present are proline and hydroxyproline (HYP), whose quantity determines the degree of tenderness, cutting and chewing resistance offered by meat. Also from the degradation of these amines derives the formation of N-nitrosamines, compounds well known for their carcinogenic properties. On one side proline is a precursor of N-nitrosamines, on the other side hydroxyproline is an inhibitor.

HYP has recently been studied during uterine involution process. The HYP trend in serum is indeed an innovative method of monitoring the immediate post-partum uterine collagen remodeling, an essential event for the normal resumption of cyclic ovarian activity.

Our data, in a comparison between Varzesi and Holstein, have shown that the time of return of normal uterine architecture in Varzesi are faster and that the reproductive physiology is characterized by an early resumption of cyclic ovarian activity and early fecundation possibility: that would allow farmers to achieve the target of a calf/year, as an indicator of welfare, reproductive efficiency and mammary correct function.

Restoration of endangered niche breeds can undoubtedly give a boost to local products and to conservation of livestock biodiversity; FAO sustains livestock biodiversity as a "safety net for the future", mainly in developing countries, as reported in a recent document, FAO, 2010. These principles can also be extended to developed countries with the aim to better exploit local resources and preserve relic breeds from an impending extinction- which would mean the loss of a priceless legacy. In the forthcoming years, the peculiar nutritional and nutraceutical aspects present in milk and in dairy products deriving from biodiversity farms will hopefully show up.

CHAPTER 6

Summary

6. Summary

In the last 40 years, the first goal pursued by dairy cattle community has been to increase individual production from a quantitative point of view, by high yielding dairy cows selection (BLAP). This mono-aptitude criterion has caused a decline in reproductive performances worldwide (eg increase in calving interval). The comparative physiological study of autochthonous cattle to value "Ancient Autochthonous Biodiversity" opens up interesting prospects for livestock to comply the FAO 2050 target, as these animals are guarantors of a good milk production, excellent and typical cheese making, peculiar characteristics of rusticity, frugality, longevity and resistance to multiple diseases. Therefore they appear to be good candidates for traditional food production. The study of autochthonous breeds lactation and reproduction physiology could be strategic to improve knowledge and develop a more rational dairy livestock in view of reduced availability of cereals and plant proteins for livestock in the next 40 years.

From a productive point of view the most variable component was analyzed. The importance of fat composition of milk in human and animal nutrition has in the last years come rapidly to the fore. Several nutritionist Associations recommend the intake of higher levels of unsaturated fatty acids (PUFA and *cis*-MUFA) in milk despite of saturated fatty acid levels: increases in PUFA and *cis*-MUFA in milk fat can be reached by enhancing the activation of the enzyme Δ9-desaturase or by selecting subject with high desaturating ability. In this research, two endangered local bovine breeds of Northern Italy, Varzese and Cabannina, were considered for the PUFA and *cis*-MUFA content and compared with the PUFA and *cis*-MUFA levels in Friesian cows bred in the same conditions. The milk collections were performed from the 40^{th} to the 180^{th} day of lactation. Milk

fat was analyzed by gas chromatography and desaturase indices for C14, C16 and C18 fatty acids were determined. Varzese and Cabannina milk fat contains significantly overall higher levels of some PUFA and *cis*-MUFA with respect to Friesian, and higher desaturase indices, mainly C16 desaturase and the total desaturase indices. The results rise the importance of the recovery and the reevaluation of local breeds, not only for the biodiversity *per se*, but also in order to bring out peculiar characteristics of niche milk.

From a reproductive point of view serum hydroxyproline (HYPRO) trend was considered for the uterine involution monitoring. Postpartum period is a physiological process between birth and complete uterine involution, essential to bring the female genital apparatus back to favorable conditions for optimal embryonic development and implantation, resumption of ovarian cyclicity and future reproductive efficiency; the latter is a parameter that unequivocally determines the career of a cow in the herd.

In this research, the same cows involved for milk analysis, were subjected to blood collection and hydroxyproline determination. Blood samples were performed the day of birth (day 0), 1,2, 3, 4, 5, 10, 15, 20, 25, 30. The two autochthonous breeds showed lower starting points and average concentrations than the Friesian cows. Only the Cabannina cows showed a peak in day 4 statistically higher than the value at day 0. All breeds showed at day 40 after birth a completely uterine involution and so it seems obvious that HYPRO patterns are characteristic of each breed. It remains to clarify the origin of HYPRO district that could not come solely from the uterine matrix, given the ubiquitous nature of collagen. These results can be considered as a starting point to try to clarify the mechanisms of uterine involution process, the intrinsic and extrinsic factors of the animals and any phenomena that would affect reproductive performance of cows.

CHAPTER 7

Acknowledgements

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