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Physiological approach to the study of autochthonous cattle breeds of Northern Italy

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"Between animal and human medicine there is no dividing line, nor should there be. The object is different but the experience obtained constitutes the basis of all medicine."

Rudolf Virchow (1821–1902)

Index

Abstract

This PhD project is focused on the physiological characterization of some autochthonous bovine breeds of Northern Italy, using many biological parameters in order to make a comparison with the cosmopolitan breeds. Despite the lower milk production, these breeds present other interesting and peculiar features, such as rusticity, frugality, good food conversion, high product quality (milk and meat), good fertility and good resistance and resilience against the diseases. We applied a multidisciplinary approach to study the physiological bases of the interesting traits present in the local breeds, studying three main issues: the metabolism of the transition period, the milk composition and technological properties and the ethological traits.

a) Biodiversity of the transition period

i) Study of the milk ketone bodies assessment in Modenese breed and Holstein with a characterization of physiological, reproductive and productive performances.

The objective of this study was to characterize some productive, reproductive and metabolic parameters (ketone bodies) in the Italian autochthonous cattle breed Modenese, comparing them with those of Holstein and their crossbred (F1=Modenese x Holstein; F2=Modenese x F1) breed in the same farm, in order to understand if there is a different metabolic picture that can influence the reproductive performances. Milk samples have been collected at different times of lactation (20, 40, and 90 days in milk (DIM)) and analyzed by gas chromatography-mass spectrometry to obtain the the concentration of ketone bodies. In all time points, the Modenese breed showed a significant ($p<0,05$) lower concentration of ketone bodies. The F1, F2 and Modenese showed also better reproductive performances when compared to Holstein, with a significant lower ($p<0,05$) length of days open period.

ii) Comparative evaluation of the transition period: Italian autochthonous breed vs. Holstein

We evaluated the metabolic indicators, milk protein profiles and the milk microbiota in 6 Holstein Friesian and 4 Rendena cows reared on the same farm and under the same management conditions. Quarter milk samples and blood were collected from all cows at dry-off (T1), 1 day after calving (T2), 7-10 days after calving (T3) and 30 days after calving (T4). Blood samples were used for the analysis of plasma metabolites such as: glucose, total cholesterol, urea, inorganic phosphorus, total protein, albumin, total bilirubin, aspartate aminotransferase (GOT), γ-glutamyltransferase (GGT), creatinine, NEFA, ß-OH-butyric acid (BHBA), thiol groups (SHp) and ferric reducing antioxidant power (FRAP). Quarter milk samples were subjected to bacteriological culture, protein profiles and characterization of the milk microbiota. Holstein cows showed a more severe fat mobilization, lower muscle mobilizationand systemic inflammatory response at T2 and T3 in comparison with Rendena cows. Significant differences were seen in the general composition of the milk microbiota of the two breeds. Concerning the milk protein abundance profile, pronounced differences were seen in the colostrum (T2), with significantly higher amounts of protective molecules (immunoglobulins and other immune-related proteins) in Rendena.

b) "Milks" biodiversity

i. Fatty acid profile, desaturase and atherogenic indices in milk of Holstein Friesian and Italian autochthonous cattle breeds

In this study the characteristics of the fatty acid profile in local Italian bovine breeds (Cabannina, Varzese, and Valdostana) are compared with those of Friesian, , a conventional breed, during the first period of lactation. The local breeds show a general trend to have higher unsaturated fatty acid percentages, as well as lower desaturase indices (related to C14, C16 and C18) and atherogenic index, with respect to Friesian cows.

ii. NMR-based metabolomics as a tool to characterize the milk biodiversity

In the present study we investigated the variations of the metabolic profiles during lactation in milks obtained from Friesian and autochthonous breeds from Northern Italy by 1H-NMR metabolomics. Furthermore, the external factors influencing the milk composition were minimized: the cows were bred on the same farm, were fed with the same diet, and were matched by for the lactation interval and lactation stage. Our results showed a difference in milk composition between breeds and in relation to stage of lactation.

iii. Milk microbiome characterization between healthy Holstein Friesian and Rendena cows

The aim of this study was to compare the milk microbiota in 6 Holstein and 3 Rendena cows reared on the same farm under the same management conditions, with a special focus on the transition period. Four time points (dry-off, 1 day after calving, 7-10 days after calving and 30 days after calving) were considered. The taxonomic profiles of cosmopolitan and local breeds were dominated by *Firmicutes*, mostly represented by the *Streptococcus* genus, followed by *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria*. In both Rendena and Holstein the most abundant species was represented by *Str. thermophilus*, a lactic acid bacterium widely used in the fermentation of dairy products. However, the microbial populations were profoundly different in the two breeds along all the time points: the Rendena milk samples showed lower biodiversity and more stable microbial ecosystem.

c) Ethological biodiversity

i. Cattle Personality biodiversity in autochthonous Italian breeds: a pilot survey

This study assessed personality in five different cattle breeds (Bos taurus), two cosmopolitan (Holstein and Brown) and three Italian autochthonous, through a personality questionnaire completed by handlers (milkers). The objective was to determine whether this method could detect differences in personality, including breed, age and sex differences. Milkers' assessments found breed and individual differences in the animals studied. Differences in personality traits resulted to be quite evident between individuals but consistent within each breed.

With these results, we have shed some light on the physiological mechanism at the base of the interesting features showed by the autochthonous Italian cattle breeds. This can contribute to the re-evaluation of bovine local breeds and their products, in order to recover the continuously reducing numbers, and enhance the quality of derived products, with positive effects on farm economy and biodiversity.

Riassunto

Questo progetto di dottorato ha avuto come scopo la caratterizzazione fisiologica di alcune razze autoctone bovine del nord Italia, tramite l'utilizzo di diversi parametri biologici che sono stati confrontati con quelli ricavati da alcune razze cosmpolite. Le razze autoctone, nonostante la minore produzione di latte, presentano altre caratteristiche interessanti e peculiari come la maggior rusticità, frugalità, la miglior conversione alimentare, l'alta qualità dei prodotti (latte e carne), buona fertilità e buona resistenza e resilienza alle malattie. Allo scopo di comprendere i meccanismi fisiologici alla base di queste interessanti caratterische, abbiamo applicato un approccio multidisciplinare basato su tre tematiche principali: il metabolismo del periodo di transizione, la composizione del latte e le sue proprieta tecnologiche e lo studio dei tratti etologici.

a) Biodiverstià del periodo di transizione

i. Studio della concentrazione dei corpi chetonici nel latte di Modenese e Holstein e caratterizzazione delle performance fisiologiche, riproduttive e produttive.

L'obiettivo di questo studio è stato quello di caratterizzare, all'interno della stessa stalla, alcuni parametri produttivi, riproduttivi e metabolici (corpi chetonici) della razza bovina autoctona italiana Modenese, confrontandoli con quelli della razza Holstein e dei loro incroci (F1 = Modenese x Holstein; F2 = Modenese x F1) allo scopo di determinare la presenza di un differente quadro metabolico che possa influenzare le prestazioni riproduttive di questi animali. Campioni di latte sono stati raccolti in diversi momenti della lattazione (20, 40, 90 giorni di lattazione) e analizzati mediante gascromatografia-spettrometria di massa per determinare la concentrazione di corpi chetonici. In tutti i time points, la razza Modenese presenta una concentrazione di corpi chetonici inferiore. F1, F2 e Modenese hanno mostrato anche migliori prestazioni riproduttive rispetto all'Holstein, con una lunghezza per periodo parto concepimento significativamente minore ($p<0,05$).

ii. Valutazione comparativa del periodo di transizione: Rendena vs Holstein

All'interno della stessa azienda sono stati valutati il metabolismo, i profili proteici e il microbiota del latte di 6 Holstein e 4 vacche di razza Rendena. A tale scopo, campioni di sangue e di latte di ogni singolo quarto mammario sono stati prelevati in quattro differenti time points: messa in asciutta (T1), giorno del parto (T2), 7-10 giorni di lattazione (T3) e 30 giorni di lattazione (T4). I campioni di sangue sono stati utilizzati per l'analisi di svariati metaboliti plasmatici, quali: glucosio, colesterolo totale, urea, fosforo inorganico, proteine totali, albumina, bilirubina totale, aspartato aminotransferasi (GOT), γ-glutamiltransferasi (GGT), creatinina, NEFA, ß-idrossibutirrato (BHBA), gruppi tiolici (SHp) e potere antiossidante di riduzione ferrica (FRAP). I campioni di latte invece sono stati sottoposti a coltura batteriologica, caratterizzazione del profilo proteico e del microbiota. I risultati hanno mostrato come nel periodo compreso tra il T2 e il T3 le Holstein soffrano, rispetto alle Rendene, di una più intensa mobilizzazione dei grassi, una minore mobilizzazione proteica e una maggiore risposta infiammatoria sistemica. Inoltre, differenze significative sono state osservate nella composizione generale del microbiota del latte delle due razze. Per quanto riguarda il profilo di proteine del latte, sono state osservate differenze importanti nel colostro (T2): le Rendene possiedono quantità significativamente più elevate di molecole protettive (immunoglobuline e altre proteine immuno-correlate).

b) Biodiversità dei latti

i. Studio del profilo acidico e degli indici aterogenetico e di desaturasi nel latte di Holstein e di razze autoctone Italiane

In questo studio, il profilo degli acidi grassi del latte di alcune razze bovine locali italiane (Cabannina, Varzese e Valdostana) è stato confrontato con quello della razza cosmopolita Holstein. I risultati mostrano come le razze locali possiedano percentuali più elevate di acidi grassi insaturi (UFA,

MUFA, PUFA), nonché indici di desaturasi (correlati a C14, C16 e C18) e aterogenico inferiori, rispetto alle Holstein.

ii. Utilizzo della risonanza magnetica nucleare per caratterizzare la biodiversità dei latti

Nel presente studio, tramite l'utilizzo della risonanza magnetica nucleare, sono state studiate le variazioni dei profili metabolici nel latte appartenente a due gruppi di animali, un gruppo composto da Holstein e un secondo gruppo composto da diverse razze autoctone del Nord Italia. Tutti gli animali appartenevano alla stessa azienda e quindi sottoposti allo stesso tipo di alimentazione e di condizioni ambientali . I nostri risultati hanno mostrato come ci sia una variazione dei metaboliti tra i due gruppi presi in considerazione. Inoltre, all'interno dello stesso gruppo si sono evidenziate differenze a seconda del momento della lattazione.

iii. Caratterizzazione del microbiota in bovine sane di razza Holstein e Rendena

Lo scopo di questo lavoro è stato quello di confrontare il microbiota del latte in 6 vacche Holstein e 3 Rendene allevate nella stessa azienda, durante il periodo di transizione. Sono stati considerati quattro time points (messa in asciutta, 1 giorno dopo il parto, 7-10 giorni dopo il parto e 30 giorni dopo il parto). I profili tassonomici delle razze cosmopolite e locali sono risultati dominati dal phylum *Firmicutes*, per lo più rappresentati dal genere Streptococcus, seguito da *Proteobacteria, Bacteriodetes* e *Actinobacteria*. Sia nella Rendena che nella Holstein, la specie più abbondante è risultata quella dello *Str. thermophilus*, un batterio lattico ampiamente utilizzato nella trasformazione dei prodotti caseari. Tuttavia, in tutti i time points, le popolazioni microbiche sono emerse profondamente differenti nelle due razze: la Rendena presentava una biodiversità inferiore e un più stabile ecosistema microbico.

c) Biodiversità etologica

i. Biodiversità nella personalità delle razze bovine autoctone italiane: un'indagine pilota

Questo studio ha valutato la personalità in cinque diverse razze bovine (Bos taurus), due cosmopolite (Holstein e Brown) e tre autoctone italiane, attraverso un questionario sulla personalità completato da operatori (mungitori). L'obiettivo è stato quello di determinare se questo metodo potesse rilevare differenze di personalità includendo differenze di razza, età e sesso. Le differenze nei tratti della personalità sono risultate piuttosto evidenti tra individui ma coerenti all'interno di ciascuna razza.

Con i risultati di questo lavoro di tesi abbiamo fatto luce su alcuni dei meccanismi fisiologici alla base delle carattesitiche peculiari tipiche di alcune razze autoctone italiane del nord Italia. Ciò può contribuire alla rivalutazione di queste antiche razze e dei loro prodotti, permettendo un recupero di questi animali con effetti positivi sull'economia zootecnica e sulla biodiversità.

Chapter 1

Introduction

1.1 Definition and general aspects about Biodiversity and Agrobiodiversity

Biodiversity is defined by the Food and Agriculture Organization of the United Nations (FAO) as the "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (Thomas B. et all 2014).

The term "biodiversity" was proposed for the first time in occasion of the *"National Forum on Biological Diversity"* held in Washington in 1986 and is attributed to E.O. Wilson (Sarkar S., 2002). The term derives from the fusion of the expression "biological diversity".

The biodiversity can be consideratd as the main tool that allows nature to remain synchronized with the speed of environmental changes. The reduction or absence of genetic variability leads to a decrease (or disappearance) of homeostatic capacity or self-government of the biological system, with the risk of losing information that can no longer be recovered (FAO, 2015).

After several decades of neglect, finally, the theme of the loss of biodiversity is becoming increasingly important and all the international institutions decided to take to the field, particularly after the recent global food crises. The United Nations General Assembly declared the 2011-2020 period as the United Nations Decade on Biodiversity. The European Parliament has stated that: “The loss of the biodiversity has devastating economic costs for society which until now have not been integrated sufficiently into economic and other policies” (European Commission, 2011). The safeguard of the agricultural biodiversity is one of the key points of the Milan Charter, the most important document produced during Universal Exposition hosted by Milan (EXPO2015), which traces the guidelines for the future of the agriculture (Cull, N. J., 2015).

The outcomes of recent meetings of global leaders, indicate a consensus on the need to increase food production to feed a still growing population, but also on the need to ensure sustainability of that increase. Numerous global challenges are affecting the way agricultural production needs to be addressed, today and

in the future. Examples of such global challenges include population increases (over 9 billion in 2050; UN, 2008), urbanization (in 2050 the 70% of the world's population will live in cities; UN-HABITAT, 2008), an increasingly degraded environment, an increasing trend towards consumption of animal protein (Figure 1) and, of course, climate change. Climate change is likely to be the first major driver of change in agriculture, requiring changes in production methods in order to reduce the water use consumptions for irrigation and the energy required for food production (FAO, 2007a).

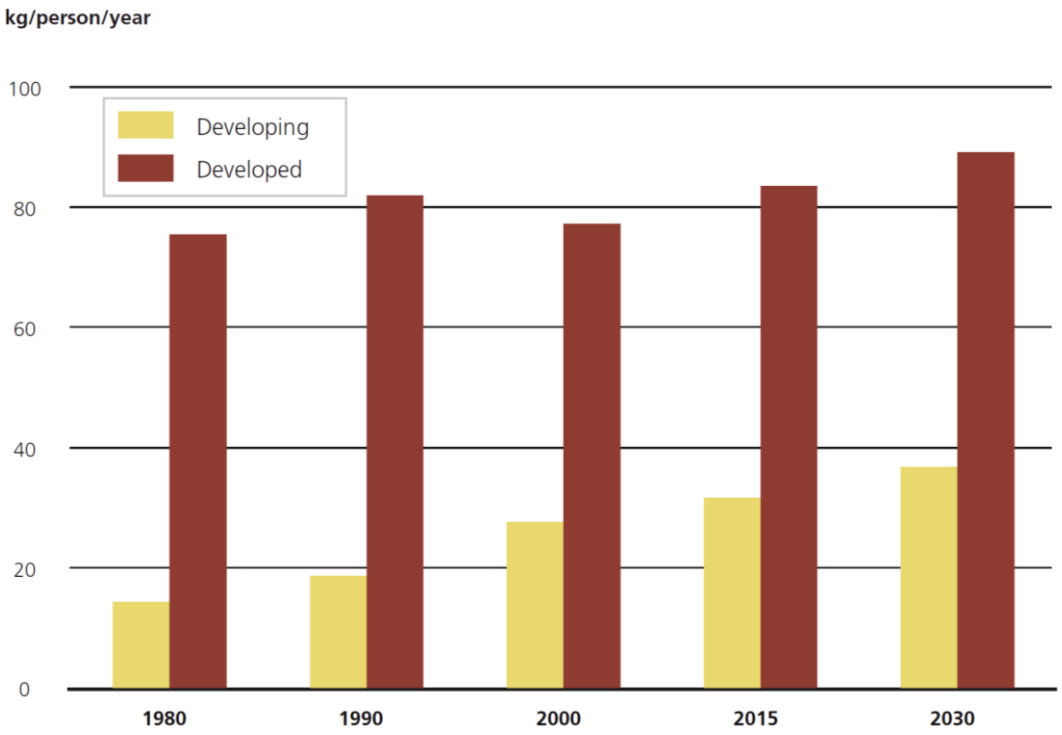

Figure 1: Changes in the meat consumption of developing and developed countries (FAO, 2007a)

The second major driver will be the sustainability of food production, in particular for the products of animal origin, because with the increase of livestock productivity (due to improved genetics, management, and industrialization) has increased also the competition with society for the food sources (cereals and human-edible vegetal protein) (Swensson et al., 2017) (Figure 2).

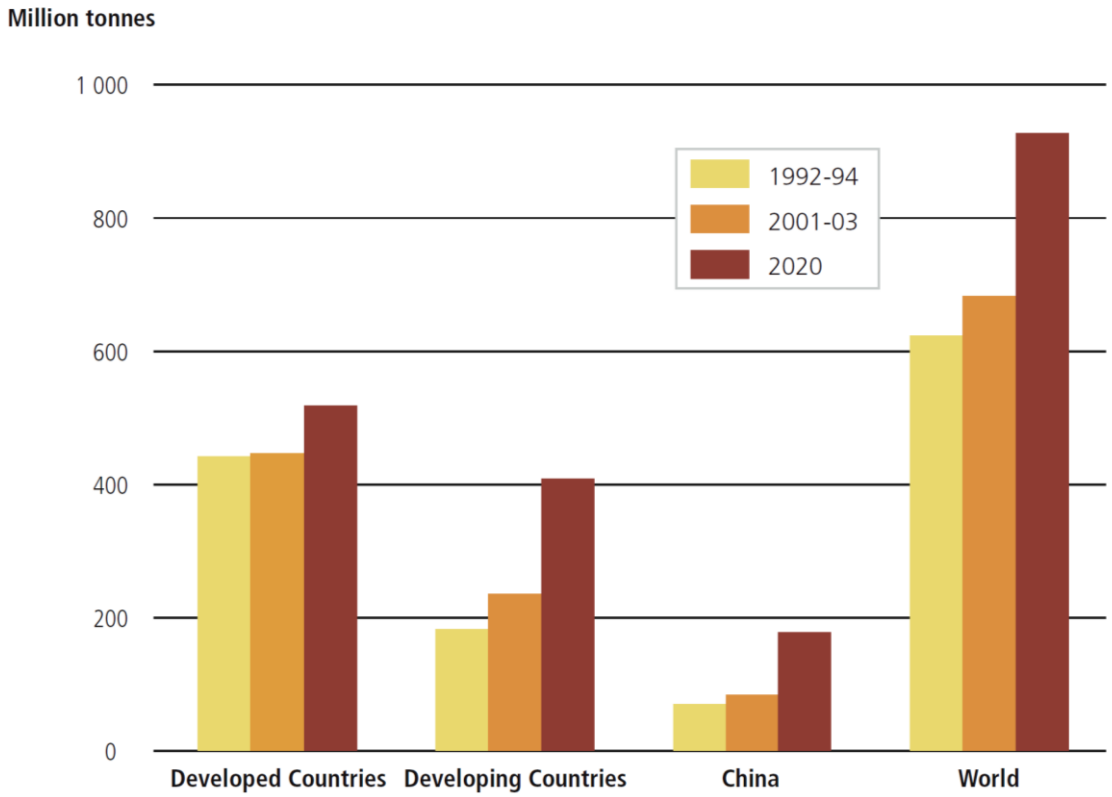

Figure 2: Changes in the quantity of cereals used as feed (1992-1994 and 2020) (FAO, 2007a)

In order to address the twin objectives of environmental sustainability and food security, the agricultural production systems need to focus more on the effective conservation and management of biodiversity and ecosystem services. Conserving the biodiversity does not just mean keeping the diversity but also means preserving and enhancing the unique cultural heritage that, with the pretext of development, run the risk of being destroyed and become extinct quickly.

In recent years in agriculture has been introduced also another term, the agrobiodiversity, which is defined by the FAO as the "variety and variability of animals, plants and micro-organisms that are used directly or indirectly for food and agriculture, including crops, livestock, forestry and fisheries". It also includes the diversity of non-harvested species that support production (soil micro-organisms, predators, pollinators), and those in the wider environment that support agro-ecosystems (agricultural, pastoral, forest and aquatic) as well as the diversity of the agro-ecosystems.

1.2 Livestock Biodiversity

Diversity has long been considered, in the human history, as a kind of disvalue. From this point of view also the animal husbandry has not been an exception: the breeder has always aimed to the homologation of the genetic pools of animals, fixing the characters that it believed useful and discarding those useless or harmful, thus operating for uniformity a loss of diversity.

A limited number of species of mammals and birds are kept by humans and used in agriculture and food production. These animals are the result of domestication processes that have been ongoing for almost 12 000 years. Over time, domesticated livestock species have evolved into distinct subgroups or "breeds" through a variety of formal and informal processes.

The composition of livestock populations has never been static. Over time, breeds emerged, were crossed to develop new breeds, and disappeared.

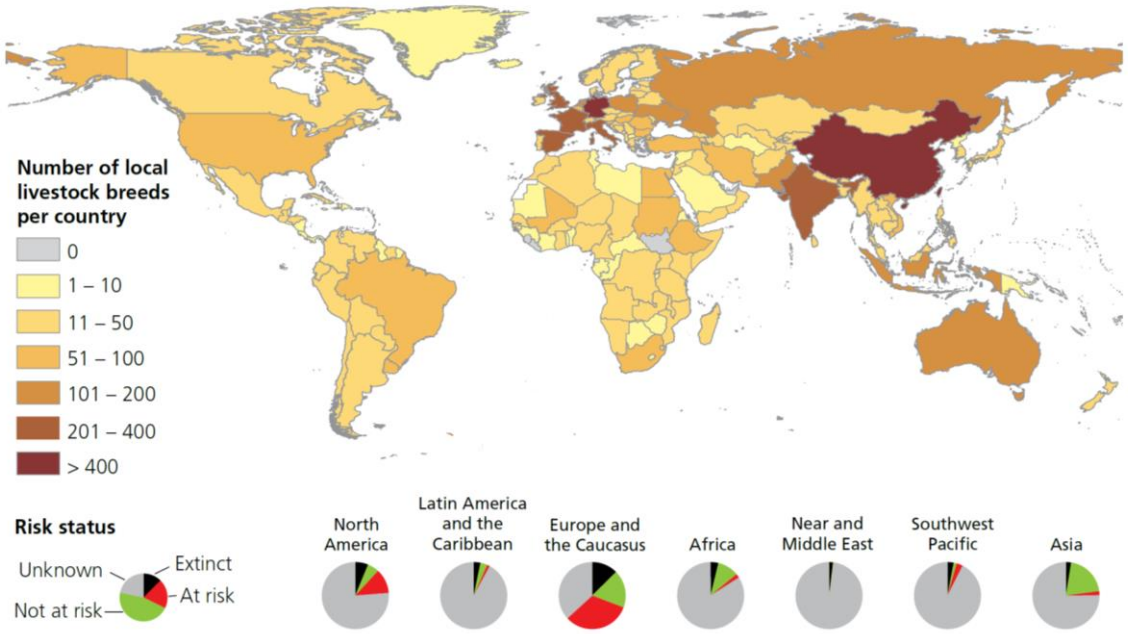

Figure 3: Livestock diversity around the world (FAO, 2015)

However, diversity always prevailed, and today, we can count 8,774 breeds (FAO, 2012), that represent the world's livestock genetic resources. Out of this total, 7,718 are local breeds (i.e. breeds present in only one country).

They have been shaped by nature and by human interventions to meet demands in the relatively short term. However, over the longer term, they will need to be drawn upon to meet future challenges related with climate change, emerging diseases, pressure on land and water, and shifting market demands, which make it more important than ever to ensure animal genetic resources are protected and used sustainably.

Therefore, today we know that the biodiversity is threatened, for this reason it is important to create strategies to promote conservation and sustainable use, and to ensure that these measures are based on appropriate knowledge and skills.

The adoption of the Global Plan of Action for Animal Genetic Resources marked 2007 as an important year, the international community recognized the vital importance of the world's livestock biodiversity. This led many countries to create policies, programs and institutional frameworks to promote the sustainable management of livestock diversity. In 2007, when FAO published the first global assessment, less than 10 countries reported having established a gene bank. Today the number has risen to 64 countries, and an additional 41 countries are planning to establish such a gene bank, according to the new report. However, many weaknesses remain, particularly in developing regions. Many countries note that improvements are constrained by a lack of financial resources.

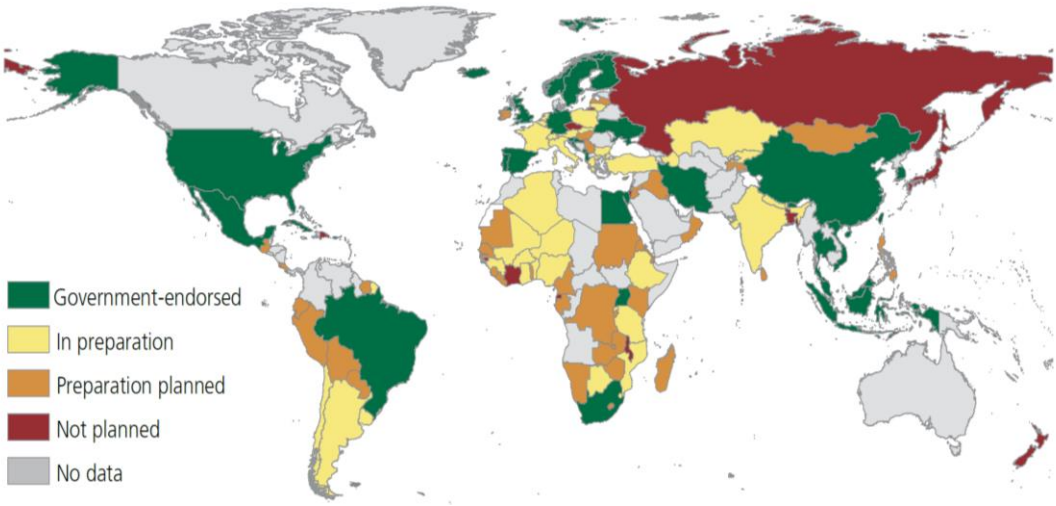

Figure 4: Status of national strategy and action plans for animal genetic resources (FAO, 2015)

Europe and the Caucasus, and North America are the two areas in the world with the highest proportion of at-risk breeds. In absolute terms, the highest number of at-risk breeds can be found in Europe and the Caucasus. Both areas are characterized by highly specialized livestock industries that tend to use only a small number of breeds for production.

Between 2005 and 2014 the percentage of the world's livestock breeds classified as being at risk of extinction increased from 15 to 17. In addition to this, a further 58 percent of breeds are classified as being of unknown risk status because no recent population data are available, this cause an underestimation of number of breeds classified as at risk (figure 5). Nearly 100 livestock breeds have gone extinct between 2000 and 2014 (FAO, 2007).

Livestock-sector trends need to be identified and monitored more effectively, for prompt and effective action to prevent the genetic erosion and breeds extinction. While there is broad agreement among stakeholders regarding the range of factors that constitute potential threats to animal genetic resources, the magnitude of these threats and the ways in which they combine to affect particular breeds in particular circumstances are often unclear.

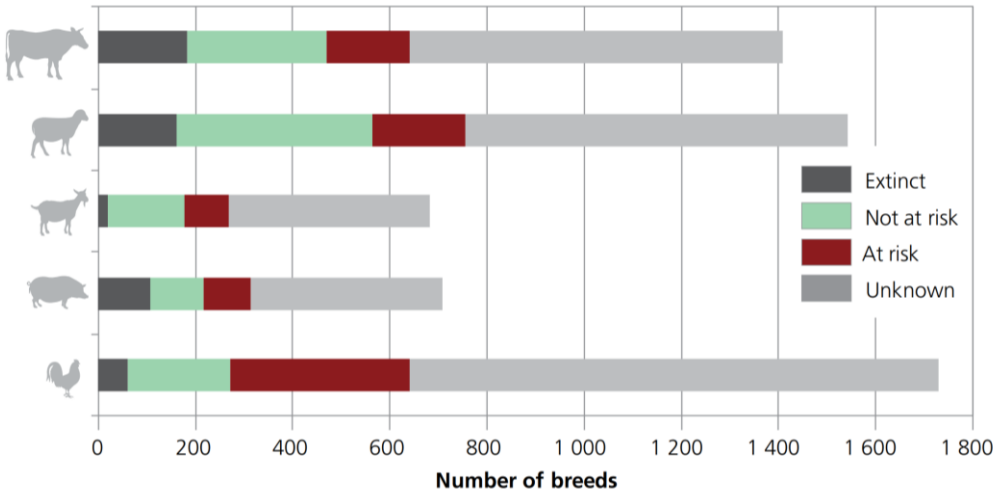

Figure 5: Risk status of the world's livestock breeds (FAO, 2015)

The main cause of genetic erosion is considered the indiscriminate use of the cross-breeding using non-native breeds. The animal genetic diversity is

threatened also by the decline of traditional livestock production systems, the weak policies and institutions regulating the livestock sector.

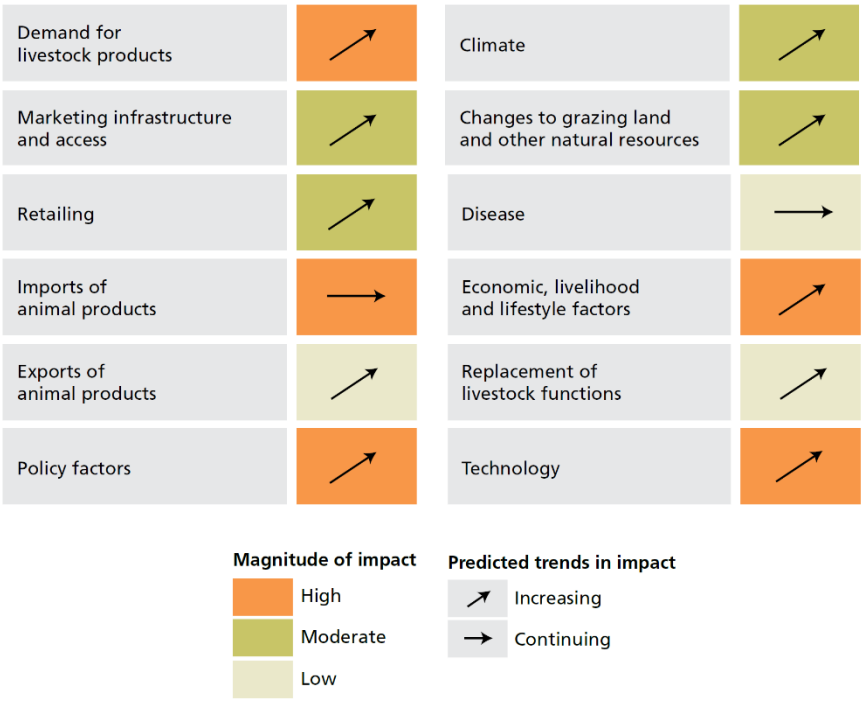

Figure 6: Drivers of change in animal genetic resources management (FAO, 2015)

1.3 Animal genetic resources and adaptation

One of the key features of animal biodiversity is that it enables livestock to be breed in a wide range of production environments, including areas where crops cannot be grown. As a result of natural and artificial selection, livestock populations acquire characteristics that facilitate their survival, reproduction and production in their respective native environments. In other words, they become adapted to local conditions. The ability of the livestock industry to reduce stressors present in the animal environment – extremes of temperature, feed shortages, diseases, etc. – has increased over the years, but the conditions in which animals are raised continue to be very diverse. Particularly in rural and pastoralist systems, animals often face difficult production conditions and have to rely on their adaptive characteristics.

In the early 1990s FAO began to build up the Global Databank for Animal Genetic Resources, which now forms the backbone of DAD-IS. Besides all the

data related with population sizes, morphology, etc., DAD-IS allows countries to enter textual descriptions of their species and breeds' adaptations (Fiugre 7).

Region	Number of breeds*	Heat	Cold	Humidity	Extreme diet	Water scarcity	Mountainous terrain	Dry environment	General hardiness
Africa	212	32	0	7	1	8	2	20	51
Asia	261	40	12	2	12	4	22	6	24
Southwest Pacific	399	8	0	0	0	2	0	0	1
Europe and the Caucasus	147	28	17	2	13	2	79	9	94
Latin America and the Caribbean	44	12	3	0	3	2	9	8	11
North America	19	2	0	0	0	1	0	1	2
Near and Middle East	33	3	0	1	0	5	1	3	2
World	**1 115**	**125**	**32**	**12**	**29**	**24**	**113**	**47**	**185**

Figure 7: Adaptations in cattle breeds as recorded in DAD-IS database of FAO. (FAO, 2015)

The genetic diversity originated by century of natural and human selection play a key role not only in the adaptation to extreme environment condition and stressors but also in the susceptibility of some species and breeds to the diseases. The information entered by countries into DAD-IS includes many anecdotal reports of such adaptations (Figure 8).

Disease/parasite	Number of reported resistant or tolerant breed populations* per species							
	Buffalo	Cattle	Goats	Sheep	Pigs	Horses	Deer	Camelids
Unspecified	8	74	22	32	27	36		1
Trypanosomosis		48	22	18	2	3		
Tick infestation/burden	1	24		5			1	
Tick-borne diseases (unspecified)	1	26	1	5			1	
Anaplasmosis		2						
Piroplasmosis/babesiosis						1		
Heartwater/cowdriosis		2		2				
Theileria		2						
Internal parasites	3	1	2	16	1	3	1	
Fascioliasis		1						
Bovine leukosis		11		1				
Foot rot		1		13				
African swine fever					6			
Tuberculosis		13	3	1				
Brucellosis	1	7	3	2				
Foot-and-mouth disease	2	1						
Total	**16**	**236**	**54**	**94**	**36**	**43**	**3**	**1**

Figure 8: Number of mammalian breed populations recorded in DAD-IS as having resistance or tolerance to specific diseases or parasites (FAO, 2015).

Diseases are one of the major constraints to livestock productivity and profitability worldwide. With the evolution of the livestock industry several disease-control strategies have been crated, including chemical or biological treatments, vaccination and preventive management. Each of these approaches has its strengths, weaknesses and limitations. Another way to address the diseases problem is to utilize genetic approaches, which can serve either to substitute or to complement other disease-control strategies.

The genetic approaches to disease control present some advantages, in particular, the long duration of the effect, the possibility of broad spectrum effects (resistance and resilience to more than one disease) and the possibility of using genetics strategies in concert with other approaches (FAO, 1999: FAO, 2015). In addition, genetic changes should, theoretically, be less subject to pathogen resistance and, nowadays, this factor could play a crucial role due to the huge problem of the pathogen antimicrobial resistance.

A great number of different strategies, including breed substitution, cross-breeding and within-breed selection can be involved in the genetic management of disease. Whatever strategy is chosen, genetic diversity (biodiversity) in the targeted livestock populations is a fundamental precondition (FAO, 2015). The preservation of multiple breeds increases the options available for matching breeds to production environments, including the disease challenges present in these production environments. Is it important also the maintenance of the genetic diversity within-breed diversity, because this allows an individual selection. Furthermore, at individual animal level, increased genetic diversity may allow for a more robust immune response to a wider range of pathogen strains and species (FAO, 2015).

Even if, in the present situation, a genetic strategy is not immediately required in order to face a current animal health problem, maintaining an higher diversity in the genes underlying resistance means maintaining a fundamental resource for combating the effects of possible future pathogen evolution (FAO, 2015). Recently, several scientific papers have highlighted the presence of

differences in resistance, tolerance or immune response to specific diseases in different breeds (Figure 9).

Disease/parasite	Breed(s) or genotype(s) showing the favourable phenotype	Compared to which breed(s) or genotype(s)	Experimental conditions	Results	Reference
Theileria annulata	Sahiwal cattle	Holstein	Artificial infection of isolated monocytes	Less severe clinical signs in the Sahiwal, gene expression profile of monocytes differs between the two breeds	Glass and Jensen, 2007
Trypanosomosis	N'Dama × Kenya-Boran cattle	Kenya-Boran	Field challenge	N'dama cross-breed more trypanotolerant, especially females	Orenge *et al.*, 2012
Tuberculosis	Zebu cattle	Holstein	Natural and artificial infection	Zebu have fewer clinical signs and decreased morbidity	Ameni *et al.*, 2007; Vordermeier *et al.*, 2012
Fasciola gigantica	Buffalo	Ongole cattle	Artificial infection	Buffalo have 1/5 the number of flukes Ongole cattle have	Wiedosari *et al.*, 2006
Rhipicephalus microplus	Nguni cattle	Bonsmara	Natural infection	Leukocyte profile differs between infected Nguni and Bonsmara	Marufu *et al.*, 2011
Rhipicephalus microplus	Braford, Brangus, Nelore cattle	Charolais	Natural infection	Fewer ticks carried by the Braford, Brangus and Nelore	Molento *et al.*, 2013
Haemonchus contortus	Caribbean hair sheep	Wool sheep	Artificial infection	Caribbean Hair sheep have higher PCV, lower FEC, higher IgA than the wool sheep	MacKinnon *et al.*, 2010
Haemonchus contortus	Gulf Coast Native sheep	Suffolk	Pasture-based infection	Native lambs have more robust immune response to infection	Shakya *et al.*, 2009
Fasciola gigantica	Indonesian Thin Tail sheep	Merino	Artificial infection	Type1 immune response makes Indonesian Thin Tail more resistant	Pleasance *et al.*, 2011
Porcine reproductive and respiratory syndrome (PRRS)	Miniature pigs	Pietrain pigs	Artificial infection	Virus replication in the miniature pigs only 3.3% of that in the Pietrain	Reiner *et al.*, 2010
PRRS	Meishan pigs	Duroc, Hampshire	Artificial infection	Meishan have less PRRS antigen in their lungs	Xing *et al.*, 2014
Marek's disease	Erlang Mountain chickens	Commercial broiler	Artificial infection	Erlang show reduced clinical signs and faster clearance of virus	Feng *et al.*, 2013
Infectious bursal disease virus	Aseel chickens	Commercial	Artificial infection	TH1 immunity, upregulation in the Aseel	Raj *et al.*, 2011
Avian influenza	Fayoumi chickens	Leghorn	Artificial infection	Resistance to infection in the Fayoumi	Wang *et al.*, 2014
Newcastle disease	Naked-neck chickens	Frizzle- and smooth-feathered chickens	Artificial infection	Naked-neck shows lower mortality	Bobbo *et al.*, 2013

Note: FEC = faecal egg count; PCV = packed cell volume; IgA = immunoglobulin A; TH1 = type 1 T helper cell.

Figure 9: Examples of studies indicating breed differences in resistance, tolerance or immune response to specific diseases (FAO, 2015).

1.4 Bovine Biodiversity: European scenario

The close contact between cattle and humans started in Central Europe approximately 7500 years ago (Benecke., 1994) and in the Northern parts of Europe a thousand years later (Gkliasta et al., 2003; Cymbron et al., 2005). Since then cattle have played a fundamental role in food and utensils production as well as in social, cultural and political development of the European societies and individual farmers (Hiemstra et al., 2010)

Currently, 277 local cattle breeds are present in Europe (FAO, 2007a), which represent about 30% of the world's FAO-registered local cattle breeds. Worldwide 16% of cattle breeds have become extinct (FAO, 2007a) and a further 16% are at risk (critical or endangered). Despite all the efforts to obtain a complete inventory of cattle breeds the status of 30% of cattle breeds still unknown. In Europe, 27% of cattle breeds are at risk and another 9% having an uncertain status (FAO, 2007b).

The decline of bovine biodiversity started during the 21st century, primarily due to increased agricultural industrialization and the increased request for animal products (Matson et al., 1997., Tilman 2002., Hammer and Khoshbakht., 2005, Tscharntke et al., 2005, Frison et al., 2011). Over the last 50 years, with advances in mechanization and the increasing availability of chemical inputs (pesticides, herbicides, drugs), it became more profitable for farmers to produce a limited set of crops (in monocultures) and livestock species (Matson et al., 1997, Swift et al., 2004).

In Europe we have assisted to the abandonment of native cattle breeds, triple purpose animals (work, milk, and meat), in favor of few breeds that have been intensely selected only on one productive character: milk or meat (Kukučková et al., 2017). As a result of this process today the dairy sector is dominated by mainly three breeds, Holstein, Brown Swiss and Jersey, which received the epithet of “cosmopolitan breeds” due to their worldwide distribution (Cossio, M. L. T. et al 2012).

This mono-aptitude selective program greatly increased the milk production but caused a problem in several other sectors. Disease incidence, including reproductive and metabolic disorders, udder health, locomotion problems, (Sørensen et al., 2007, Roxström et al., 2001a; Roxström et al., 2001b; Carlén, 2004) has increased in conjunction with genetic merit for yield (Nash et al., 2004). In addition, as a result of the dramatic increase in milk yield, we assisted to a decline of the animal longevity (Oltenacu and Algers., 2005) and the products' quality (Schennink et al., 2007, Roxström et al., 2001a).

Another factor, that should not be underestimated, is the dramatical increase of the "energy and financial voracity" of the dairy farm. To sustain the production, the producers have to provide expensive diets (based on starch and protein meals) and great health and structural investments are required to address the high recurring diseases (Ingvartsen et al., 2003; Collard et al., 2000; Carlén et al., 2004).

1.5 Bovine Biodiversity: Italian scenario

The geographical conformation of the Italian territory, extremely variegated, and the history of this country, unified only in 1861 and before then divided into many kingdoms and republics with different populations, determined over the centuries a development of an extreme variability of the bovine biodiversity (Figure 10). The union among bovine biodiversity (different breeds means different milks), territories (different animal feed) and different traditions led to the creation of the thousands of products that characterize the worldwide famous Italian diet.

Cattle breeds/populations		
Abruzzese (extinct)	Grigia alpina	Pisana
Agerolese	Grigia di Val d'Adige (extinct)	Podolica
Bardigiana (extinct)	Grigia di Val di Fiemme (extinct)	Pontremolese
Bianca Val Padana	Grossetana (extinct)	Pugliese del Veneto (extinct)
Bruna Italiana	Jersey	Pustertaler Sprinzen
Bruna Vecchio Ceppo	Limousin	Reggiana
Burlina	Lucana (extinct)	Rendena
Cabannina	Marchigiana	Romagnola
Calabrese (extinct)	Maremmana	Romana (extinct)
Calvana	Modenese	Sarda
Camandona (extinct)	Modicana	Sardo Bruna
Carniella (extinct)	Mölltal (extinct)	Sardo-modicana
Charolais	Montana	Sicilian
Chianina	Oropa	Valdarno (extinct)
Chianino-Maremmana	Ossolana (extinct)	Val di Chiana (extinct)
Cinisara	Pasturina	Valdostana Castana
Demonte (extinct)	Perugina (extinct)	Valdostana Pezzata Nera
Frisona	Pezzata Rossa Italiana	Valdostana Pezzata Rossa
Frisona Italiana	Piemontese	Valtarese (extinct)
Friuli (extinct)	Pinzgauer	Varzese Ottonese
Garfagnina	-	-

Figure 10: List of Italian cattle genetic resources described in the DAD-IS database of FAO. (Bittante ., 2011)

However, over the last century, also the Italian livestock industry has suffered the influence of the worldwide policies, that, in order to increase the food productivity (milk and meat), to cope with the increasing human population, led to a reduction of the biodiversity (Fortina et al., 2002).

This process received a boost after World War II, during the 1963 was promulgated the Zootechnic Law № 126, which imposed the use, for bovine

reproduction, of semen belonging only to the more productive cosmopolitan breeds. In less than 50 years we assisted an almost complete substitution of the Italian native bovine breeds, with mainly, two cosmopolitan breeds the Holstein Frisian and Brown Swiss (Felius et al., 2011)

Despite all, some native breeds have survived, mainly thanks to the efforts of many small traditional farmers, especially belonging to the several rural areas that characterize the Italian territory. It is exactly the link between the territory, breeds characteristics and food products that allowed these breeds to survive despite the agricultural-livestock economy has moved to an industrial model. According to the Italian Breeders Association (A.I.A) today on the Italian territory are bred 35 different bovine breeds and 1 buffalo breed (Mediterranean buffalo) (Figure 11). For 19 of these breeds, the most representative on the Italian territory (some are cosmopolitan, and some are native), all the genetic information are recorded inside of genealogical books (Italian Holsten Frisian, Brown, Pezzata Rossa Italiana, Reggiana, Jersey, Rendena, Grigio Alpina, Valdostana Pezzata Rossa, Valdostana Pezzata Nera, Valdostana Castana, Piemontese, Chianina, Marchigiana, Maremmana, Romagnola, Podolica, Limousine, Charolaise, Pinzgauer). The other 16 breeds are categorized as Italian Autochthonous breeds with limited diffusion (Agerolese, Burlina, Cabannina, Calvana, Cinisara, Garfagnina, Modenese, Modicana, Mucca Pisana, Pezzata Rossa d'Oropa, Pontremolese, Pustertaler, Sarda, Sardo-Bruna, Sardo-Modicana and Ottonese-Varzese). These breeds didn't receive any kind of intense genetic selection in order to improve a specific character, for this reason they still present double (milk and meat) or triple attitude (besides the milk and meat, these animals were used for the work, thanks to their great muscle efficiency). They are characterized by a demographic contraction that, in some cases, is very severe. An official genealogical register is responsible for the safeguard and preservation of these breeds that are not included in any national selection program (Mastrangelo et al., 2018).

Figure 11: Representation of the different cattle breeds reared in Italy and their aptitude (milk or meat) and distribution in the territory. (Italian Breeders Association, A.I.A)

Despite the apparent “low production”, to these animals are attributed other very important characteristics. All these native breeds show great rusticity, frugality and longevity. They are able to adapt themselves to marginal habitats and not intense production systems, maintaining adequate milk and meat production, of elevated quality, using diets based on forage or pasture deficient of proteins and cereals. These features make these animals the first choice in marginal areas like mountain, wood and foothill grazes, where they can guarantee an economic return to farmers. Furthermore, they have a fundamental role in the preservation of the habitat and landscape integrity, reducing fire and flood risk, promoting seedling establishment by reducing biomass accumulation (Ajmone-Marsan et al., 2001; De Haan et al., 1997).

In the last years, due to the increasing sensibilization to the farm biodiversity theme, several research groups have started to study the products derived from these animals. The milk of some of this breeds presents interesting technological and nutritional characteristics. In particular, Rendena milk showed small fat globule with high specific surface, which is important for digestion process and, when compared with the Holstein milk, a shorter coagulation time with a production of firmer curd, making this milk more suitable for cheese processing (Varotto et al., 2015). Furthermore, the milk fat contained high levels of cis-monounsaturated fatty acids (cis-MUFAs) and a higher atherogenic index, which are deeply studied for their beneficial effects on cardiovascular disease risk and blood lipid profiles (Lichtenstein et al., 2006). Similar characteristics have been shown also in the milk of other breeds, the Cabannina (Communod et al., 2012) and the Grigio Alpina (Alpine Gray) (Gottardo et al. 2017). In a different study, the Modenese milk presents elevated values of casein and titratable acidity which influence positively the cheese yield (De Marchi et al., 2009). The same milk showed also an interesting mineral profile for human nutrition with higher total Ca and P when compared with the Holstein breed. This characteristic could help to cover the daily requirement of essential minerals, maintaining bone health and preventing osteoporosis (Petrera et al., 2016).

The Italian native breeds, as well as being described as rustic animals, possess also other interesting functional traits, such as good fertility (Figure 12) , disease resistance and resilience, and longevity (Gandini et al., 2017). These functional traits could have a large impact on profitability of dairying, due to lower input costs for animal management and replacement costs (Groen et al., 1997). Unfortunately, not all the physiological mechanisms underlying these characteristics are known, and the veterinary scientific community have to put much more efforts in this field.

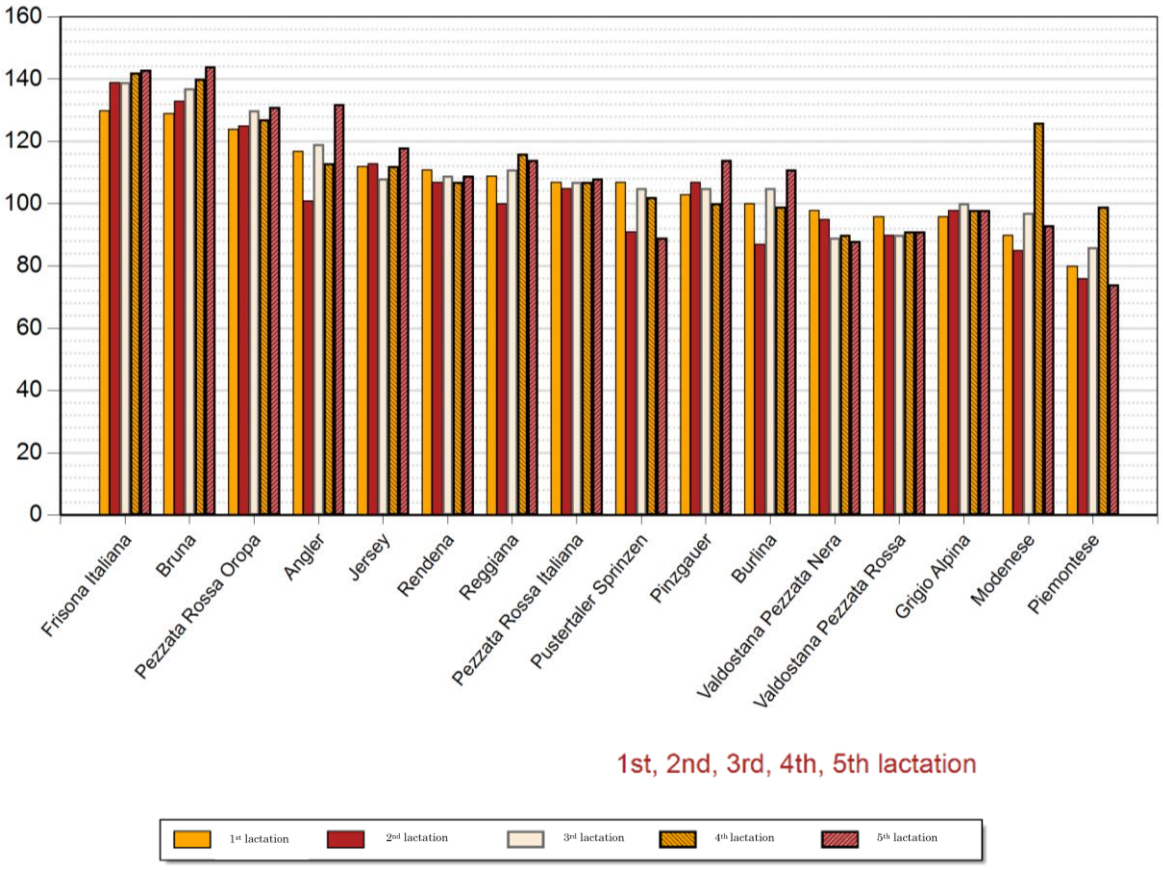

Figure 12: Median of days open for the first 5 calving intervals divided by breed (A.I.A., 2016)

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Chapter 2

Objectives

2.1 Comparative evaluation among Breeds.

The aim of this project is to obtain a characterization of the Italian autochthonous breeds of Northern Italy using many physiological parameters in order to make a comparison with the high productive cosmopolitan breeds. This investigation aimed to understand which are the physiological regulatory mechanisms that are at the base of the great rusticity, good fertility and good resistance-resilience against the diseases of these breeds, in order to increase the scientific knowledge in the perspective of developing a more efficient and sustainable dairy breeding.

Chapter 3

Native breeds studied

3.1 Cabannina

Origin and diffusion

This cow was bred in the Apennines, in the area of Genovesato and can be currently found mainly in its area of origin: the plain of Cabanne, after which it has been named. Here in Val d'Aveto, the particular climatic conditions and the need to use as pasture inaccessible areas dominated by shrubs do not allow (and make it economically not profitable) replacing it with other breeds.
This cow has always been bred for its ability to use the spontaneous natural resources of the territory, with very few integrations from the outside. This genetic type is the result of biological evolution, and consequently its products (milk, cheese) are the most representative of the production area.

Morphological characteristics

Cabannina cow is a small-size cow: males reach at withers 125cm and females 118cm. The average weigh is about 400 kilos. The head is small; it has medium-long horns, thin, white at the base and with a black tip, heading outwards, upwards, and slightly backwards; the mantle is dark brown, sometimes light brown with shades. The back is characterized by a very light stripe (cream color) with reddish shades. The limbs are characterized by good muscles giving the animal a great agility. Its features of great and tough grazing cow able to climb or enter steep areas covered by thick bushes make this cow ideal for the territory of Liguria.

Economics characteristics

Though the amount of milk it produces is small (3000 kg per lactation), it is of excellent quality, ideal for cheesemaking and rich in the scents and flavors of its grazing environment. The Cabannina is an excellent grazer, able to make the most of even the harshest environments and the poor, shrubby pastures of the Ligurian Apennines. The Presidium farmers produce a sweet dairy product, known as dolce di latte, a kind of aged ricotta (sarazzu) and various raw-milk cheeses, made without the use of starter cultures. These include the traditional prescinsêua (fresh or aged), formagetta (to be consumed after 15 days) and u cabanin (which ages for at least 40 days). The encouraging results of a scientific study on the nutritional quality of Cabannina beef have also convinced several farmers to start producing the meat as well.

http://www.parks.it
http://www.agraria.org
www.slowfood.com

3.2 Modenese-Bianca Val Padana

Origin and diffusion

This breed seems to have been originated from the light golden -coated cattle quite similar to present day Reggiana, at several steps crossbred with Podolico grey cattle. It takes its name from the area of distribution around the provinces of Modena, Reggio Emilia, Mantova, Ferrara, Bologna, where it was highly appreciated for its very good milk production.
The population reached a maximum of about 120,000 cows around 1940. From early fifties, we observed a substitution process with Brown Alpine and Holstein cattle (to give an example, in the forties in the Modena province 100% of cattle was Modenese, in 1968 26% only), followed in the '70-'80 by a reduction of dairy cattle farming substituted by fruit plant cultivations. Beginning '80: 650 cows. In 2000: a minimum of 300 cows. In 2006, 550 cows, in approximately 50 herds. Today approximately 650 cows, 60% farmed in herds mixed with Italian Friesian. Milk is sold to local market or industry, linked to the production of parmigiano reggiano cheese, anonymous and Modenese branded (a limited amount).

Morphological characteristics

Females adult: 125-140 cm height (official breed standard); average b.w. 650 kg.; white coat. Males adults: 130-160 cm height (official breed standard); average b.w. 980 kg.; white coat with grey areas on neck, shoulders, hips. Breed aptitudes: Double purpose, with major emphasis on milk (4,700 in 305 days of lactation; with 3.3% fat and 3.4% protein).. Another typical characteristic of some Italian white breed is the so-called 'cut', that is a pink coloured reversed V in the centre of the dark grey wide muzzle..

Economics characteristics

This breed was originally used for milk, labour and meat but throughout the years it was mainly selected for milk production. Conservation activities started in the eighties with limited results. Since 1997 we observe a renewed interest of the local administrations, Emilia Romagna Region and Modena Province, in collaboration with the breeder associations APA and Associazione Nazionale Bovini Razza Val Padana. Conservation activities include among others: inbreeding control, cryo-conservation (semen from 54 bulls is stored for about 20,000 doses), development of a branded Modenese Parmigiano Reggiano cheese made with Modenese milk only and of a branded Modenese meat. Two cooperatives for production and valorization of the branded products were recently created: "Valorizzazione prodotti bovini di razza Bianca Valpadana Modenese", and "Bianca Modenese società cooperativa agricola". Slow Food set up a "presidium" on the breed. Most farmers benefit from the EU agri-environmental subsidies, approximately 150 Euro per adult cow/year.

http://www.regionalcattlebreeds.eu
http://www.agraria.org

3.3 Rendena

Origin and diffusion

The area of origin is Rendena Valley (Trentino). Today this breed is mainly spread in the provinces of Padua, Trento, Vicenza, Verona and other northern Italy provinces. Very rustic, it is suitable to the most difficult grazing areas or in the mountains. Herd book since 1976, it is now slightly increasing after the 80s decrease. It is a very long lasting breed indeed

Morphological characteristics

The Rendena is a local double attitude breed, both meat and milk, with great tendencies to the second production, element that strongly characterizes its morphological aspect. The coat is smooth and uniform with different nuances of brown, darker in males where it can become almost black. Peculiar of the breed are the ivory hair in the ears, the light stripe on the back, light horns, white at the base and black on the point. Bones in general are strong but not big. Height: 130 cm; weight: 500-550 kg

Economics characteristics

The average production is about 4800 kg per lactation; this is an extremely positive number because the production is obtained with minimum concentrated food, even in the more extreme area and with the 70 % of cows that pasture for 100 and more days during summer time. In the farms in plain, characterized by a correct management, the production goes over 6000 kg of milk per lactation with good percentages of fat and proteins. The Rendena grows veals that are highly requested on the market, as also cows that weight 400-450 kg at the age 12-13 months.

www.campigliodolomiti.it
http://www.agraria.org
http://www.anare.it/

3.4 Valdostana Pezzata Rossa - Pezzata Nera - Castana

Origin and diffusion

Is the autochthonous breed from Valle d'Aosta, where almost all heads are bred (85%). It is either for milk or meat, with a fairly good milk production considering the size of the animals (on average 500 kg alive), their frugality as well as their quite good meat production and good butchery yield.
There are three breeds which differ by their morphological characteristics, coat, milk and meat production and temperament. These are: Valdostana Pezzata Rossa, Valdostana Pezzata Nera and Castana. There are two genealogical books, one to register Valdostana and Pezzata Rossa heads and the other to register either Valdostana Pezzata Nera or Castana heads.
In 1937 the National Association of Valdostana Breeders was founded A:N:A Bo.Ra.Va. (www.anaborava.it)

Valdostana Pezzata Rossa

It is the most common breed in Val d'Aosta (in 2003 there were 13,241 heads registered). It is marked by a red Pied coat shading from a light to a dark red with white head, abdomen, parts of limbs and tail. Like all red pied breeds grazing in the Mount Blanc valleys, this one comes from red pied cattle dating back to the end of the fifth century (Burgundi). One of the typical Italian dairy breed which is appreciated for its quality meat and its strength. Particularly docile and strong, it is very apt to harsh climate and also resistant to ordinary pathologies. Long-lived, frugal, this breed is able to live on coarse forage.

Economics characteristics

Valdostana Pezzata Nera and Castana together with the Swiss Hérens belong to the authochtonous cattle which originated in the Alps, coming probably from 'Bos brachyceros'. Brachycephalic breeds stand out for their lively temperament, their rusticity. They both belong to the same Genealogical Book but being different in the coat. Milk production is lower than in the Valdostana Pezzata Rossa. The coat of Pezzata Nera has a black and white pigment to built up the typical pied, while Castana has a uniform black and red pigment shading from black to tawny. In 2003 there were 7,094 heads registered. It has similar features as Pezzata Rossa, but it is usually less developed, more rustic, stronger and more harmonious. These animals show a lively quite aggressive temperament when grazing: they establish a sort of hierarchy within the herd by fighting uncruelly, though. It is just by exploiting this peculiarity that since over 50 years breeders organize fighting between their heifers (Batailles de Reines) in order to point to the strongest and give it the title of Reìna (queen).

Pezzata Rossa

Castana

Pezzata Nera

3.5 Varzese or Tortonese or Ottonese

Origin and diffusion

The Varzese is the only autochthonous breed from Lombardia: its origin is around the Apennines situated in five regions, Lombardia, Emilia, Toscana, Liguria and Piedmont. Probably it reached Italy following the barbarians during the sixth century. In the late 1950s, there were an estimated 20-25,000 head of the Varzese cattle, in less of 50 years the number has dropped to 200 head becoming endangered.
Since 1985 the Registry Office of autochthonous cattle breeds and ethnic groups of limited diffusion has been founded, in order to protect those Italian cattle breeds at risk of extinction and to safeguard this genetic heritage.

Morphological characteristics

The cows belonging to 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 weight of 550 kg), and their characteristics of rusticity, frugality, fertility and longevity make these animals the first choice in marginal areas like mountain, wood and foothill grazes. This breed can easily reach 10 births and the goal of one calf/year in a very poor management regimen. Born to be primarily used for work, Varzese oxen were famous for their strength, endurance, docility and for their resistant hoofs. Even the cows were employed for rapid and light job.

Economics characteristics

Varzese breed is appreciated for the production of meat and milk, used today to make excellent cheese including the valuable cheeses named "Nisso", “Robiola”, “Montebore” and "Molana" (all produced in Oltrepo regions, located in the southern areas of the Po River)

Chapter 4

Biodiversity of the transition period

4.1

Study of the milk ketone bodies assessment in Modenese breed and Holstein with a characterization of physiological, reproductive and productive performances

The data shown in this chapter refer to the already published paper:

Curone, G., Zanini, M., Panseri, S., Colombani, C., Moroni, P., Riva, F., Faustini, M. (2016) Milk Ketone Bodies Assessment in A Local Italian Cow Breed (Modenese) vs. Holstein and Characterization of its Physiological, Reproductive and Productive Performances. International Journal of Health, Animal Science and Food Safety, Vol 3, No 1s (2016). https://doi.org/10.13130/2283-3927/7072

Introduction

In the last 60 years the bovine genetic selection has been focused only on the milk production criterion, neglecting the metabolic and muscularity aspects; the Holstein, Brown and Jersey are proof. This mono-aptitude selective criterion has caused a decline in several aspects, the main ones being reproductive performances (*Washburn, S. P. et al 2002, Royal, M. D., et al. 2002, Lucy, M. C. 2001, Esposito et al 2014*) and disease resistance and resilience (metabolic syndrome, ketosis, mastitis and foot diseases) *(Roxström A. et al., 2001a; Roxström A. et al., 2001b, Sørensen A.C. et al.2007, Carlén E. et al 2004)*. The higher production of milk does not correspond to a real gain for the farmer, because the metabolic problems, reproductive disorders and inflammatory problems are increasing both the direct costs, such as the costs for veterinary and medicinal products, and the indirect costs, influencing in a negative way the lactation curve (*Cools S. et al . 2008*) and the economic gain. An adequate reproductive performance of the lactating herd is a major component of profitability in dairy farms *(Ribeiro E.S. et al., 2012).* Reproduction is the essential physiological stage to obtain a higher milk production efficiency. Without the effect of the pregnancy and calving it is not possible to reach an optimal development of mammary system and milk production. The scientific studies have identified the optimal cow lactation period around 305 days and the calving to conception interval (days open) around 85 days (*Hafez, B., & Hafez, E. S. E., 2000*). These physiological data are far from situation of the modern dairy farm where more than 50% of cows exceed the 305 days (*Steri R. et al 2012, Vargas B. et al., 2000; González-Recio O. et al., 2006; Cole J.B. et al., 2009*) and the days open period is around the 150 days (official national analysis Italian Breeder Association AIA, 2014).
The lengthening of these physiological times depends on many factors that affect the post-partum period, where about 50% of the cows suffer from metabolic or inflammatory disorders that influence the reproductive performance (*LeBlanc S., 2010*). During the days from calving to conception

(days open period) are diagnosed the 75% of the diseases that can be found throughout the whole productive life of the cow (*LeBlanc S., 2010*) such as the retained placenta, metritis, endometritis, lameness, puerperal collapse, hypomagnesemia, fatty liver, ketosis, abomasal displacement left and right, hypoplasia ovarian and ovarian cysts. Many of these diseases have as trigger the negative energy balance (NEB) typical of the early lactation period (first 4-6 weeks) caused by the high energy demand required for the milk production associated with the physiological suppression of appetite *(Santos J. E. P. et al., 2014)*. The energy balance, in addition to predispose to many disease, causes also a direct effect on the reproductive performance in the cows *(Butler WR., 2003)*. During the NEB we have an increase of the NEFA and the beta-hydroxybutyrate (BHBA). The NEFA high concentration (≥0,7 mM) reduces the resume of the ovarian cyclicity before the 50 days *(Ribeiro ES. et al 2013)*, while the elevated BHBA concentration are negatively associated with the probability of pregnancy after the first postpartum artificial insemination *(Walsh RB. et al 2007)*. Another marker of NEB is the milk protein percentage. As showed by Fulkerson et al. (2001), the cows with low milk protein percentage suffered from more severe and prolonged NEB compared to cows with higher milk protein percentage. Milk protein percentage has been reported to be positively associated with cow fertility, the cows with a higher milk protein percentage in early lactation have a better reproductive performance (*Yang L. et al 2009, Morton J.M. et al 2001*). Most of the studies about the reproductive disorders are focused on the cosmopolitan breeds, Holstein and Brown Swiss, because they are one of the major reported reasons for culling in dairy herds with lameness and udder health (*Waiblinger S. et al. 2004; Wathes RB. et al. 2007; Cozler YL.et al. 2009*). Analyzing the Italian autochthonous cattle breed, we found a totally different situation. Some studies have demonstrated that these animals have an early reactivation of the ovarian activity after the calving that permits a precocious insemination with a reduction of the days open period under 100 days *(Communod R. et al 2010)*. Not all the physiological mechanism underlying this phenomenon are

known, but as showed by Communod et al (2010), one important factor is the uterine involution. They demonstrated that the Cabannina and Varzese breeds (two local breeds of the northern Italy) have a shorter timeframe for uterine involution compared to the Holstein. These results could explain the early resumption of ovarian activity and the early fecundation opportunity typical of these breeds: in fact, the onset of first detectable 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 *(Communod R. et al 2011)*. In Italy, a great biodiversity in term of autochthonous cattle breed is present. There are 16 breeds officially recognized and most of them are located in the northern/centre sides of Italy. The objective of this study was to characterize some productive, reproductive and metabolic parameters (ketone bodies) in the Modenese breed, comparing them with those of Holstein breed in the same farm in order to understand if there is a different metabolic situation that can influence the reproductive performances.

Animals and samples

Comparison between Holstein and Modenese was carried out in the farm 'Società emplice Agricola Cornetti Alessandro e f.llis.s.' located in Quinzano d"Oglio (CR), the peculiarity of this farm is that it has implemented a plan to replace the entire original Holstein herd with Modenese breed using the crossbreeding methods.

The reproductive (Open Days Period and number of Services Per Pregnancy) and productive (percentage and kg of protein between the 40th and 90th days in milk/DIM) data have been recovered by the consultation of the farm registers and the APA (Provincial breeder association) data.

Milk samples

A total of 19 animals were sampled for this study:

- 4 Holstein
- 6 Modenese
- 5 F1 (Modenese x Holstein)
- 8 F2 (Modenese x F1)

All these animals were in the second lactation and their calvings were in the same period (within 10 days) and none has undergone treatments in order to obtain the estrus synchronization. The milk sample have been taken at different time of lactation (T1=20th DIM, T2=40th DIM, T3=90th DIM). Milk samples have been collected from the whole udder during morning milking and from a lactometer.

Milk samples analysis

Reagents and standards

Acetone and cyclohexanone (used as internal standard -IS) were purchased by Sigma (Steinheim, Germany).

Sample preparation and Headspace–Solid-Phase Microextraction (HS-SPME):

All samples were prepared by weighing exactly 5 g of milk in a 20 ml glass vial, fitted with cap equipped with silicon/PTFE septa (Supelco, Bellefonte, PA, USA), and by adding 10 µl of the IS solution in ethanol (0.2 mg ml-1). Standard solutions of acetone in water, containing increasing amounts of analyte (from 2 to 300 µg ml-1) and of 10 µl of IS solution were added to a fresh milk sample to obtain the calibration curve. A temperature of 10°C was selected as extraction and equilibration temperature, in order to prevent

possible matrix alteration. To keep the temperature constant during analysis, the vials were maintained in a cooling plate (CTC Analytics, Zwingen, Switzerland). At the end of the sample equilibration time (1 h), a conditioned (1,5 h at 280°C) 85 µm Carboxen/polydimethylsiloxane (CAR/PDMS) StableFlex fibre (Supelco, Bellefonte, PA, USA) was exposed to the headspace of the sample for analyte extraction (15 min) by CombiPAL system injector autosampler (CTC Analytics, Zwingen, Switzerland). Recovery was studied by adding 100 µg ml-1 of acetone to a milks ample and by comparing the results obtained from the sample and the spiked one.

Gas chromatography-mass spectrometry apparatus and conditions:

Analyses were performed with a Trace GC Ultra coupled to a quadrupole mass spectrometer (MS) Trace DSQ (Thermo-Fisher Scientific, Waltham, MA, USA) and equipped with an Rtx-Wax column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, Bellefonte, PA, USA).

Oven temperature program was: from 35°C, hold 8 min, to 60 °C at 4 °C min-1, then from 60°C to 160°C at 6°C min-1 and finally from 160 to 200 at 20°C min-1. Helium was the carrier gas, at flow rate of 1 ml min-1. Carry over and peaks originating from the fibre were regularly assessed by running blank samples. After each analysis fibres were immediately thermally desorbed in the GC injector for 5 min at 250°C to prevent contamination.

MS operated in electron impact (EI) ionization mode at 70 eV. Ion source temperature was 250°C. Selected ion monitoring (SIM) was used as data acquisition mode, the ions chosen being 43 and 58 for acetone and 55 and 98 for cyclohexanone, respectively. The dwell time was set to 100 ms. Identification of acetone and IS was carried out by comparing GC retention time with those of standard compounds. Concentration of acetone was determined in triplicate as relative area (analyte area/IS area) (Ra) using the calibration curve.

Data analysis

Ketone bodies concentrations mean values over the three periods were evaluated with a two-way ANOVA, considering the period and the year as fixed factors. On days open, number of services per pregnancy, % of proteins in milk, and kg of proteins in milk, a Spearman correlation analysis was applied. The statistical significance was set at $p<0.05$.

Results

Analyzing the data obtained regarding the values of the ketone bodies in three different time of lactation (20 days, 40 days, 90 days) concerning only the pure breeds Holstein and Modenese, it was found that the lactation period has never been significant for the concentration of ketone bodies, while there is an increasing trend in the samples taken at 40 days from calving (figure 1). This period coincides with the beginning of the peak of lactation (*Hafez, B., & Hafez, E. S. E., 2000*). This tendency is valid for all three ketone bodies.

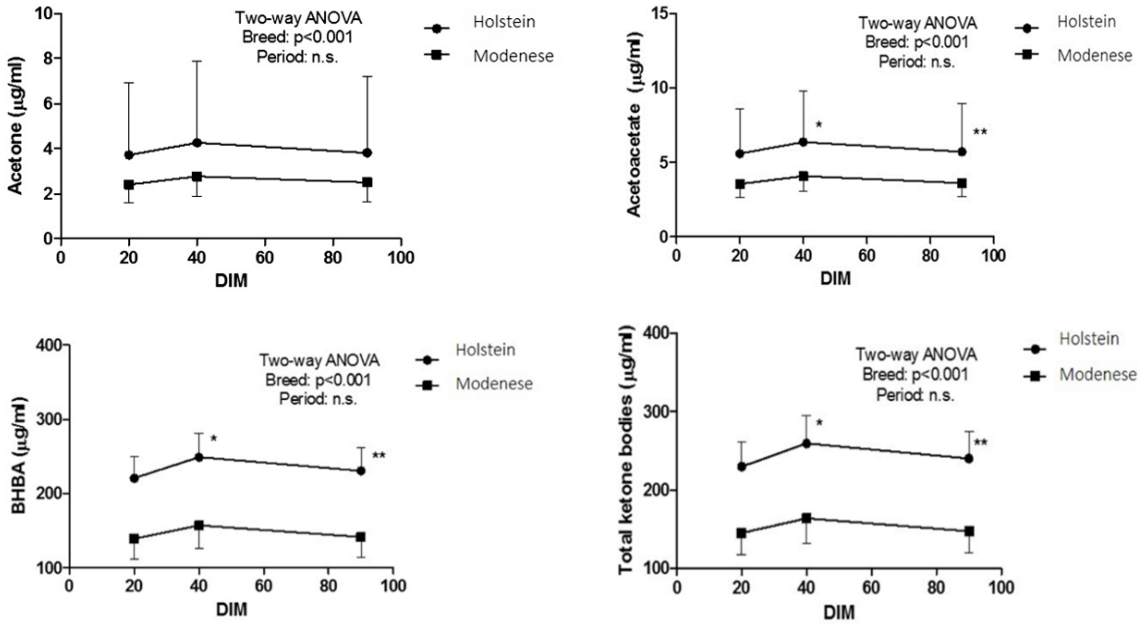

Figure 1: *The figure show the graphics relative to the comparison of the ketone bodies milk concentration in two group of animals, Modenese vs Holstein, this data are referred to three different time of lactation (20 days, 40 days, 90 days). **- p<0-05*

Comparing the ketone bodies concentration in the three periods (20th,40th,90th days in milk) (Figure 1) between different genetic lines (Holstein against the Modenese) there are big statistically significant differences ($p < 0.05$) for the acetoacetate and BHBA at the 40th and 90th days in milk (DIM) while, for the acetone the difference found isn't statistically significant. The explanation of these results could depend on two factors. The first factor, as it is easy to find in the literature, is the higher production of the Holstein that causes many difficulties to the energy metabolism, in particular the carbohydrate metabolism (*Veerkamp R.F. et al., 2003*). The second factor could be referring to the fact that the Holstein are characterized by a smaller amount of muscles. The muscular system, especially the skeletal muscle, is physiologically able to oxidize the ketone bodies (*Schaff C. et al. 2013, Brennan K.M. et al. 2009*) and to make bioavailable a larger quantity of glycogen as well as to release a big amount of amino acids that can best support the neoglucogenesis (*Van Der Drif SG. et al. 2012*). Therefore, the muscular system has an important role in the reduction of the intensity of the NEB and in the mitigation of the negative effects of the ketone bodies released during the NEB period. The perfect energetic metabolism of the Modenese breed (as showed by the ketone bodies concentration) is essential to ensure good reproduction efficiency, since it allows an optimal functioning of the hypothalamic -pituitary -ovarian axis (*Judd, S. J., 1997, Iwata, K. et al 2011*) and the entire female genital system.

Variable	F1	F2	Modenese	Holstein	2013	2014	Statistical significance per year
Days open (dd)	99.95 ± 7.81[a]	114.77 ±10.47[a]	89.24 ± 12.51[a]	215±30[b]	147.7 ±15	125 ± 21.5	0.025
Number of Service Per Pregnancy	1.73 ± 0.12[a]	1.63 ± 0.16[a]	1.70 ± 0.19[a]	3.35±0.2[b]	3.1± 1.7	3.3 ± 2.1	0.16
% protein	3.25 ± 0.04[b]	3.44 ± 0.05[a]	3.38 ± 0.06[ab]	3.35±0.1[b]	3.15 ± 0.03	3.32 ±0.14	0.23
Kg prot (kg)	50.80 ± 1.[a]	38.08 ±2.23[b]	32.24 ± 2.66[b]	41.33±10.40[ab]	40.37 ±1.58	43.31 ± 1.39	0.14

Table 1*: mean values ± standard error of the parameters evaluated in the study for the three genetic groups and for the years 2013-14. The table shows the significance statistics for the groups considered . Superscripts indicate statistical differences with p <0.05 between genetic groups : different letter corresponds to a difference at p < 0.05.*

The first variable analyzed was the Days open (table 1), the results show a statistically significant differences between different genetic lines, and the Modenese/crossbreed have the Days open period significantly lower. For the factor year, there is a statistically significant difference between the year 2013 and the year 2014. The evaluation of the days open period evidences that in F1, F2 and Modenese the length in average is between the 80-105 days, in full compliance with the provisions of the reproductive physiology of dairy cows (*Hafez, B., & Hafez, E. S. E., 2000*) and allow to reach the goal of one calf per year, this goal is impossible in the Holstein were the average of days open is 215±30 days. The good reproductive efficiency of Modenese and crossbreed is also showed by the number of service per pregnancy that is < 2 against the value of 3.35 of the Holstein, this difference is a statistical significant. The factor year was not statistically relevant.

The productive parameters analyzed were the milk proteins percentage and kilograms. For the percentage of milk proteins we found a statistically significant difference between the averages of the different genetic lines, the F2

have percentages of protein significantly higher than the Holstein and F1, while the Modenese have an intermediate value. The year does not influence this parameter. The milk protein percentage is an important indicator for both the energy balance and the reproductive efficiency (*Yang, L. et al 2010*). Elevate milk production is associated with reduction of the glucose blood levels, and glucose shortage would result in a reduction of synthesis of milk protein; thus, low milk protein percentage would be found in cows with high milk production and low fertility (*Yang, L. et al 2010*). The percentage of protein in the milk arrives at levels of excellence in particular regarding to the generation F2, values that are even higher than the purebred Modenese. This finding could be explained as a phenomenon of the synergy of the cross between F1 and pure breed. Finally, we analyzed the kg of proteins produced, that has emerged to be influenced in a statistically significant manner by the different genetic lines. The factor year, also in this variable, has not been shown statistically relevant. The analysis of kg of milk protein produced show that the F1 have the higher values, this is correlatable to their higher milk production (about a 30 % higher) due to the greater influence of the generation progenitor (Holstein).

Variables	Modenese	F1	F2	Holstein
Days open/SPP	0.72**	0.7**	-	-
%proteins/SPP	-0.51**	-	-	-
%protein/days open	-	-	-	-
Kg protein/SPP	-	-	-	-
Kg protein/days open	-	-	-	-
Kg protein/%protein	-	-0.22**	-	-

Table 2*- Correlations between productive/reproductive parameters for the examined breeds. Only significant coefficients are reported. **- p<0-01.*

Conclusions

This study has allowed to obtain a series of information about the physiological and metabolic profile of the Italian autochthonous cattle breed Bianca Val Padana / or Modenese. This autochthonous breed, reared for centuries in a large area of the Po Valley, demonstrates that it maintains peculiar and interesting physiological parameters due to his poor genetic selection. His good metabolic trim (low level of ketone bodies) associated with the optimal days open period allow to reach the goal of one calf per year, that is difficult to obtain in a normal Holstein herd. The data processing of the Modenese and its crossbreed concerning the days open period, percentage of protein in milk and number of inseminations indicate that it is a very valid breed from the point of view of the reproduction and production. The few heads of Holstein, used for this study, showed a higher milk ketone bodies concentration than the Modenese and its crossbreed, whereas the animals were bred in the same conditions. The comparative study between the races in the same breeding conditions is a fundamental tool to understand the correct reproductive and productive physiology of dairy cattle. A better resilience against the negative energy balance and his adverse effects of Modenese cattle could be one of the phenomena underlying their better reproductive efficiency, as shown from the data in this study.

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4.2

Comparative evaluation of the transition period: Italian autochthonous breed vs. Holstein

The data shown in this chapter refer to the already published paper:

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Introduction

The achievement of high production levels in dairy farms poses great difficulties on numerous subjects to adapt to the environment. This translates into increased culling rates, reduction of life expectancy, more frequent occurrence of variegated and multifactorial diseases and increased use of veterinary drugs. In dairy cattle, a dramatic improvement of milk quality in terms of somatic cell count reduction has been correlated for many years with a milk yield increase in Holstein Friesian (HF) cows. According to the technical report of the Italian Breeders Association (AIA), the average milk yield in 1,095,576 lactating Italian Holstein Friesian cows amounted to 9,325 kg in 2015, with average contents of 3.67% and 3.25% for fat and protein, respectively. The impact of these performances on animal welfare and health has been considerable. The genetic ability to increase milk production seen during the last years has been associated with a higher risk of metabolic and infectious diseases, as well as with reduced fertility. However, less is known about the biological mechanisms behind these relationships (Oltenacu and Broom, 2010). In keeping with this, cows alive in North-Eastern USA at 48 months of age went from 80% in 1957 to 13% in 2002; in the same farms and in the same period the mean calving interval went from 13 to 15.5 months (Oltenacu P.A. and Broom D.M., 2010). In the calving period, high-yielding dairy cattle are probably more susceptible to common environmental stressors in terms of housing, hygiene and feeding conditions. This may have crucial repercussions on disease occurrence early in life and on the subsequent milk production levels (Bach, 2011).

In agreement with the above data, a large meta-analysis study (Ingvartsen et al., 2003) demonstrated an unfavorable genetic correlation between milk yield and incidence of mastitis, and to a lesser extent of ketosis, ovarian cysts, and lameness. Therefore, a high correlation can be demonstrated between metabolic stress in high-yielding dairy cattle and mastitis occurrence. The highest peak of new intramammary infections (IMI) is usually recorded in the first 2-3 weeks after calving (Green et al., 2002), which partly accounts for the highest culling rates in the first 2 months of lactation (Pinedo et al., 2010). This is in contrast

with the low prevalence of clinical mastitis in some autochthonous cattle breeds, such as Rendena (REN) (Curone et al. 2016; Gandini et al. 2007). This breed is native of the Rendena Valley in Northeastern Italy (Trentino), but it can now be found in many areas of Northern Italy, and it is particularly suitable to pastures given its small size (withers height of 130 cm for females and 135 cm for males with a maximum adult weight of 5.5 q). Rendena is a rustic and double-purpose animal, mainly used for milk production, that approximately reaches 5000 kg per lactation, with an average content of 3.4% and 3.3% of fat and protein, respectively (ANARE 2012). The first calving usually occurs around the 30th month of age and the average number of inseminations is 3.3 with approximately 106 calving to pregnancy "open" days (ANARE 2012; Mazza et al 2014).

Bovine Mammary Epithelial Cells (bMECs) lining the inner surface of the mammary gland are crucial for the early defense against intramammary pathogens. bMECs constitute a physical barrier and produce several antimicrobial substances and inflammatory mediators: Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1ß), Granulocyte macrophage colony-stimulating factor (GM-CSF), Interleukin-8 (IL-8), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), lactoferrin, serum amyloid A, and cyclooxygenase-2, to name a few (Zbinden C. et al., 2014). Whenever bMEC fail to control intramammary infections, granulocytes go into action and typical clinical signs of mastitis can be detected.

The above observations raise several fundamental questions: what underlies mastitis resistance in local breeds? How can we effectively investigate it? Most importantly, can we define markers of innate immune response in the mammary gland discriminating mastitis resistant dairy cattle? These issues make a case for an *ad hoc* experimental study, in which high and low-yielding dairy cows are reared under the same conditions and carefully compared for fundamental parameters of the innate immune response to mastitis pathogens. To this aim, we identified a farm meeting these requirements, where a balanced number of healthy HF and REN dairy cows were kept under the same environmental and

farming conditions, and we applied a multidisciplinary approach to compare their innate immune response patterns, metabolic parameters, milk protein profiles and milk microbiota composition. The study was encouraged by local and regional Italian projects for the valorization of autochthonous breeds.

Materials and Methods

Animals

We collected samples from 6 Holstein Friesian (HF) and 4 Rendena (REN) cows, housed in the same farm in Pavia (Italy), under the supervision of expert bovine practitioners. Cows were housed with a tie-stall housing system, and were milked using a pipeline milking system twice daily. No dry cow therapy was used. Cows were fed ad libitum with a total mixed ration without silage using alfalfa hay, straw and concentrated feed with mineral and vitamin supplementation. All the cows remained clinically healthy along all the study duration, and no signs of disease were observed (mastitis, laminitis, endometritis and metabolic disorders). The cows (6 HF and 4 REN) were all among 2 and 4 lactations, with an average of 3.6 for HF and 2.7 for REN. The average milk yield was significantly higher in HF compared to REN (HF=5,366 kg vs REN=3,769; p=0.0147). The percentage of milk fat (HF=3.52% vs REN=3.37%) and protein (HF=3.02% vs REN=3.08%) content was comparable in the two breeds.

This study complied with Italian laws on animal experimentation and ethics (Italian Health Ministry authorization n. 628/2016-PR).

Samples

Quarter milk samples and venous blood were collected from each animal at the following time points: dry-off (T1), 1 day after calving (T2), 7-10 days after calving (T3) and 30 days after calving (T4).

Blood (10 ml) was collected from the tail vein in an EDTA-vacutainer at each time point and centrifuged 20 minutes at 2000 rpm at 4°C. Plasma was collected and immediately stored at -20°C until use.

Before milk sampling, teat ends were carefully cleaned. First streams of foremilk were discharged, and then approximately 150 ml of milk was collected aseptically from each quarter into sterile vials. Samples were delivered to the laboratory at 4°C and immediately processed. Milk (50 ml) was centrifuged for 10 minutes at 2000 rpm at 4°C. The fat layer (milk fat globules, MFG) was collected in 2 ml sterile tubes with 1.5 ml TRIreagent and vortexed for 2 minutes. Fat samples were stored at -80°C until RNA extraction. When the remaining fat layer was completely removed, 1 ml of skim milk was transferred to a 1.5 ml sterile tube and immediately stored at -20°C for lysozyme determination. The cell pellet was washed in PBS with 0.25 mM EDTA, centrifuged for 10 minutes at 1500 rpm at 4°C and finally lysed in 2-4 ml of TRIreagent and stored at -80°C until use. The rest of the milk was stored in 3 aliquots for proteomic analysis (50 ml), metagenomics analysis (15 ml) at -80°C and for bacteriological analysis and somatic cell count (SCC; 10 ml) at -20°C.

Plasma metabolites

Plasma metabolites were analyzed at 37°C by an automated clinical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA), using the methodologies previously reported (Calamari et al. 2016). Commercial kits were used to measure glucose, total cholesterol, urea, inorganic phosphorus, total protein, albumin, total bilirubin, aspartate aminotransferase (GOT), γ-glutamyltransferase (GGT), and creatinine (Instrumentation Laboratory SpA, Werfen, Monza, Milan, Italy), NEFA (Wako, Chemicals GmbH, Neuss, Germany), ß-OH-butyric acid (BHBA, kit Ranbut, Randox Laboratories Limited, Crumlin, County Antrim, UK), thiol groups (SHp, Kit from Diacron srl, Grosseto, Italy). The ferric reducing antioxidant power (FRAP) assay was assessed by adapting the colorimetric method of Benzie and Strain (1996) to the clinical auto-analyzer ILAB 650 (Instrumentation Laboratory, Lexington, MA).

Bacteriological analysis and somatic cell counts

For bacteriological analysis, quarter milk samples were allowed to thaw at room temperature. Ten microliters of milk were plated onto blood agar plates containing 5% defibrinated bovine blood. Plates were incubated aerobically at 37°C and evaluated after 24 and 48 hours. Bacteria were identified according to the guidelines of National Mastitis Council (NMC, 1999). For each quarter , SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN).

Metagenomic analysis

Five ml from each quarter milk were centrifuged at 2000 rpm for 10 min at 4°C, the supernatant was discarded and the pellet was resuspended with one ml of saline solution (NaCl 0.9%) and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was discarded and the bacterial DNA was extracted from the samples as described previously (Cremonesi et al., 2006). DNA quality and quantity was analyzed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was stored at -20°C until use.

Bacterial DNA was amplified using the primers described in literature (Caporaso et al., 2011), which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25-µl volumes per sample. A total of 12.5 µl of Thermo Master Mix 2X (Thermo Scientific) and 0.2 µl of each primer (100 µM) were added to 2 µl of genomic DNA (5 ng/µl). A first amplification step was performed in an Applied Biosystem 2700 thermal cycler, as follows: samples were denatured at 98 °C for 30 seconds, followed by 25 cycles with a denaturing step at 98 °C for 30 seconds, annealing at 56 °C for 1 minute and extension at 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes. Amplicons were cleaned-up with Agencourt® AMPure® XP (Beckman, Coulter Brea, CA) and the libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol (http://supportres.illumina.com/documents/documentation/chemistry_documen

tation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf, Illumina). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (KapaBiosystems, Inc. MA, United States) pooled in equimolar proportion and sequenced in one Miseq (Illumina) run with 300-base paired-end reads.

After sample loading, the MiSeq system provides on-instrument secondary analysis by the MiSeq Reporter software (MSR) classifying organisms from V3 and V4 amplicon using a database of 16S rRNA data. The classification is based on the Greengenes database (http://greengenes.lbl.gov/) and the output of this workflow was a classification of reads at several taxonomic levels (kingdom, phylum, class, order, family, genus, and species). Alpha diversities were calculated according to different microbial diversity metrics (i.e.: Shannon index, observed species).

Cathelicidin and Lysozyme determination

Cathelicidin was assessed by a pan-cathelicidin sandwich ELISA as described previously (Addis et al., 2016a, 2016b). For enabling logarithmic visualization, a correction factor of 0.1 was added to OD450 values to obtain the adjusted OD450 value (AOD450). Lysozyme in samples of fat and cell-free bovine milk was assessed by the lyso-plate assay as previously described (Osserman and Lawlor, 1966), but in this case the reaction was carried out at 37°C, for 18 h, in a humidified incubator.

Electrophoretic and densitometric analysis of milk proteins

The proteins corresponding to 0.1 μL of each milk sample were separated by SDS PAGE using precast Any kD polyacrylamide gels (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA, USA) and digitalized with an ImageScanner III (GE Healthcare, Little Chalfont, UK). Band intensities were compared with the Quantity One 1-D analysis software (Bio-Rad) as described previously (Ghisaura et al., 2016; Salvatore et al., 2014). For defining peaks and

calculating relative peak abundances, band areas were estimated using Origin-Pro 8 SR0 software (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA, USA). In order to specifically assess physiological differences between breeds, only samples having negative bacteriological culture, less than 1,000,000 cells/ml in colostrum (T2) and less than 500,000 cells/ml in milk (T3) were included in the evaluation.

RNA extraction, reverse transcription and Real-time PCR from milk fat globules and milk cells

Total RNA was isolated from MFG and milk cells by the TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) protocol; total RNA was extracted according to the manufacturer's directions. The concentration of RNA was determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength. One µg of total RNA from all the samples was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA obtained from each sample was used as a template for Real Time PCR in an optimized 25 □l reaction volume using Sybr Green chemicals, as previously described (Riva et al., 2010). The primer pairs were designed using the Primer Express Software (Applied Biosystem, Foster City, CA, USA) and purchased from Invitrogen (Carlsbad, CA, USA). Their sequences are listed in Table I.

Gene	Protein	Sequence	Gene Bank gi-number
CD45 (Trevisi et al. 2014)	Bovine membrane tyrosine phosphatase (CD45)	F: CTCGATGTTAAGCGAGAGGAAT R: TCTTCATCTTCCACGCAGTCTA	GI:9944227
GAPDH (Trevisi et al. 2014)	Glyceraldehyde-3-phosphate dehydrogenase	F: GGCGTGAACCACGAGAAGTATAA R: CCCTCCACGATGCCAAAGT	GI:89573946
PTX3	Pentraxin 3	F: TCCATCCCACTGAGGACCC R:TCTCCAGCATGGTGAAGAGCT	GI:402691645
IL-1R8	Interleukin-1 receptor 8	F: TCCGGAACATCAGCTCCTCT R: CCGCCAGCCCAGCTC	GI:982972365
TNF-α	Tumor necrosis factor alpha	F: TCTTCTCAAGCCTCAAGTAACAAGT R: CCATGAGGGCATTGGCATAC	GI:402693442
IL-1β	Interleukin-1 beta	F: GAGGAGCATCCTTTCATTCATC R:TTCCTCTCCTTGCACAAAGCTC	GI:27806570
KRT5	Bovine keratin 5	F: CAAGGTCCTGGACACCAAGT R: TCCAGCTGTCTCCTGAGGTT	GI: 56710316

Table I. Oligonucleotide primer sequences for SYBR Green quantitative RT polymerase chain reaction amplification.

Specific primers were also employed for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

IL-1ß, TNF-α, CD45 and KRT5 genes were investigated in milk cells, whereas PTX3 and IL-1R8 genes in MFG. A duplicate, no-template control (NTC), was included in each plate. Real-time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) as previously described (Riva et al. 2010). The expression of bovine target genes was normalized using the calculated GAPDH cDNA expression (mean) of the same sample and run. The relative quantification of each gene was calculated with the method of the "delta Ct" (Schmittgen et al. 2008). The value obtained was multiplied by "10000" in order to obtain the Arbitrary Unit.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 for Windows (IBM, Armonk, NY, USA), GraphPad Prism 6 (La Jolla, CA, USA) and PROC MIXED of SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). For the statistical analysis of the bacteriological data, all quarter samples were included. For the statistical analysis of the metabolic parameters, all plasma samples were included. In order to analyze the milk parameters (SCC, cathelicidin, lysozyme, microbiota, gene expression of immune related genes) under physiological conditions, only healthy quarter samples were evaluated. The healthy quarter samples were defined as follows. For T1 and T2: negative bacteriological culture growth (udder pathogens) (given that at T1 and T2 an increase in SCC is typically observed, the SCC threshold was not applied to the dry off milk and colostrum samples) (McDonald and Andersen 1981a and b); for T3 and T4: SCC < 200,000 cells/ml (threshold used in high yielding HF cows as a reliable indicator of mastitis; Bradley and Green, 2005) and negative bacteriological culture growth. Quarter samples showing microbial growth at T1 and T2 and SCC > 200,000 cells/ml and/or positive bacteriological culture growth at T3 and T4, were identified as suspected IMI or subclinical mastitis samples, and were analyzed separately for the gene expression of immune related genes. Student's t test was used to compare HF and REN gene expression and cathelicidin at each time point. A P value < 0.05 was considered statistically significant. Plasma inflammometabolic parameters and lysozyme were analyzed using a pair wise comparison. The model for the time-course data analysis contained breed, day, and their interactions as fixed effects, and cow nested within breed as random effect. A spatial power (SP = POW) covariance structure was used. The pair wise comparison has been done using least significant difference (LSD) test. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Results

Different metabolic pathways are evident in HF and REN

We investigated different metabolic parameters in plasma samples. Results are plotted in Figure 1. No differences were observed between HF and REN at any time point in the following parameters: glucose, cholesterol, urea, phosphorus, ceruloplasmin, albumin, AST/GOT and GGT (data not shown). In both breeds NEFA increased after calving, but in HF the peak was more marked at T2 and T3 ($P<0.01$), suggesting a more severe fat mobilization.

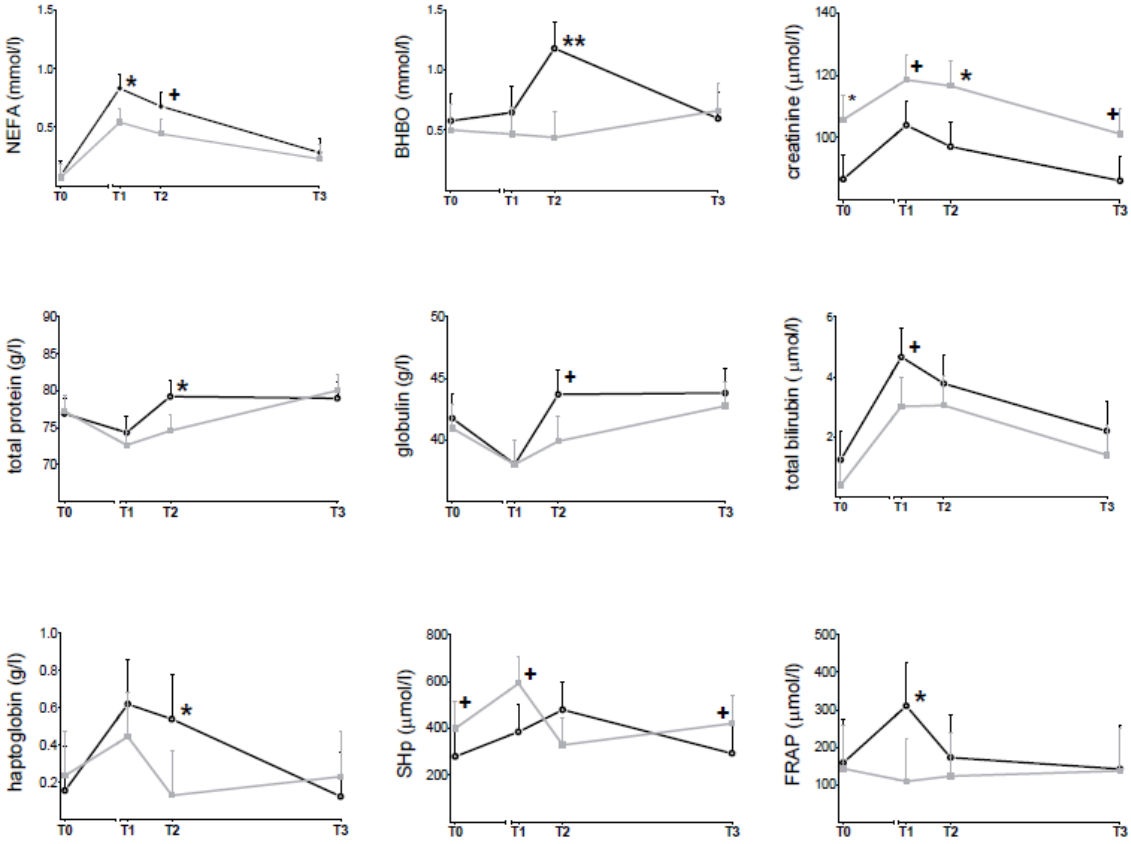

Figure 1. Metabolic profile.

The BOHB increased after calving only in HF, and the difference with REN reached the maximum at T3 ($P<0.01$). Conversely, REN showed an higher concentration of plasma creatinine in comparison with HF ($P<0.05$) for the whole period. HF showed a more severe systemic inflammatory response in comparison with REN in terms of positive acute phase markers (haptoglobin, total proteins, and globulins) mainly at the beginning of lactation at T2 and T3.

Also, total bilirubin was higher in HF vs. REN after calving (P<0.10 at T2), but in this case the difference between the breeds was already evident before the parturition and remained also during the lactation. REN showed a higher concentration of thiol groups (index of the sulfhydryl compounds, SHp) in comparison to HF for the whole period, except for T3. On the other hand, HF showed a significant increase of FRAP (index of total antioxidant) at T3 (P<0.05 vs REN).

Udder health based on microbial culture

Udder health was defined at the quarter level. Contagious pathogens (*Staphylococcus aureus* and *Streptococcus agalactiae*) were never detected. The most common microorganisms isolated in milk were environmental streptococci and Coagulase-negative staphylococci (CNS). Culture-negative status was observed in 2 cows. At T1 and T2 HF cows presented a lower percentage of positive bacterial culture results, while at T3 and T4 we observed the opposite, with a higher percentage of samples with positive bacterial culture in HF *vs* REN (Table II).

Table II. Bacteriologically positive samples at the different time points in the two breeds.

	HF	**REN**
T1	33.3%	40.0%
T2	33.3%	41.7%
T3	20.8%	12.5%
T4	29.2%	12.5%

Healthy HF milk shows higher levels of cathelicidin and a higher SCC

All the healthy quarter milk samples from both breeds were evaluated for SCC and cathelicidin abundance as mastitis markers. In fact, SCC above 200,000 cells/ml is typically considered as a trustworthy indication of mastitis (Bradley and Green, 2005). Recently, we have demonstrated that the measurement of milk cathelicidin can reliably indicate mastitis with improved diagnostic

performances than SCC (Addis et al., 2016a and b). Therefore, both markers were assessed in this work. Results were assessed separately for all lactation time points (Figure 2). Table III reports the respective median and interquartile range (IQR) values.

As a result, the median and IQR values of both SCC and cathelicidin were higher in HF *vs* REN milk at all times. As expected, the values of both markers were lowest in T3 and T4 and highest in T2. In the case of SCC, only the HF T2 median was above the 200,000 cells/ml diagnostic threshold (Figure 2). In the case of cathelicidin, the T2 of both breeds had median values above the threshold, again with higher values for HF. In T3 and T4, 75% of the result distribution was always below threshold for both markers (Figure 2).

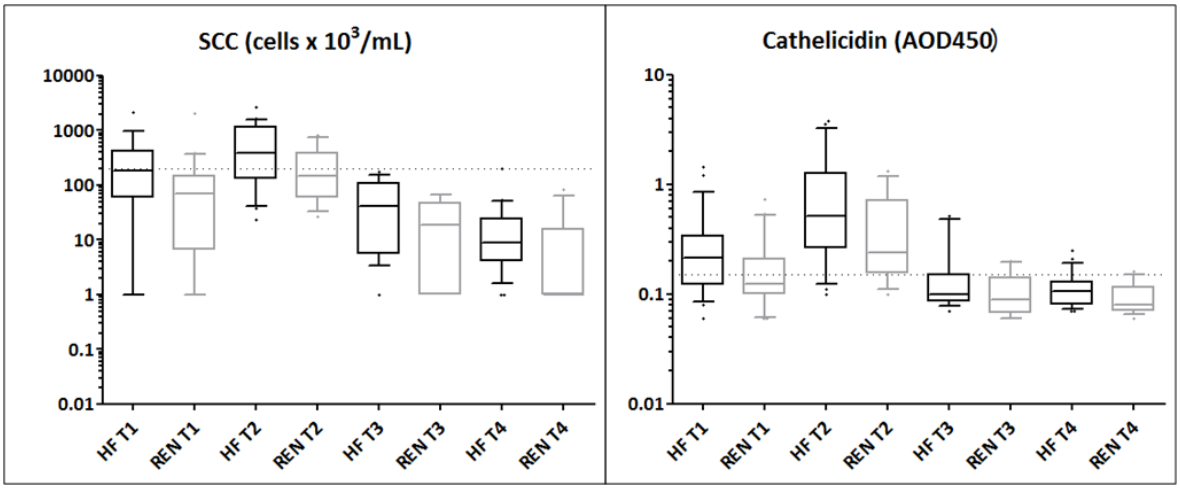

Figure 2. Cathelicidin and SCC evaluation in colostrum and milk. SCC was determined by an automated fluorescent microscopic somatic cell counter in colostrum and milk quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4; it is expressed as 10^3 cells/ml. Cathelicidin was measured by sandwich ELISA in colostral and milk quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4; it is expressed as AOD 450.

Table III. Medians and interquartile ranges (in parentheses) of SCC and cathelicidin in milk of the two breeds.

	HF			REN		
Milk sampling	**N**	**SCC (cells/ml)**	**Cathelicidin (AOD450)**	**N**	**SCC (cells/ml)**	**Cathelicidin (AOD450)**
Whole lactation	88	78,000 (9,000/290,500)	0.150 (0.100/0.380)	60	34,500 (1,000/83,000)	0.110 (0.080/0.180)
Dry (T1)	24	184,000 (58,500/418,800)	0.215 (0.120/0.340)	20	71,000 (6,500/148,500)	0.125 (0.100/0.207)
Colostrum (T2)	25	382,000 (130,000/1,173,000)	0.515 (0.263/1.258)	12	147,000 (57,250/394,300)	0.240 (0.152/0.722)
Mature (T3)	17	147,000 (5,500/108,500)	0.100 (0.085/0.150)	14	41,000 (1,000/48,000)	0.090 (0.067/0.140)
Mature (T4)	22	19,000 (4,000/24,000)	0.105 (0.080/0.130)	14	9,000 (1,000/15,500)	0.080 (0.070/0.115)

The milk microbiota of Rendena cows displays a lower biodiversity

A more detailed knowledge of the healthy milk microbial communities and their interactions in physiological conditions might provide useful information on the factors influencing milk quality and udder health. Therefore, a NGS approach based on 16 S metagenomics was applied to the milk of HF and REN during the peripartum period. Results were assessed by pooling all lactation time points together.

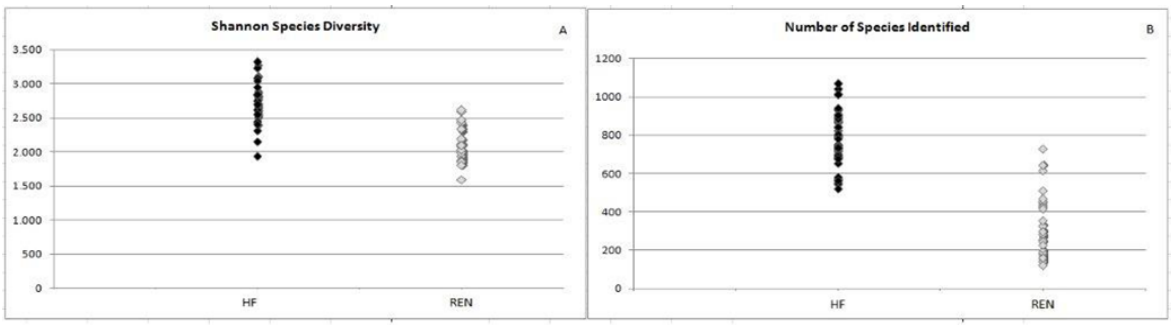

Figure 3. **Alpha diversities of the milk microbiome.** Alpha diversities of the milk microbiome collected from Holstein Friesian (HF) and Rendena (REN) cows was calculated by Shannon index (A) and observed species (B).

Based on the alpha-diversity (Figure 3) according to the Shannon Index and observed species, a lower biodiversity was present in REN milk when compared to HF milk.

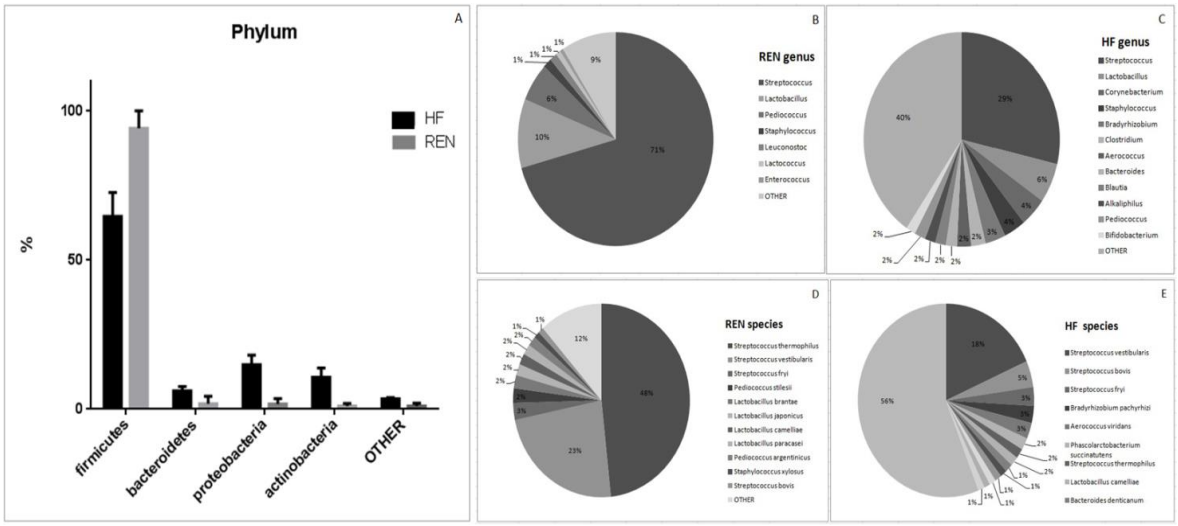

Figure 4. Milk microbiome composition in Holstein Friesian (HF) and Rendena (REN) breeds. Relative abundance of the milk bacterial phyla (A). Pie charts summarizing genus level taxa (B: HF, C: REN) and species level taxa (D: HF, E: REN).

At the phylum level, REN milk was dominated by *Firmicutes* (94%, relative abundance), while HF milk contained *Firmicutes* (65%), *Proteobacteria* (15%), *Actinobacteria* (11%) and *Bacteriodetes* (6%) (Figure 4 A). At the genus level, REN milk showed the predominance of *Streptococcus* (71%), followed by *Lactobacillus* (10%) and *Pediococcus* (6%), while HF milk was dominated by *Streptococcus* (29%), followed by *Lactobacillus* (6%), *Corynebacterium* and *Staphylococcus* (4%) (Figure 4 B and C). Among streptococci, *Streptococcus thermophilus* was the most prevalent (48%) in REN milk, in comparison with only 2% in HF (Figure 4 D and E).

The protein abundance profiles of HF and REN colostrum show visible differences

For estimating milk protein profiles at T2, T3 and T4, the same volume of each sample (0.1 microliters) was subjected to SDS-PAGE and densitometric analysis. In the case of T2 (Figure 5 A), the mean pixel density value was 5 Mpx for FH and 7.3 Mpx for REN, respectively, indicating a higher protein concentration in milk of the latter breed. The SDS-PAGE profile (Figure 5 A) showed numerous differences according to the breed, as reflected in the

corresponding densitogram analysis (Table IV). In some cases, these differences were quite relevant, such as for peaks a, e and g, all having molecular weight ranges corresponding to immunoglobulin components. Specifically, in the MW range of band a (200 kDa) we have previously identified a component of IgM (Pisanu et al., 2012), while bands e and g migrate in the MW range of the Ig heavy and light chain (55 and 26 kDa), respectively (Pisanu et al., 2012; Salvatore et al., 2014; Thomas et al., 2016).

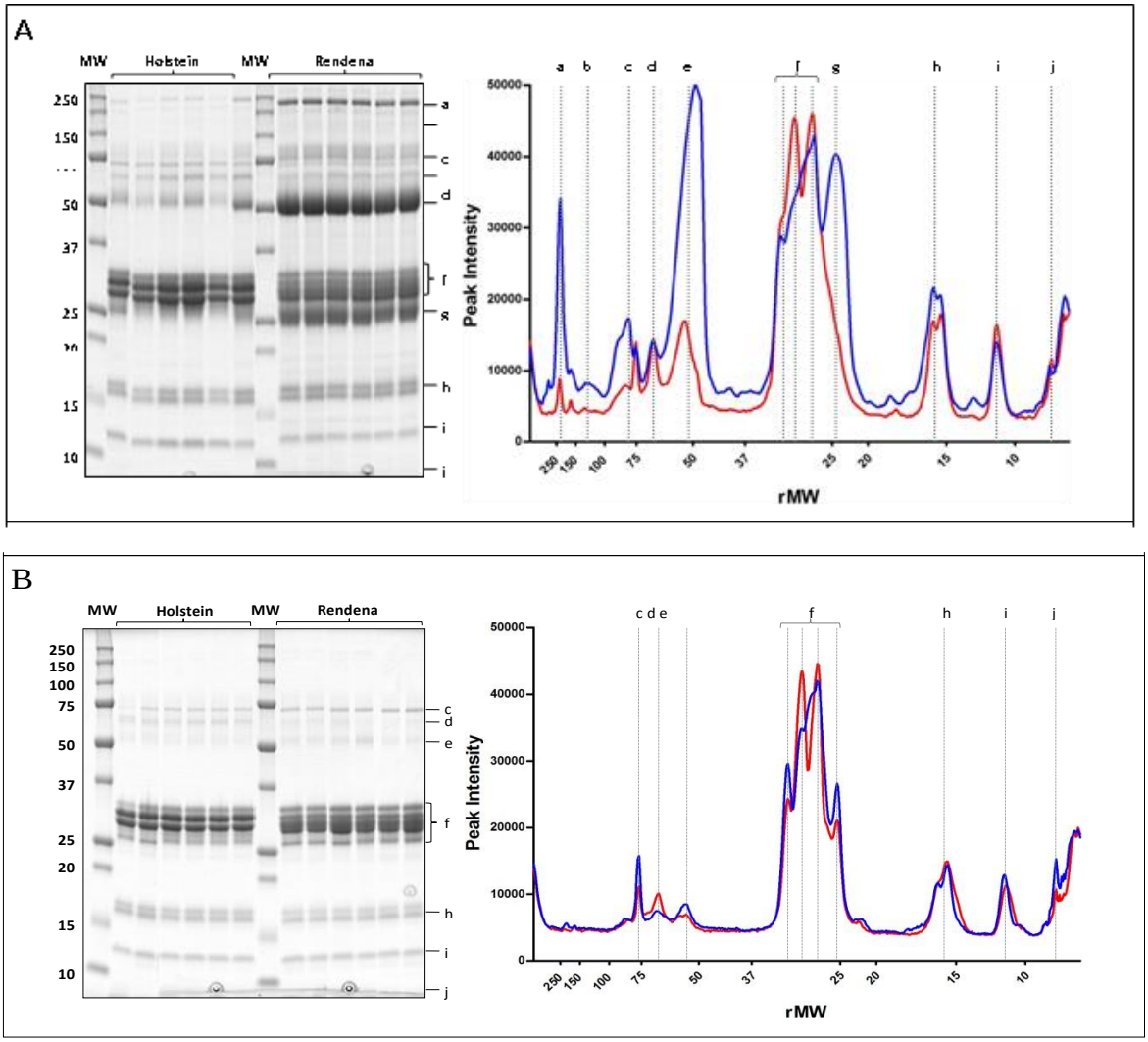

Figure 5. SDS-PAGE and densitometry of milk samples at T2 (A) and T3 (B). Left: SDS-PAGE profile of milk samples; right: corresponding densitometric profile. The letters indicate the main electrophoretic bands and the corresponding peaks in the densitogram. MW: molecular weight. rMW: relative molecular weight. Black: Holstein-Friesian; Gray: Rendena.

A higher abundance of band c, corresponding to the MW range of bovine lactoferrin, was also seen in REN milk, together with other minor bands. On the other hand, the protein profiles of T3 and T4 (Figure 5 B and Table IV) were more similar in the two breeds. At these time points, the mean pixel density values were of 4.3 Mpx for HF and 4.6 Mpx for REN, respectively, indicating similar milk protein concentrations in the two breeds (Figure 5 B). Peak area ratios were also comparable. Nevertheless, differences in the banding pattern at the MW range of caseins (Figure 5 B, group f) could still be observed, as well as differences in the shape, and therefore in relative composition, of the four peaks included in this group.

Table IV. Densitogram results. Estimated MW, areas and area ratios for peaks/bands reported in Figure 2. Areas are expressed as pixel intensity values.

Peak/band	Est. MW	Area - HF	Area - REN	REN/HF	HF/REN
			T2		
A	200	299.8	866.3	2.89	0.35
B	130	200.2	360.9	1.80	0.55
C	80	514.6	866.2	1.68	0.59
D	70	383.7	379.8	0.99	1.01
E	52	1084.5	2955.0	2.72	0.37
F	30-35	4711.8	3252.8	0.69	1.45
G	24	-	2172.5	-	-
H	16	1004.9	1312.3	1.31	0.77
I	12	575.4	473.9	0.82	1.21
J	4	292.7	367.7	0.80	1.26
			T3		
C	77	193.1	260.6	1.35	0.74
D	70	372.5	298.3	0.80	1.25
E	53	397.4	532.7	1.34	0.75
F	25-35	3727.0	3877.9	1.04	0.96
H	16	974.9	1006.8	1.03	0.97
I	12	500.9	511.7	1.02	0.98
J	4	261.6	344.9	1.32	0.76

The mammary innate immune response is different in the two breeds

In order to gain a more detailed picture on the differences between the two breeds in terms of innate immune response, lysozyme concentration and gene expression pattern of some key regulators were evaluated in milk. HF did show a significant increase in lysozyme at T2 when compared to T1 (P=0.0014). In addition, lysozyme showed a peak at T2 in REN. Interestingly, such an increase at T2 was on average much higher in REN cows, albeit with higher internal variability (Figure 6). At T1 we observed a significant difference between the

two breeds in terms of lysozyme concentration in skim milk (P=0.0141; Figure 6).

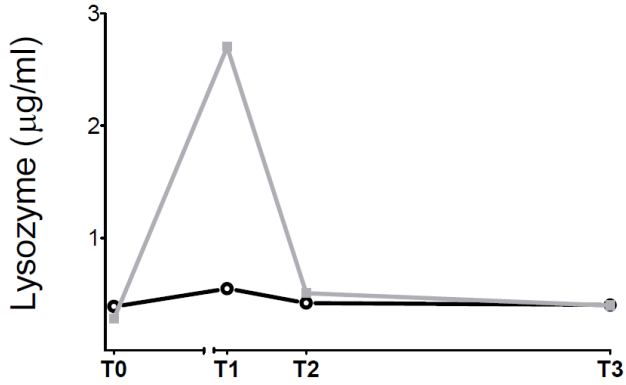

Figure 6. Lysozyme concentration in colostrum and milk. Lysozyme concentration (□g/ml) was measured in colostral and milk quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4.

By Real Time PCR, the expression pattern of PTX3 and IL-1R8 was assessed in the MFG, representative of the mammary epithelium (Brenaut P. et al. 2012; Addis et al. 2011), whereas the expression pattern of IL-1ß, TNF-α, CD45 and KRT5 was assessed in milk cells.

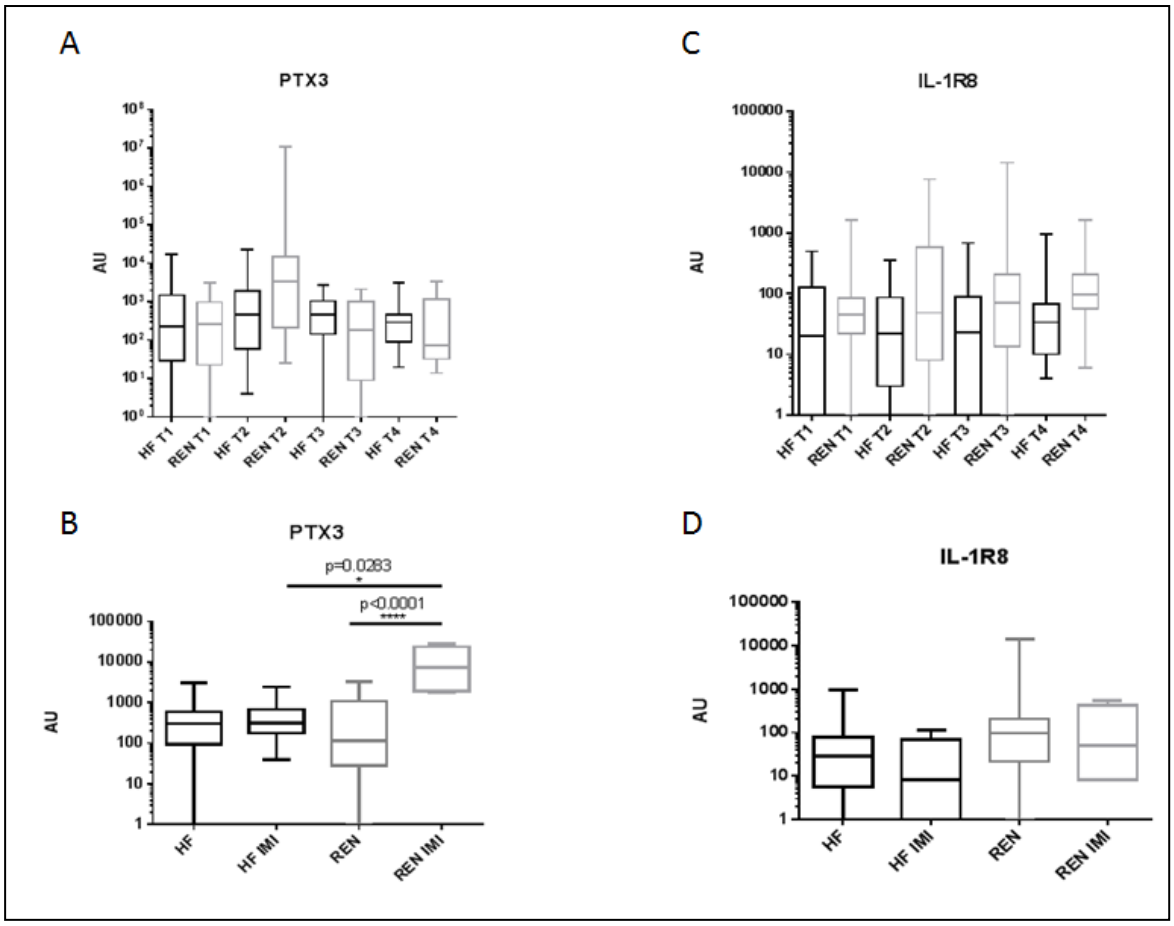

Figure 7. Expression of IL-1R8 and PTX3 in MFG. PTX3 (A) and IL-1R8 (C) mRNA expression was analyzed by Real Time PCR in MFG of healthy quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Unit. PTX3 (B) and IL-1R8 (D) mRNA expression was analyzed by Real Time PCR in MFG of suspected IMI/subclinical mastitis quarter sample from HF and REN cows at T3 and T4. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Unit.

As shown in Figure 7 (A) PTX3 showed no differences in expression levels between the two breeds for all time points, except for the colostrum (T2), at which REN showed a trend ($P<0.1$) of increased levels. Interestingly, HF seemed to be unable to up-regulate PTX3 during infection (in suspected IMI/subclinical mastitis samples), whereas REN significantly up-regulated PTX3 during infection ($P<0.0001$; Figure 7 B). Moreover, PTX3 expression level was significantly lower in suspected IMI/subclinical mastitis samples from HF compared to REN ($P=0.0283$; Figure 7 B). Non significant (NS), but constant higher expression was seen for IL-1R8 in REN at all time points when compared to HF (at T2 $P<0.1$). The same NS trend was evident in suspected

IMI/subclinical mastitis samples in which HF presented lower expression of IL-1R8 (Figure 7 D). This receptor, as expected, was slightly down-modulated in suspected IMI/subclinical mastitis samples when compared to healthy milk samples, in both breeds (Figure 7 D; Riva et al. 2012).

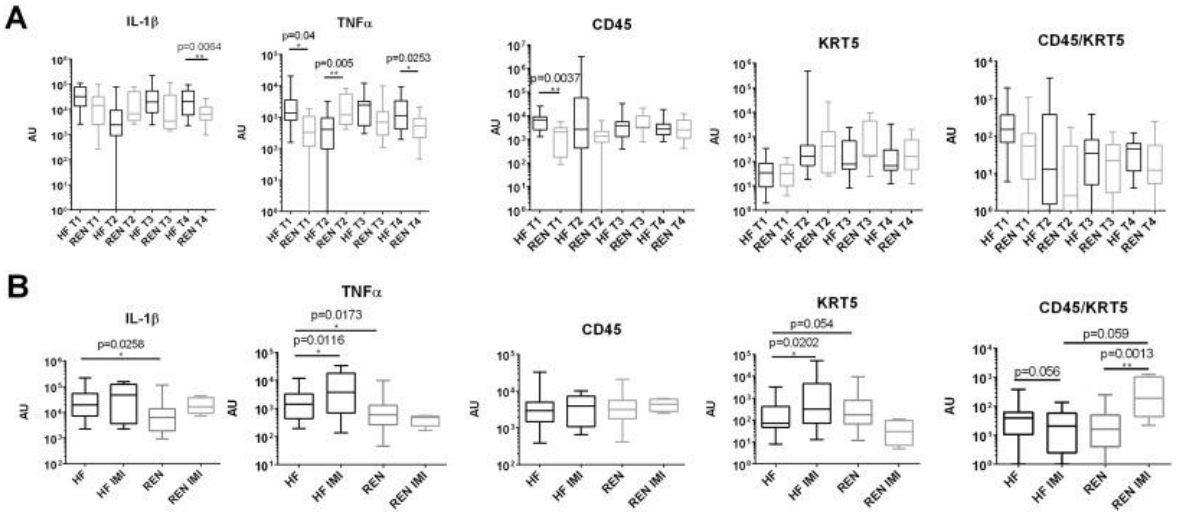

Fig. 8. Expression of IL-1β, TNFα, CD45 and KRT5 in milk cells. A) IL-1ß, TNFα, CD45 and KRT5 mRNA expression was analyzed by Real Time PCR in isolated milk cells of healthy quarter samples from 6 HF and 4 REN cows at T1, T2, T3 and T4. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Unit. CD45/KRT5 report the ratio of expression of the two messengers. B) IL-1ß, TNFα, CD45 and KRT5 mRNA expression was analyzed by Real Time PCR in isolated milk cells of suspected IMI/subclinical mastitis quarter samples from HF and REN cows at T3 and T4 and compared to the expression of the healthy quarters at the same time points. The gene expression level of each target gene was normalized to GAPDH and the results are presented as Arbitrary Units. *P < 0.05, **P < 0.01, ***P < 0.001

Proinflammatory cytokines (IL-1ß and TNF-α) presented lower expression levels in REN milk cells compared to HF at all time points but in colostrum (T2), where REN samples showed higher cytokine gene expression levels (TNF-α P=0.005; Figure 8 A). In suspected IMI/subclinical mastitis samples, proinflammatory cytokines were more expressed in HF than in REN (Figure 8 B)..

In order to investigate the cell types present in milk samples, we analyzed the gene expression of CD45 (leukocytes) and KTR5 (epithelial cells). CD45 did not present any difference in expression levels between the two breeds except for the dry off period (T1; P=0.0037), when HF showed higher levels than REN (Figure 8 A and B). KRT5 seemed to be slightly up-regulated in REN *vs* HF milk

cells (except at dry off, T1), whereas the opposite was observed in suspected IMI/subclinical mastitis samples, as shown in Figure 8 B. Interestingly, the CD45/KRT5 expression ratio was always lower in REN vs HF cows at all times (Figure 8 A). During infection (in suspected IMI/subclinical mastitis samples), the CD45/KRT5 ratio was higher in REN *vs* HF (Figure 8 B).

Discussion

Autochthonous and lower-yielding dairy cattle breeds are known to possess higher resistance to disease and resilience to intensive farming conditions when compared to high-yielding, highly selected dairy breeds such as HF, especially concerning udder health and metabolic disorders in the peripartum period (Gandini et al. 2007; Curone et al. 2016). The availability of a small group of HF and REN cows reared in the same farm and under the same conditions prompted us to carry out a multidisciplinary study aimed to investigate the traits that may underline these differences in disease susceptibility.

Taken together, the results presented here suggest that HF cows seems to develop a greater systemic and local (in the mammary gland) inflammatory condition compared to the autochthonous REN breed, although it should be considered that our findings should be validated on larger animal cohorts to allow for final conclusions.

Cows of both breeds showed the typical variations of inflammometabolic profile around calving previously described in healthy periparturient dairy cows (Bionaz et al., 2007; Trevisi et al., 2011; Trevisi et al., 2012). In particular, HF and REN cows have shown the significant mobilization of the body reserves, from the adipose tissue (peak of NEFA a T2) and from the muscle tissue (peak of creatinine at T2) and the characteristic inflammatory response immediately after the calving, as confirmed by the peak of haptoglobin at T2. The post-calving increase of globulin, total bilirubin and the similar variations of proteins produced by the liver (i.e. albumin, paraoxonase) around calving, suggest that both breeds had the same responses at the liver level (Bionaz et al., 2007; Bertoni et al., 2008). Nevertheless, all the above phenomena are more

pronounced and prolonged in HF cows, confirming that cows with a high genetic merit for milk yield have a more severe lipomobilization and suffer of a more marked inflammation after calving. Interestingly, REN cows at calving time (T2) showed also three other important differences in comparison to HF: absence of ketosis (the BOHB remained unchanged in the first week of lactation), higher muscle mass (the creatinine concentration was constantly higher in the whole period) and lower risk of oxidative stress (the concentration of thiol groups, SHp, was higher in late pregnancy and the total antioxidant concentration after calving, assessed as FRAP, was unchanged, for a lower endogenous production of these molecules). Thus REN cows have been less susceptible to oxidative damage probably for the lower production of free radicals consequent to a lower mobilization of body reserves, a better oxidation of the fatty acids in the liver, a shorter and less severe inflammatory response (Trevisi et al., 2010; Bertoni and Trevisi, 2013). Combining these data with the lower milk production, we can conclude that REN cows have not suffered an important negative energy status also in the days immediately after calving, and that the nutrients mobilized from reserves were more rich of amino acids than the HF cows.

Moreover REN cows demonstrated the ability to maintain lower levels of the mastitis markers cathelicidin and SCC, and therefore of mammary tissue inflammation; this ability is of significant interest especially in the post-partum period and it appears to be combined with the capability of REN cows to release in colostrum a higher amount of other immune-related proteins, such as lysozyme, that efficiently protect the mammary gland against pathogen infections. Based on electrophoretic and densitometric analysis of colostrum, several bands associated to immunoglobulin components are present in consistently higher amounts in REN when compared to HF cows. At 10 days after calving (T3), milk protein profiles become instead highly comparable. In addition, the differences seen in the peak group around 30-35 kDa suggest that differences may exist also in the relative abundance of caseins between the two breeds, and deserve further investigations for their implications on cheesemaking properties (Perna et al., 2016).

On the basis of our strict inclusion criteria, fundamental and significant differences in the baseline profile of local innate immunity could be detected mainly in the colostral phase. With respect to HF cows, REN cows showed stronger innate immune responses in the mammary gland (in terms of proinflammatory cytokines, PTX3 and leukocyte/epithelial cell ratio) shortly after calving. These differences in colostrum samples were never accounted for by lower concentrations of SCC in HF cows. On the contrary, these frequently outnumbered the corresponding counts of DIM (days in milk)-matched REN breed cows. Most importantly, SCC in colostrum was shown to be significantly different, leukocytes being predominant in HF but not in REN cows. These findings outline a new scenario, whereby leukocytes of HF cows are probably less efficient in the colostral phase compared with those of a mastitis-resistant, low-yielding cattle breed. The latter displayed a much stronger expression of inflammatory cytokine genes (IL-1ß and TNF-α) and concentrations of antimicrobial substances in colostrum (Lysozyme and PTX3), despite an overwhelming majority of epithelial cells. This was in sharp contrast to HF cows, that showed instead very high leukocyte infiltrations in colostrum SCC. The observed hypofunctionality of colostrum leukocytes in HF cows had an important confirmation *ex iuvantibus* in a previous study, in which a dramatic up-regulation of innate immunity parameters could be demonstrated in colostrum of HF following the parenteral injection of a very low interleukin-2 dose (Zecconi et al., 2009). The cytokine allegedly restored the previously suppressed innate immune response in the udder and caused a significant reduction of new cases of intramammary infections in the first 2 weeks after calving compared with control untreated cows (Zecconi et al., 2009). These results outline a potential important role of immunomodulators early after calving, to raise local immunity to the level required to effectively face environmental and transmissible mastitis agents. Among the parameters of the innate immunity under study, the possible role of lysozyme should be properly highlighted. Beyond being a fundamental and ubiquitous antibacterial compound, lysozyme is involved in the regulation of the inflammatory response

(Liu et al., 2006; Lee et al., 2009). HF cows consistently show much lower serum lysozyme levels compared with other cattle breeds (Trevisi et al., 2011), and concentrations outside the reference interval (1-3 □g/ml) are associated with an increased risk of disease occurrence (Trevisi et al., 2012; Amadori et al., 2015). Therefore, the much lower lysozyme response in the colostrum of HF cows should be considered as an important indicator of susceptibility to new intramammary infections, having in mind the crucial role of the early post calving period for the establishment of new intramammary infections in the herd (Fleischer et al. 2001). The above differences were shown to be restricted to the colostral (T2) phase and were no longer detectable later on.

Moreover, the milk microbiota of REN cows showed a lower biodiversity in terms of bacterial phyla, genera and species. This is likely to have important consequences on innate immunity in the mammary gland, having in mind that microbiota is associated with inhibition of pathogen colonization, degradation of xenobiotics and stimulation of both development and maintenance of the immune system (Addis et al., 2016c). As previously described (Quigley et al., 2013), typical cow's milk contains a significant lactic acid bacterium population (LAB) that includes *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Enterococcus*; other microorganisms, such as *Pseudomonas*, *Acinetobacter* and *Aeromonas* spp., can be present with significant different proportions. Microbiota can prevent pathogens colonization by competing for space and nutrients and exerting an outright amensalism, *i.e.* the production of substances (like lactic and short-chain fatty acids, hydrogen peroxide, orbacteriocins) toxic for other microbial species. This underlies the use of lactobacilli and other probiotics in the prophylaxis of farm animal diseases, including bovine mastitis (Nader-Macias et al., 2008; Espeche et al., 2012). Interestingly, REN milk samples showed a much higher prevalence of *Streptococcus thermophilus*. This bacterium is a thermophilic LAB prevalently used as a starter during the manufacture of dairy products and the second most important industrial dairy starter in the production of fermented milks, yogurts and many cheese types (Quigley et al., 2013). *Streptococcus thermophilus* high concentration in REN

milk therefore makes it more suitable to dairy processing. Moreover *Streptococcus thermophilus* might exert a protective function against mammary pathogens by competing for space and nutrients, or secreting antibacterial molecules or stimulating the mammary immune response (Rigobelo et al. 2015) The above findings can be viewed in a conceptual framework that includes the different data sets in coherent cause/effect relationships. The greater metabolic stress of HF cows and the related higher NEFA peak early after calving, as well as the higher inflammation and oxidative stress, could be the foundation of some crucial downstream processes affecting the profile of innate immunity in the mammary gland. In particular, high NEFA levels can affect the proliferation of lymphocytes and the release of interferon-gamma and IgM after treatment with mitogens (Lacetera et al., 2004). Also, they severely depress both vitality and oxidative burst of bovine neutrophils (Scalia et al., 2006). NEFA can directly signal through Toll-like receptor (TLR) 4, being thus an important component of metabolic stress. This can be sensed by the innate immune system following tissue acidosis, osmolarity changes, hypoxia, Reactive Oxygen Species (ROS) accumulation, altered ATP/AMP ratio, shortage of amino acids (Amadori et al., 2016). Moreover, also the more severe inflammation in the weeks after calving, measured with higher concentration of positive acute phase proteins and mainly with lower concentration of negative acute phase proteins, has been linked at the impairment of the immune system and to a reduction of performances of dairy cows (Bertoni et al., 2008; Trevisi et al., 2010; Trevisi et al., 2016). Therefore, it is conceivable that high NEFA levels and other poorly defined metabolic products after calving could inhibit in HF cows a badly needed local innate immune response in the colostral phase. This response is likely to prime the epithelia of the mammary gland to an effective and time-limited response to mastitis agents. In agreement with a previous study on innate immunity in the dry period, environmental stressors after dry-off could amplify such biological effects after calving by affecting the "memory" of the innate immune system (Quintin et al. 2014; Amadori et al., 2015; Trevisi et al., 2016). In the framework of such a response to metabolic stress, innate immunity in the mammary gland

of HF cows would be less pronounced and effective in the colostral period and, probably, also poorly controlled later on, after exposure to mastitis pathogens. As a result, bMEC could fail to control the colonization of pathogenic bacteria; leukocytes would then go into action underlying the typical signs of subclinical and clinical mastitis cases. On the other hand, the lower expression in MFGs of IL-1R8, a negative regulator of TLRs and ILRs proinflammatory signaling, could explain the increased expression of proinflammatory cytokines in HF cows under resting condition at T1 and T4 (Riva et al. 2012). This steady proinflammatory response could exhaust the cells (leukocytes and mammary epithelial cells), that would no longer be able to respond to a subsequent infectious challenge.

The above findings probably make a case for heterosis, or hybrid vigor, in "problem" herds, to be reappraised within rational crossbreeding systems with local, autochthonous breeds. Also, our findings indicate that fundamental effector activities of innate immunity in the mammary gland should be included in the breeding programs of HF cows, and given adequate priority by the scientific community. Therefore, immunostimulation in the colostral period could be a badly needed choice to effectively face mastitis pathogens in a period in which prevalence of new IMI is highest. This conclusion is strengthened by the contradictory results of the current *S. aureus* vaccines for dairy cattle, which question the very foundation of a vaccination strategy against mastitis agents (Scali et al., 2015). Immunostimulation should be inserted in a wider strategy, aimed at minimizing the impact of the metabolic stress in the first DIM (Carbonneau et al., 2012), keeping high hygiene conditions and proper BCS levels in the dry period, providing satisfactory housing and feeding conditions during the lactation period, adopting protocols of early and predictive diagnosis of production diseases (Trevisi et al., 2012; Bertoni and Trevisi, 2013; Amadori et al., 2015). This kind of integrated approach can be conductive to a substantial reduction of antibiotic usage in dairy farms, based on active involvement of farmers, veterinary practitioners, dairy extension specialists and veterinary

authorities towards better qualification of the food chains and improved consumers' awareness.

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Chapter 5

“Milks” Biodiversity

5.1

Fatty acid profile, Desaturase and Atherogenic indices in milk of Holstein Friesian and Italian autochthonous cattle breeds

The data shown in this chapter refer to the already published paper:

FAUSTINI M, CHIESA LM, MUNARI E, CURONE G, COLOMBANI C, ET AL. (2016) A SURVEY ON MONO-, POLYUNSATURATED FATTY ACIDS, DESATURASE INDICES AND ATHEROGENIC INDEX IN THE MILK FAT OF LOCAL BREEDS (CABANNINA, VARZESE AND VALDOSTANA) REARED IN NORTHERN ITALY. J DAIRY VET ANIM RES 3(6): 00101. DOI: 10.15406/JDVAR.2016.03.00101

Introduction

In the past decades, milk has been considered a mere supplier of nutrients: although its importance was considered paramount for the development and growth of newborns, a number of aspects regarding the biological functions of milk were still unknown. Several positive functional properties of milk derive from fatty acids (FA), mainly unsaturated fatty acids (UFA), either monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids (Mills et al., 2011). The importance of such acids in human nutrition is evidenced in a huge background of documentation, as reported by FAO on fat and fatty acids in human nutrition report (FAO, 2008). In particular, UFAs are considered functional components of food because of their positive effects on disease prevention. The ω-6 and the ω-3 fatty acids have demonstrated potential health benefits (Connor et al., 2000), by reducing the risk of cardiovascular disease (Fedacko et al., 2007; Siddiqui et al., 2008), type-2 diabetes, hypertension (Wijendran, 2004; Willett, 2007; Zhao et al., 2007), cancer (Dupertuis et al., 2007; Larsson et al., 2004) and certain neurological disfunctions (Alessandri et al., 2004; Hamilton et al., 2007), Some of them have an antimicrobial function, as showed by Clement et al. (2007) the arachidonic acid (20:4n-6), linoleic acid (18:2n-6) inhibit the germination of *Candida albicans in vitro*. In another study (Clement et al., 2008) the lauroleic acid (C12:1), 11-methyldodecanoic acid (iso-C13:0), myristoleic acid (C14:1n-5), and g-linolenic acid (C18:3n-6) showed antifungal activities against Aspergillus fumigates as well as *C. albicans*.

The vaccine milk contains about 25% of oleic acid (C18:1 *cis*-9) on the total of FA, but also appreciable percentages of myristoleic (C14:1 *cis*-9), palmitoleic (C16:1 *cis*-9) acids, and conjugates of linoleic acid (CLA). The absorption of UFA in the ruminal environment is limited by the biohydrogenation process, operated by rumen bacteria (Bauman et al., 2006). The presence of several UFAs in milk is due mainly to the presence in mammary gland of the enzyme Δ^9-stearoyl CoA-desaturase (SCD): for example, oleic acid derives from stearic acid, while myristoleic acid is synthesized from C14 (myristic acid), and palmitoleic acid

from palmitic acid (C16). The activity of SCD accounts for the synthesis of other FA,

The SCD activity can be indirectly determined by the calculation of simple desaturase indices calculated on the base of the substrate: product ratios or analogue calculations (Kelsey et al., 2003). Desaturase indices vary noteworthy between and within subjects, with a heritability of 0.2÷0.3 (Soyeurt et al., 2008; Garnsworthy et al., 2010).

SCD exerts its activity in a non-equal manner: the preferred substrates are C16 and C18 fatty acids (Ntambi and Miyazaki, 2004); the preferred desaturase index is the C14 index ($\Delta^9$14), since almost all C14:1 cis-9 acid is synthesized by the mammary gland. $\Delta^9$14 correlates positively with SCD activity in milk somatic cells (Feng et al., 2007).

Italy, is particularly rich in biodiversity, especially is reported a high number of autochthonous cattle breeds. Three northern breeds, namely Cabannina, Varzese and Valdostana have been appreciated for their milk and characteristics, especially for the rusticity, frugality, fertility and longevity (Communod et al., 2010; Communod et al., 2011). These features make these animals the first choice in marginal areas like mountain, wood and foothill grazes. The great rusticity of these breeds is also noticeable in its low susceptibility to the metabolic or inflammatory disorders/diseases (Curone et al., 2016). The autochthonous breeds are better adapted to the local environment, climate, feed and pathogens compared to the cosmopolitan breeds. These breeds have unique and peculiar features resulting from the interaction of its genetic background and the environmental conditions where they live.. Unfortunately, the animal husbandry of the 21st century has brought a decline in biodiversity of bovine breeds, due to the abandonment of autochthonous cows in favor of more productive cosmopolitan breeds (Holstein, Brown Swiss and Jersey) only following the milk production increase goal.so these breeds –at least Cabannina and Varzese- are endangered. Nowadays, Cabannina breed accounts for about 120 brood-females, and Varzese breed for 210 brood-females (Regione

Lombardia, 2007). Valdostana breed accounts for about 13000 brood-females (ANABoRaVa, 2013).

Materials and methods

Animals

The research took place in three dairy farms located in Northern Italy with similar management and feeding conditions.: a total number of 129 multiparous post partum cows with eutocic delivery have been enrolled. The cows were of four different breeds: Friesian (n=30), Cabannina (n=30), Varzese (n=30) and Valdostana (n=39) and were milked twice a day. All subjects were in good health status, as verified by periodic veterinary observations. All cows received a similar diet composed of poliphyte hay and integration of concentrates (flour of cereals and *Leguminosae* (*Fabaceae*), soy-free) Animals were chosen in order to have three classes of lactation stage: milk collections were carried out starting from 40±10 days (group A), 70±10 days (group B), and 130±10 days (group C). All milk samples were taken during the morning milking into a glass tube, immediately forwarded to the laboratory and frozen at -80°C until analysis.

Milk fat determination and fatty acid profiles

The milk fat content of each sample was determined by the UV spectrophotometrical method proposed by Forcato *et al.* (2005): 30-60 µl of milk are added of ethanol and stored for a time ~1h at -20°C in order to precipitate interfering proteins and peptides. The supernatants are read at 208 nm wavelength in a UV/Vis spectrophotometer.

Milk fat matter was extracted from the thawed samples by the method described by Bligh and Dyer (1959) modified by Manirakiza et al. (2001). Briefly, 1 mL of each sample are transferred in a 15 mL Falcon test tube and 3.75 mL of a chloroform: methanol 1:2 solution are added. After 10 minutes of vortex processing, 1.25 mL of chloroform are added to the mixture, and the sample is

vortex-stirred for 1 min. Finally, 1.25 mL of microfiltered distilled water are added and the resulting suspension is vortex mixed again for 1 minute.

Samples are centrifuged at 2000 rpm for 10 minutes at 20°C in order to obtain three phases: the supernatant, clear, composed of fat dissolved in chloroform; the intermediate one, mainly composed of proteins and the lowest density one formed by the aqueous medium.

The chloroform-containing phase was filtered with paper and collected in a previously weighted test tube containing anhydrous sodium sulfate, in order to eliminate possible water residues. One hundred and fifty µl of nonadecanoic acid (C:19) were added to each sample as an internal standard for gas chromatography. The solution was dried under mild nitrogen flow and the solid matter quantified.

Samples prepared as descripted above, were stored at -80°C till GC analysis were performed. For this purpose, the sample was dissolved in chloroform:methanol 2:1 solution with a ratio of 1mL for 1 mg of extract.

Sample were derivatized as described by Moltó-Puigmartí et al. (2007) with some modifications: each sample was incubated with 0.5 mL of sodium methoxide at 80°C for 10 minutes, then they were cooled at 37°C, prior to a new incubation with 0.5 mL of boron trifluoride at 80°C for 3 minutes; finally the sample was cooled at room temperature.

After the addition of 0.5 mL of hexane, samples were shaken for 1 minute, then 0.5 mL of NaOH saturated solution and anhydrous Na_2SO_4 were added to obtain 3 phases.

The supernatant was collected, dried under mild nitrogen flow and suspended in 1mL of hexane. 0.5µl of this solution were injected in a gas chromatograph Trace GC 2000 Series equipped with a GC column (Teknokroma 100 mT plus C max 150/prog 250°C, High Polar, Flow N2 0,5 ml/min (0,25 mm x 0,20 µmm Film) and a pre-column of 5 mT (ID = 0.25) deactivated). A programmed temperature run was used: the initial oven temperature was 70°C, isothermal for 2'30". Then, temperature was increased at a rate of 3°C min^{-1} to 240°C. The total analysis time was 95 min. Gaschromatographic peaks were identified by

comparing the retention times of sample with a 37 methylester fatty acid mix (FAME Mix 37, Supelco) and 9-11 and 10-12 linoleic acid conjugate methyl esters (Matreya).

Fatty acids and desaturase indices

On the total peak fatty acids profile, the percentage of 18 unsaturated/polyunsaturated fatty acids (UFA) have been determined by integration of chromatograms by the software Azur for Windows platform. The following UFA were determined:

C14:1*cis* (myristoleic)
C16:1cis (palmitoleic)
C18:1*trans* (elaidic)
C18:1*cis* (oleic)
C18:2*trans* (linolelaidic)
C18:*2cis* (linoleic)
C18:3n6 (γ-linolenic)
C18:3n3 (α-linolenic)
CLA 9-11 (C18:2 *cis*-9 *trans*-11, conjugated linoleic acid)
C20:1n9*cis* (*cis*-11eicosenoic)
C20:2*cis* (*cis*-11,14 eicosadienoic)
C20:3n6*cis* (*cis*-11,14-eicosatrienoic)
C22:1n9*cis* (erucic)
C20:3n3*cis* (*cis*-11,14,17-eicosatrienoic)
C20:4n6 (arachidonic)
C22:2 (*cis*-13,16-docosadienoic)
C20:5n3 (*cis*-5,8,11,14,17-eicosapentaenoic)

Furthermore, the percentages of C14, C16 and C18 fatty acids were taken into account in order to calculate the desaturase indices.

Beside the single fatty acid percentages, the total percentage of unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty

acids (PUFA), and the UFA to saturated fatty acid ratio (UFA/SFA) were calculated.

Desaturase indices (Δ^9) were calculated as reported by Schennink et al. (2008) from the percentages, and total desaturase index ($\Sigma\Delta^9$) was calculated according to Mele et al. (2007) on C14, C16 and C18 fatty acids; briefly, the individual Δ was calculated as $\sum Cx{:}n/\Sigma(Cx+Cx{:}n)*100$, where x is the number of carbons of fatty acid, and n is for the number of double bonds. The total desaturase index was calculated as:

$$\Sigma\Delta^9=(C14{:}1+C16{:}1+C18{:}1)/(C14+C14{:}1+C16+C16{:}1+C18+C18{:}1)\cdot 100.$$

Atherogenic index (AI) of milk was calculated as follows (Ulbricht and Southgate, 1991):

$$AI=[C12{:}0+4(C14{:}0)+(C16{:}0)]/\sum(MUFA+PUFA)$$

Statistical analysis

Data concerning the percentages of fatty acids, the desaturase and atherogenic indices were analyzed by a mixed model analysis of variance, considering the lactation period as the fixed factor, and the subject as random factor. Differences between breeds were evaluated by the Tukey test for multiple comparisons. Data are resumed by mean±standard deviation. Statistical significance was set for all tests at $p\leq 0.05$.

Results

Fatty acid or Index %	Holstein Friesian (n=30)	Cabannina (N=30)	Valdostana (n=39)	Varzese (n=30)	Breed p	Time p	Time trend
C14	15.0±1.5	15.29±1.8	14.9±1.8	14.2±1.4	n.s.	n.s.	-
C14:1	0.9 ± 0.30^{b}	1.09 ± 0.3^{a}	1.0 ± 0.3^{ab}	0.9 ± 0.3^{ab}	**	***	↑
C16	41.7 ± 4.6^{a}	37.5 ± 3.32^{b}	36.0 ± 2.8^{b}	36.5 ± 3.8^{b}	***	n.s.	-
C16:1	1.9 ± 0.8^{ab}	2.0 ± 0.7^{ab}	1.5 ± 0.8^{b}	2.0 ± 0.90^{a}	*	n.s.	-
C18	9.4±2.3	9.4±2.2	10.0±1.4	10.4±2.2	n.s.	***	↓
C18:1t	0.7 ± 0.3^{b}	0.7 ± 0.2^{b}	1.6 ± 0.4^{a}	0.8 ± 0.4^{b}	***	n.s.	-
C18:1c	17.3 ± 4.3^{c}	20.1 ± 3.7^{b}	20.3 ± 3.1^{b}	23.2 ± 3.6^{a}	***	n.s.	-
C18:2t	0.2±0.1	0.2±0.1	0.1±0.3	0.2±0.2	n.s.	n.s.	-
C18:2c	1.70 ± 0.60^{b}	2.5 ± 1.5^{a}	1.7 ± 0.5^{b}	1.80 ± 0.8^{b}	**	n.s.	-
C18:3n6	0.001±0.005	0.001±0.005	0.03±0.1	0.05±0.30	n.s.	n.s.	-
C18:3n3	0.2 ± 0.4^{b}	0.6 ± 0.6^{a}	0.6 ± 0.3^{a}	0.4 ± 0.4^{ab}	**	*	↑
CLA 9-11	0.1 ± 0.2^{c}	0.3 ± 0.2^{ab}	0.4 ± 0.3^{a}	0.2 ± 0.3^{bc}	***	***	↑
C20:1	0.09 ± 0.2^{b}	0.07 ± 0.2^{b}	0.2 ± 0.3^{a}	0.04 ± 0.10^{b}	***	n.s.	-
C20:2	0.01±0.03	0.01±0.02	0.01±0.01	0.01±0.04	n.s.	n.s.	-
C20:3n6	0.02 ± 0.03^{b}	0.04 ± 0.04^{ab}	0.04 ± 0.02^{a}	0.04 ± 0.04^{ab}	*	**	↑
C22:1	0.02±0.03	0.01±0.03	0.01±0.01	0.01±0.02	n.s.	*	↓
C20:3n3	0.01±0.02	0.02±0.04	0.02±0.02	0.03±0.08	n.s.	n.s.	-
C20:4	0.01 ± 0.02^{b}	0.02 ± 0.03^{b}	0.07 ± 0.05^{a}	0.02 ± 0.03^{b}	***	n.s.	-
C22:2	0.002 ± 0.01^{b}	0.003 ± 0.01^{b}	0.01 ± 0.02^{a}	0.001 ± 0.02^{b}	***	n.s.	-
C20:5	0.01 ± 0.02^{b}	0.01 ± 0.01^{b}	0.03 ± 0.02^{a}	0.01 ± 0.01^{b}	***	n.s.	-
UFA	23.3 ± 4.8^{b}	27.8 ± 4.4^{a}	27.9 ± 4.0^{a}	29.4 ± 5.6^{a}	***	n.s.	-
MUFA	21.1 ± 4.6^{b}	24.2 ± 4.2^{a}	24.9 ± 3.7^{a}	28.6 ± 5.4^{a}	***	n.s.	-
PUFA	2.2 ± 1.0^{b}	3.6 ± 1.7^{a}	3.0 ± 0.7^{a}	2.8 ± 1.4^{ab}	***	n.s.	-
UFA/SFA	0.3 ± 0.09^{b}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	***	n.s.	-
Δ^{9}14	5.5 ± 2.0^{b}	6.7 ± 1.4^{a}	6.5 ± 1.3^{ab}	6.2 ± 2.3^{ab}	*	***	↑
Δ^{9}16	3.9 ± 1.8^{b}	5.1 ± 1.9^{ab}	4.1 ± 2.1^{ab}	5.3 ± 2.2^{a}	*	n.s.	-
Δ^{9}18	65.0±5.4	68.2±4.4	67.0±2.8	67.0±13.9	n.s.	**	↑
$\Sigma\Delta^{9}$	23.1 ± 4.9^{b}	27.1 ± 4.1^{a}	27.4 ± 3.8^{a}	29.2 ± 6.0^{a}	***	n.s.	-
AI	4.8 ± 1.2^{a}	3.9 ± 0.8^{b}	3.7 ± 0.8^{b}	3.3 ± 0.7^{b}	***	n.s.	-

Table 1. Mean percentages of the fatty acids taken into account for the survey. Different superscripts indicate a $p<0.05$ difference between breeds (Tukey post-hoc test). Asterisks indicate the statistical significance of the effect (breed/time): *-$p<0.05$; **-$p<0.01$; ***-$p<0.001$, n.s.- not significant; arrows on the "time trend" column mark an ascending or descending trend for the variable ($p<0.05$).

The three groups of cows had a mean milk production of 24.17±6.84 kg/d (Friesian), 14.24±4.36 kg/d (Cabannina), 11.84±4.59 (Varzese), and 9.33±4.50 kg/d (Valdostana); the mean fat content was 3.53±1.01 %, 4.05±1.14 %, 4.27±0.87 %, and 3.63±0.81 % for Friesian, Cabannina, Varzese and Valdostana, respectively.

Several variables changes over the breeds, while only a few variables show significant changes between lactation periods (table 1). A number of differences between breeds are evidenced, in particular between local and Friesian, with higher percentages of UFA, MUFA, PUFA, and a higher UFA/SFA ratio. Varzese cows account for the higher UFA content (about 29%, table 1), while PUFA percentages are significantly higher in Cabannina and Valdostana breeds. MUFAs percentage in milk fat is higher in local breeds with respect to Friesian, with no differences between Cabannina, Varzese and Friesian. Among MUFAs, Cabannina cow yield a milk with higher levels of C14:1, C18:2*cis*, C18:3n3, while Varzese milk fat shows higher percentages of C18:1*cis*. Valdostana breed remarkably yielded the higher percentages of a -*trans* FA, C18:1*trans*, with respect to the other breeds; the same breed had notably levels of C18:3n3, CLA 9-11, C20:1, C20:4, C20:5 (table 1).

Concerning the Δ^9 indices, the $\Delta^9$14 had the higher values in Cabannina breed (about 6.7, table 1), whereas the highest $\Delta^9$16 mean value (5.3%) was found in Varzese. No differences have been noted for the $\Delta^9$18. The $\Sigma\Delta^9$ index was, for the three local breeds, significantly lower (mean values 3.3 to 3.9%) when compared to Friesian (mean 4.8%, table 1). The opposite conclusions could be drawn for the AI, significantly higher in Friesian (mean AI=4.8): Cabannina, Varzese and Valdostana breeds maintain lower levels of AI, without differences between groups (mean AI from 3.3 to 3.9, table 1).

The influence of lactation period is limited for all the considered parameters: a significant increasing trend is observed for C14:1, C18:3n3, CLA 9-11, C20:3n6, $\Delta^9$14, and $\Delta^9$18. On the other side, negative trends over time are seen for C18 and C22:1.

Discussion

The results here reported shows that cattle biodiversity is an important feature –at least at the fatty acids level- for the maintaining and the preservation of the local breeds. The breeds of autochthonous cattle analyzed in the present work show a number of differences from a cosmopolite breed (i.e. Friesian), with an overall higher percentage of UFA, MUFA, and PUFA. Several fatty acids differ between breeds: in particular, the levels of C14 (myristic acid) in all breeds are similar to that reported by White et al. (2001) for Holstein, whereas the unsaturated analogue C14:1 (myristoleic acid) resulted, with respect to the data reported by White et al. (2001), higher in all of the breeds. The percentage of C16 fatty acid in the examined breeds is higher than the percentages reported by White et al. (2001) for Friesian (31.19÷31.67 %) with higher values in Friesian cow than in the other breeds.

The percentages on the single fatty acids are generally in the ranges reported by Jensen (2002), as for oleic acid (C18:1*cis*), with the exception of Friesian, with mean values below the lower limit. Linoleic acid (C18:2*cis*) is –for all breeds- in the range referred by Jensen (2002), while linolenic acid (C18:3) in Friesian and Varzese resulted lower than the limits reported by Jensen (2002). Unsaturated long-chain fatty acids (>20C) are overall more represented in local breeds with respect to Friesians, in particular C20:1 (mainly present in Valdostana), C20:3n6 (in Cabannina, Valdostana and Varzese), C20:4 (in Valdostana), C22:2 (in Cabannina), C20:5 (in Valdostana).

Desaturase indices are generally higher in local breeds than in Friesian, with the exception of $\Delta^9$18, that did not differ between breeds. $\Delta^9$14 indices are quite lower than that referred by Kay et al. (2005), with significant differences between breeds, since Friesian cows show lower values of the index when compared to the other breeds. The same conclusion can be drawn for $\Delta^9$16, lower in our samples than in those reported by Kay et al. (2005).

The $\Delta^9$18 did not change among breeds, although a temporal increasing trend ($p<0.01$) was observed.

Concerning the total desaturase index ($\Sigma\Delta^9$) resulted significantly higher in local breeds than in Friesian cows; overall, the evaluated $\Sigma\Delta^9$ is lower than that calculated by Kay et al. (2005). The desaturase indices $\Delta^9 14$ and $\Delta^9 18$ increase significantly with lactation (table 1). These results partially contrast with the findings of Kay et al. (2005), reporting significant variations for all of the indices. The actual desaturase capacity can be, therefore, a matter of discussion for the direct determination of the activity for this enzyme.

The atherogenic index was significantly lower in local breeds (mainly Varzese) when compared to the Friesian; in this breed, the AI values are comparable to those reported by Nantapo et al. (2013), while in the other examined breeds the mean values of AI are sensibly below the value of AI= 4.0.

Conclusions

The results confirm, at least partially, that local breeds produce a different milk with respect to a cosmopolite breed as Friesian, so the biodiversity is not only related with the breed but also with the 'milks'. The fatty acid proportions in local breeds milk show significantly higher percentages of unsaturated acids, either mono- or polyunsaturated; these features reflects on the desaturase indices, with the highest mean values in local breeds than in Friesian, and on Atherogenic index, strongly lower in local breeds. The differences in such characteristics can enhance the value of milk in human health terms, due to deep differences in fatty acid composition. These features can contribute to the re-evaluation of bovine local breeds and their products, in order to recover the continuously reducing heads, and enhance the quality of derived products, with positive effects on health,little breeding farm economy and biodiversity.

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5.2

NMR-based metabolomics as a tool to characterize the milk biodiversity

The data shown in this chapter refer to the already published paper: TOMASSINI, A., CURONE, G., SOLÈ, M., CAPUANI, G., SCIUBBA, F., CONTA, G., MICCHELI, A., VIGO, D., (2018, APRIL 14). NMR-BASED METABOLOMICS TO EVALUATE THE MILK COMPOSITION FROM FRIESIAN AND AUTOCHTHONOUS COWS OF NORTHERN ITALY AT DIFFERENT LACTATION TIMES. NATURAL PRODUCT RESEARCH, PP. 1–7. TAYLOR AND FRANCIS LTD. HTTPS://DOI.ORG/10.1080/14786419.2018.1462183

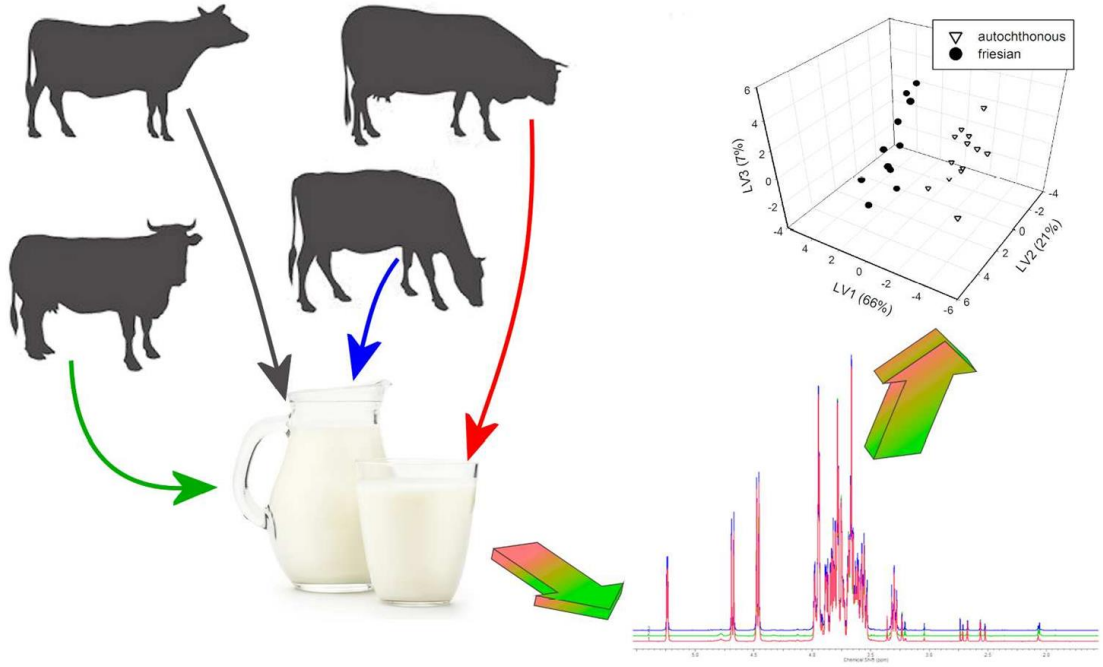
Introduction

Milk has been described as an almost perfect food and cow milk represents a worldwide popular component in both infant and adult nutrition. From a chemical point of view, milk is a complex mixture of several substances ranging from oligosaccharides, proteins and lipids to vitamins and mineral salts, among others. (Solomons, 2002; DuPuis, 2002) and is consumed both fresh and processed into dairy products. In this regard milk composition has been associated to both the nutritional quality and to the technological processing capabilities so that a profound characterization of milk metabolic profiles may represent a basis to improve its nutritional and technological quality. However, it is known that milk composition varies in relation to many factors ranging from genetics to physiological (number and stages of lactation, cow health status, seasonal variations) and to zootechnical (feeding, climate, breeding techniques) ones so that a comprehensive knowledge of milk composition can be of paramount importance towards the optimization of processing capabilities to dairy products (Jakob et al., 1992; Lock et al., 2003; McJarrow et al., 2004; Tsioulpas et al., 2007, Le Maréchal et al., 2011). Up to now the breeding programs to improve milk production have been based on strategies that often favoured the economical parameters over milk quality, leading to an homogenisation of the cow's genetical pools and, consequently, to the risk of extinction of less producing autochthonous breeds and biodiversity loss.

The application of NMR spectroscopy to analyse milk metabolites has proven a valuable tool in studies ranging from mammary gland physiology to nutritional, quality and technological properties of milk (Sundekilde et al., 2013; Praticò et al., 2014; Cesare Marincola et al., 2012).

In the present study, the chemical composition of milk obtained from Friesian cows and different autochthonous breeds from Northern Italy has been analyzed by NMR spectroscopy. The matrices of the resulting data were analyzed by multivariate statistical methods in order to obtain a metabolic profile (metabotype) of each breed. Furthermore, the cows were breeded in the same

farm, were identically fed with a standardized diet and milk was sampled at the same lactation times, in order to minimize the inter,individual variability.



Materials And Methods

Experimental design

Milk samples were collected from a total of 28 cows ,11 Friesian and 17 autochthonous cows (5 Modenese, 6 Rendena, 3 Varzese, 3 Pezzata Rossa Italiana) matched for parity and lactation period. To minimize the metabolic variation due to external factors, the cows were grown in the same farm and were identically fed with a diet constituted by a 80% of fodder crop: lucerne hay of first cut, polifita grass hay and barley straw, and 18% of soybean, sunflower meal, corn meal, sugar beet pulp and flax cake plus vitamin, mineral supplementation.

Milk samples collection

For each cow 15 ml of milk were collected during morning milking in a Falcon tube. Before sampling, teat ends were carefully cleaned and the first streams of foremilk were discharged. A total of 52 samples were collected from

100 to 300 days of lactation. Samples were stored at ,80° C until subsequent analysis.

Sample extraction for NMR

A total of 600 μL of milk were vortexed in 3 mL of a methanol–chloroform (2:1) mixture in polypropylene tubes. A total of 400 μL of distilled water and 1 mL of chloroform were sequentially added, then the mixture was vortexed again and kept overnight at 4 °C. Polar and organic phases were separated by centrifugation at 11 000 g at 4 °C for 20 min. The extraction procedure was a modification of the method previously set up for tissues, cells and biological fluids (Miccheli et al., 1988; Ricciolini et al., 1991, Miccheli et al., 2006). The polar phases (top layer) and organic phases (bottom layer) were separately collected, dried under N2 flux and preserved at ,80 °C until NMR analysis. The dried polar samples were re-dissolved in 600 μL of D_2O phosphate buffer solution (pH 7.4) containing 2 mM (final concentration) sodium-3-(trimethylsilyl) propionate-2-3-d4 (TSP) as ^1H NMR reference. The obtained samples were transferred to 5 mm NMR glass tubes for analysis and the hydroalcoholic phase was analyzed by the acquisition of mono, and bi-dimensional ^1H,NMR spectra.

NMR spectroscopy

NMR spectra were acquired at 298 K using a Bruker Avance 400 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) equipped with a 9.4 T magnet operating at a ^1H frequency of 400.13 MHz.

The pulse sequence adopted for ^1H NMR spectra acquisition of aqueous samples was a presaturation pulse, single 90° detection pulse, acquire, relaxation delay. The relaxation delay was 7.5 s, while the presaturation pulse was applied for 2 s; the acquisition time needed to collect the 32 k points was about 5.5 s, therefore the proton nuclei different from the solvent ones were allowed to relax for 15 s, complying with the full relaxation condition after a 90 degree pulse. The length of the detection pulse was calibrated previously to the

acquisition of each spectrum, spectral width was set to 5995.02 Hz (12 ppm), 64 scans were collected for each spectrum preceded by 4 dummy scans. Total Correlation Spectroscopy (TOCSY) experiment was performed at 298 K with a spectral width of 12 ppm in both dimensions, employing a matrix of 4k x 256 data points, a repetition time of 2 s, 112 scans. TOCSY spectra were acquired with different mixing times.

The ^{1}H-^{13}C Heteronuclear Single Quantum Correlation (HSQC) spectrum was recorded with the echo–antiecho phase, selective mode, with a spectral width of 12 and 200 ppm in proton and carbon dimensions respectively, employing a matrix of 4k x 400 data points for f2 and f1 dimensions, a repetition delay of 2 s and 96 scans. The average heteronuclear coupling constant was 145 Hz.

The ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) spectrum was obtained with 12 and 220 ppm spectral widths in proton and carbon dimensions respectively, employing a matrix of 4k x 400 data points for f2 and f1 dimensions, a repetition delay of 1.5 s and 96 scans. Different long range heteronuclear coupling constant were used (2, 4 and 8 Hz), in order to observe different long range heteronuclear correlations for the same metabolite, while the direct coupling constants comprised between 130 and 170 Hz were filtered.

The Diffusion Order Spectroscopy DOSY experiments were acquired employing the ledbpgp2s pulse program with 128 scans, a spectral width of 15 ppm, 32 gradient increments, a diffusion time of 1000 ms, a gradient length of 2000 μs, 64K data points and a recycle delay of 2 s.

Signals assignment was achieved by standard 2D experiments (TOCSY, HSQC, HMBC and DOSY) on selected samples and confirmed by comparison with the literature (Lamanna et al., 2011; Osthoff et al., 2008; Sundekilde et al. 2013), web database HMDB (Wishart, Jewison, Guo, Wilson, & Knox, 2013) and in-house database.

Processing and analysis

One-dimensional (1D) NMR spectra were processed and quantified by using ACD Lab 1D NMR Manager ver. 12.0 software (Advanced Chemistry Development, Inc., Toronto, ON, Canada), whereas 2D NMR spectra were processed by using Bruker TopSpin ver.3.1 (Bruker BioSpin GmbH). The acquired NMR spectra were manually phased and baseline corrected. Proton spectra were referenced to the chemical shift of the TSP methyl resonance at δ 0.00 in D2O. Quantification of metabolites was obtained by comparing the integrals of their specific signals with the internal standard integral. Overlapped signals in the polar extract between 1.14 and 1.28 ppm, 2.03 and 2.08 ppm and between 5.35 and 5.45 ppm were measured by peak fitting analysis on the selected multiplets, using the fitting function provided by NMR Manager software. The concentrations are expressed as mmol L^{-1}.

Data analysis and statistics

Statistical procedures were carried out by using a combination of Sigmaplot 12.0 (Systat Software Inc.) and The Unscrambler X ver. 10.3 (CAMO Software, Oslo, Norway) for univariate and multivariate analysis, respectively. The amounts of metabolites were expressed as medians of concentration (mmol L,1) and ranges from the 1st to the 3rd quartile.

To evaluate the differences between different milk groups, Partial Least Square – Discriminant Analysis (PLS,DA) was applied on the data matrix, the rows of which included the sample set.

Data were mean-centred and scaled before analysis. Projection of PLS,DA was applied to maximise the discrimination between sample classes (Barker et al., 2003) and to assess the correlation between the observed data and class membership, that is, an external variable that indicates to which class the subjects belong (class value = 1).

In addition, Partial Least Square analysis was performed to assess the correlation between NMR data and lactation time, which is a continuous variable. The same pre-processing procedure used for PLS,DA was used.

Uncertainty test, based on cross validation, Jack,knifing and stability plots, was applied to test the significance of the PLS and PLS,DA models (Martens et al., 2000). Significant correlation coefficient was used to identify discriminant variables. Unpaired Student's t test or Mann–Whitney rank sum test were applied to assess the differences on metabolite levels among phenotypes. Paired Student's t test or Wilcoxon signed rank test were applied to evaluate the differences on single metabolite level between two time points, for normal and non,normal distribution, respectively. A p-value of 0.05 was considered significant. Prior to comparison, Shapiro,Wilk test was performed to assess the normal distribution of the data. Pearson Product Moment Correlation coefficient was calculated to assess significant correlation between metabolites.

Results And Discussion

The "omics" technologies, namely genomics, proteomics, transcriptomics and metabolomics, have been applied in studies regarding lactation mainly in dairy cows (reviewed in Li et al., 2017).

In this regard, NMR spectroscopy-based metabolomics allows a comprehensive identification and quantification of low molecular weight metabolites and is particularly appropriate in studies on biofluids, including milk (Hu et al., 2004; Lamanna et al., 2011; Maher et al., 2014). In the present study we first analysed the variation in milk composition in relation to the lactation stages that are commonly roughly divided in early (13 ± 1.8 days), mid (130 ± 4.6 days) and late (283 ± 3.4 days) lactation (Garnsworthy et al., 2006) to investigate any possible influence of the genetical different breeds. We chose to analyze the milk samples collected in the mid and late lactation stages to avoid the possible metabolic stress or the negative energy balance that can affect individual animals in early lactation, introducing an additional source of variation (Kessel et al., 2008).

The ^{1}H NMR spectrum of the polar phase can be divided into three main spectral regions. The aliphatic region, from 0 up to 3.5 ppm, includes amino acids (alanine, isoleucine, lysine, threonine and valine), tricarboxylic acid cycle

intermediates (citrate, succinate and a,ketoglutarate), short chain fatty acids (acetate and valerate), lactate, N,acetyl moieties, N,trimethyl moieties (choline, carnitine and acetylcarnitine) and oligosaccharides (sialic acid and fucosylated derivatives). The sugar region, between 3.5 and 5.5 ppm, includes a series of overlapped signals, due to the presence of simple (glucose, lactose and myoinositol) and complex sugars. The most intense signals (d 5.22, 4.69 and 4.47) were assigned to the anomeric protons of free lactose, superimposed to the peaks of some lactosyl units of oligosaccharides. The aromatic region includes aromatic amino acids (histidine and its methyl derivatives, phenylalanine and tyrosine) and phenolic compounds (4-hydroxyphenylacetic acid).

The DOSY experiment is reported in Supplementary Figure 2. In this figure only the signals relative to TSP, lactose and of different N-acetyl groups were considered to better display their autodiffusion coefficients. It is interesting to observe that the most abundant N-acetyl groups show an autodiffusion coefficient about half the one exhibited by lactose, while the less abundant one has a coefficient of only one fifth of lactose. This means that while all acetyl groups belongs to molecules heavier than a disaccharide, such as oligosaccharides or oligopeptides.

A further indication of the presence of N-acetyl groups with different mobility could be derived from the study of the slices of the DOSY experiment spectrum prior inverse Laplace transformation. Each slice corresponds to a specific gradient strenght, and at progressevely higher strenght the resonances of fastest species cannot refocus, and as such only the signals of the slowest molecules can be observed. In Supplementary Figure 3, for example, it is possible to identify two N,acetyl groups belonging to relatively fast molecules, which are defocused at 75% gradient strenght, and another group characterized by a lower translational mobility, the resonance of which is observable even at 100% gradient strength field. On the basis of the diffusion coefficients, we could disciminate the class of Nacetyl-groups (at 2.05, 2.08 ppm) in N-acetyl-glucosamine or Nacetyl-galactosamine containing

oligosaccharides and in N-acetylglutamine containing oligopeptides (at 2.04 ppm), having an higher hydrodinamic radius than oligosaccharides.

A total of 50 metabolites were identified and quantified. No significant variations were evidenced in the milk sampled between 100 and 200 days of lactation irrespective on the breeds by the PLS analysis, showing an almost steady composition in terms of the polar analyzed components (R2=0.48; Q2=0.18; data not shown).

On the contrary, a statistical significant variation is shown by the dataset including all the samples from every breed ranging from 200 to 300 days of lactation (R2=0.78; Q2=0.42) (Fig. 1). 9 metabolites were responsible of the observed variation, namely 1,2-propandiol, 3 hydroxybutyrate, butyrate , N – acetyl, X4, galactose-1-P and glucose-1-P with decreased concentrations and N-acetyl,glucosamine and cytidine-X-P with increased levels.

Milk production during lactation is a multiphasic process and, furthermore, milk metabolites have different sources, being synthesized by multiple cell types in the mammary gland or by different metabolisms in the whole organism contributing to the variability of milk metabolic profiles (Mc Manaman et al., 2003). Late lactation is characterized by an involution of the mammary gland with a loss of tight junction integrity and the resulting variation of metabolite fluxes via the paracellular pathway (Stelwagen et al., 2014). A loss of function and a diminished milk yield linked to increased secretory cell apoptosis and diminished secretory cell biosynthetic capacity in the mammary gland with late lactation have also been shown (Hadsell D et al. 2007).

The observed significant decrease of N-acetyl,X2, glucose-1P and galactose-1-P with the concurrent increase of N-acetyl-X4 in milk sampled from 200 to 300 days of lactation suggested significant qualitative and quantitative variations in the oligosaccharides synthesis and secretion in
milk as a function of lactation time.

It has been suggested that the levels of ß,hydroxybutyrate in milk could be used as biomarkers of subclinical and/or acute ketosis in cows, and high levels of ß-

hydroxybutyrate and butyrate have been linked to elevated numbers of somatic cells counts (Klein et al., 2012; Sundekilde et al., 2012; Klein et al., 2013). Our results showed a decrease of the levels of these two metabolites in late lactation. As we did not observe any case of ketosis that, incidentally, is more frequent in early lactation, and although we did not perform somatic cell counts, we suggest that our data are more likely associated to the energy status in dairy cows in late lactation.

Furthermore, an oxidative damage within mammary cells in late lactation could be suggested by the increase in the levels of cytidine-X-P in milk possibly associated with the RNA synthesis decline showed in prolonged lactation (Hadsell et al., 2007).

On the basis of these results, we applied a PLS-DA analysis on the NMR data matrices to disentangle potential differences of milk composition among the breeds while keeping the lactation stages between 100-200 and 200-300 days.

At 100-200 days of lactation, significant differences between Friesian and autochthonous breeds were shown (Fig. 2; R2=0.93; Q2=0.57). Nine metabolites were involved, namely valine, acetate, phosphorylcholine and glucose–1P and citrate, carnitine, fumarate, hippurate and fucose with higher and lower levels in the Friesian cows, respectively. Acetate, citrate, carnitine, fumarate, hippurate and fucose were found to be statistically different at the univariate analysis, too (Suppl. Table 1).

The PLS-DA analysis could also distinguish the Friesian and autochthonous milks after 200,300 days of lactation (Fig.3; R2=0.99; Q2=0.65), with lower levels of acetylcarnitine, carnitine and fumarate and higher levels of ribosyl and cytidineXP in the Friesian and autochthonous breeds, respectively. Carnitine, fumarate and ribosyl were also statistically different at the univariate analysis (Suppl. Table 1).

Citrate, phosphocholine, carnitine, acetate and hippurate have been associated with milk coagulation properties, thus being metabolites of

paramount importance in the evaluation of milk technological quality and processing capabilities (Sundekilde et al., 2011; Harzia et al., 2012).

In agreement with our results, good coagulating milks have been characterized by increased levels of choline and acetate and decreased levels of citrate and carnitine, and different milk metabolite profiles characterized by differences in the relative abundance of these metabolites have been demonstrated in milk between two cow breeds (Sundekilde et al., 2011; Harzia et al., 2012). In this regard, our results suggested different technological characteristics, (faster coagulation time with a bigger casein clot dimensions) of Friesian milk than autochthonous breeds milk from 100 days of lactation.

In particular citrate, that is associated with the whey fraction, is the most abundant organic acid in milk either complexed with calcium and magnesium ions or in free form. Changes in its concentration have important effects on the physico-chemical properties of milk. High levels of citrate are capable of disrupting the casein micelle structure thus affecting the coagulation parameters. It is well known that the coagulation properties are also influenced by other factors like pH, ions availability, as well as lactation time, climate, and cow's nutritional and health status (Salaun et al. 2005, Udabage et al., 2000, Skeje et al., 2007, Tsioulpas et al., 2007).

However, in our experimental set both Friesian and autochthonous cows were reared in the same farm and consequently in the same climate, were equally fed and their milks were sampled at equivalent lactation stages, thus ruling out these external sources of variation. Furthermore, our data did not show variations of citrate levels in relation to lactation stages, in agreement with previous results (Klein et al., 2010, Garnsworthy et al., 2010), this suggests that the observed variations could be ascribed to genetical differences of the studied breeds.

Interestingly, our results could be evaluated from a nutritional point of view beside the processing capabilities into dairy products. In fact choline, that is crucial in human development as well in adult life, and carnitine, whose deficiency has been associated to systemic effects on the infants, were among

the metabolites discriminating the Friesian and autochthonous breeds. Therefore, NMR-based metabolomics of milk could represent an informative tool from a nutritional perspective when selecting milks for premature children or to be used in infant formulas.

Figures

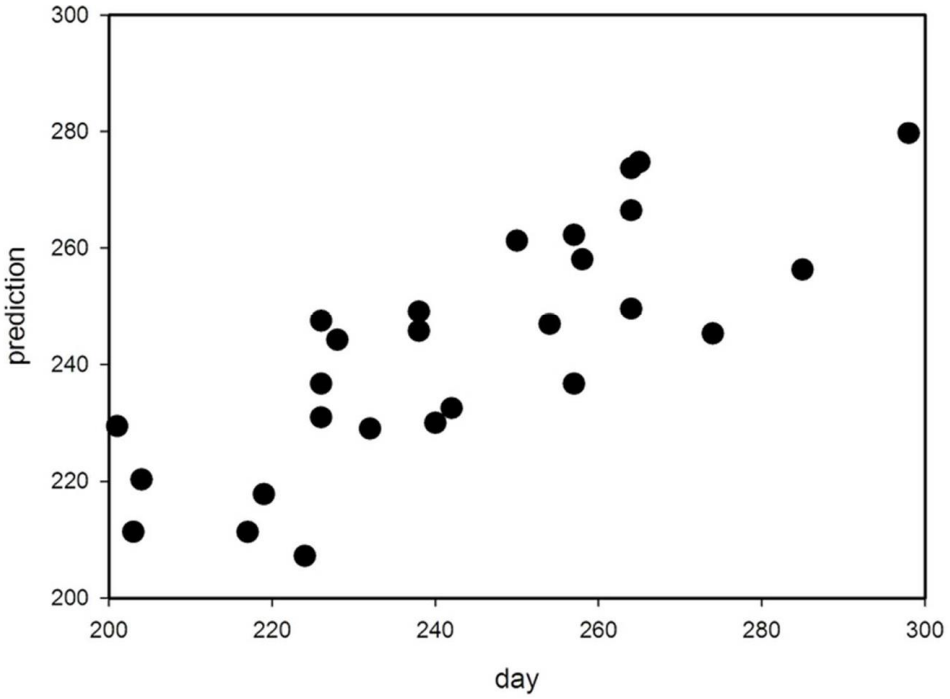


Fig.1 – PLS model for the lactation time (200-300 days of lactation)

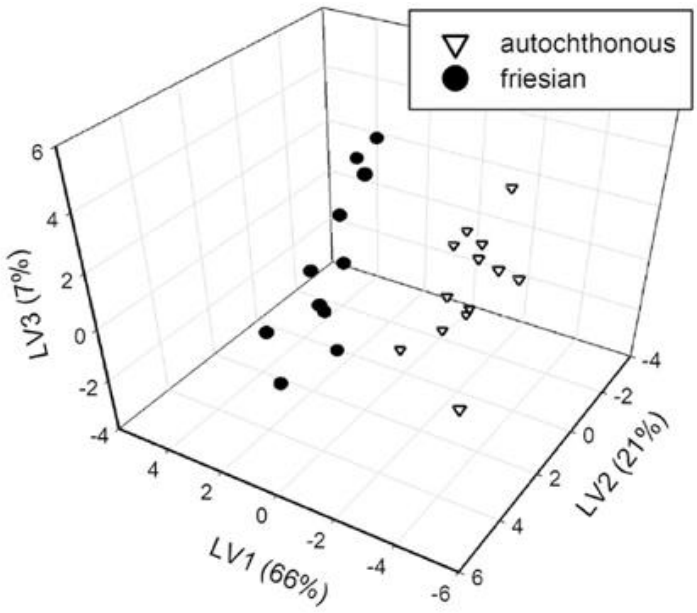


Fig.2 :PLS-DA 3D score plot of autochthonous and Friesian cow milk at 100-200 days of lactation (LV= latent variables)

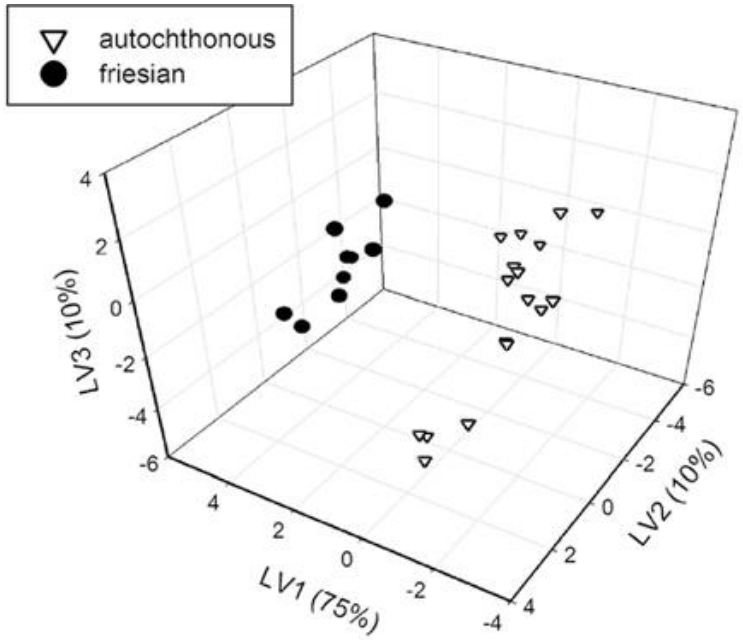
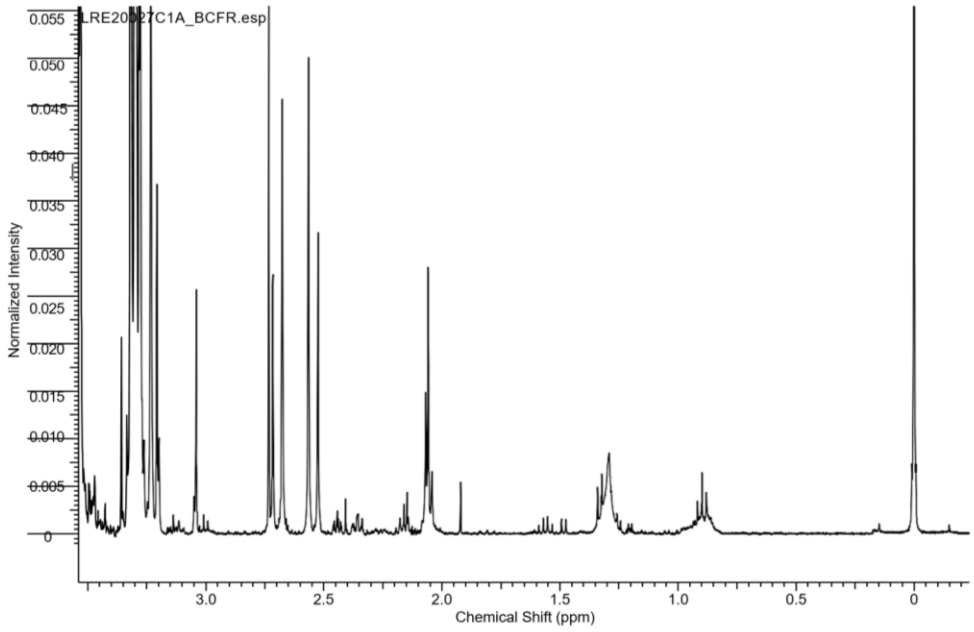


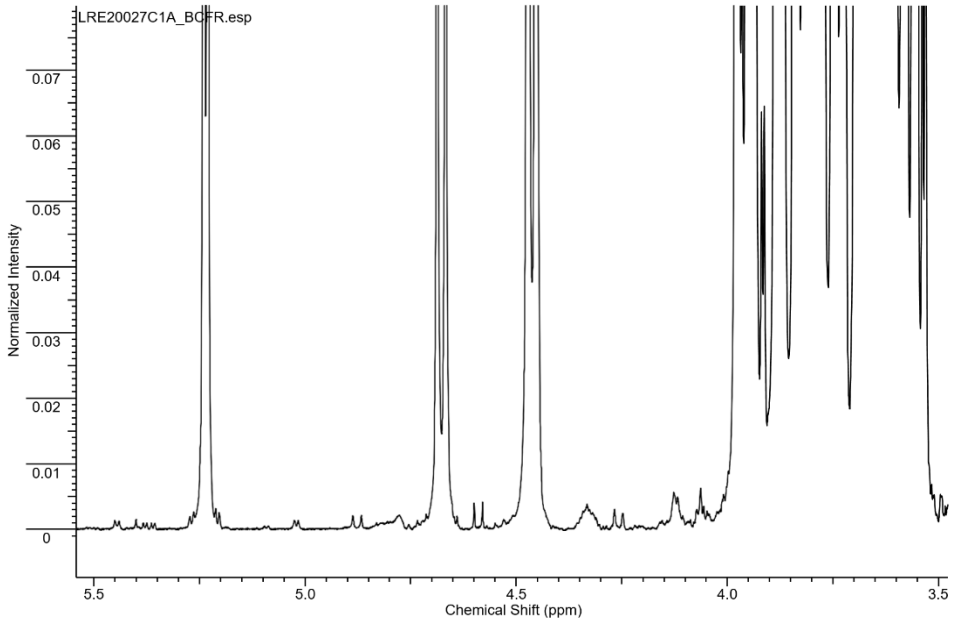
Fig.3: PLS-DA 3D score plot of autochthonous and Friesian cow milk at 200-300 days of lactation (LV= latent variables)

Supplementary Figure 1 – A representative ^{1}H-NMR spectrum of cow milk. a) 0- 3.5 ppm region; b) 3.5- 6 ppm region; c) 6- 8.5 ppm region.

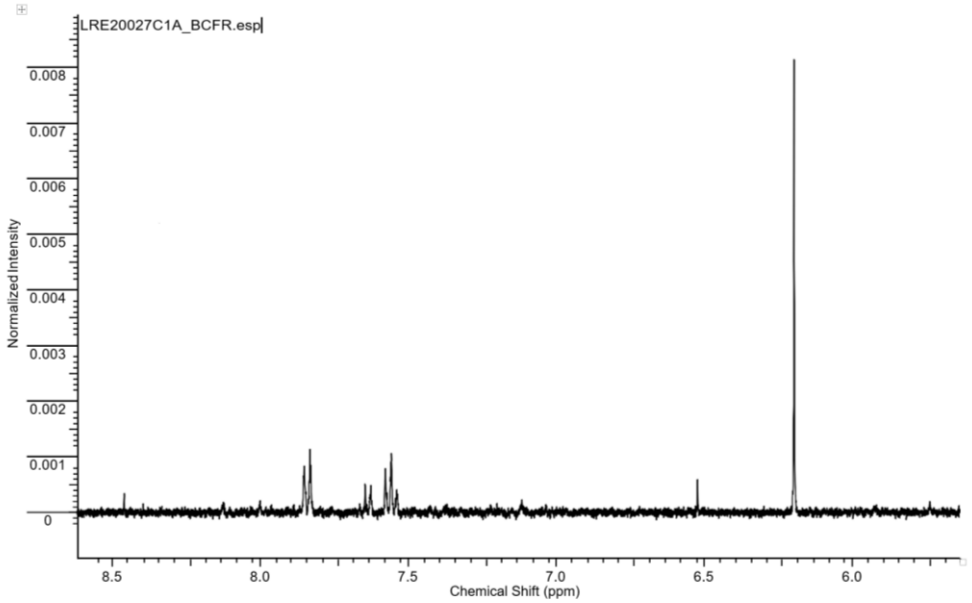
a) Region from 0 to 3.5 ppm



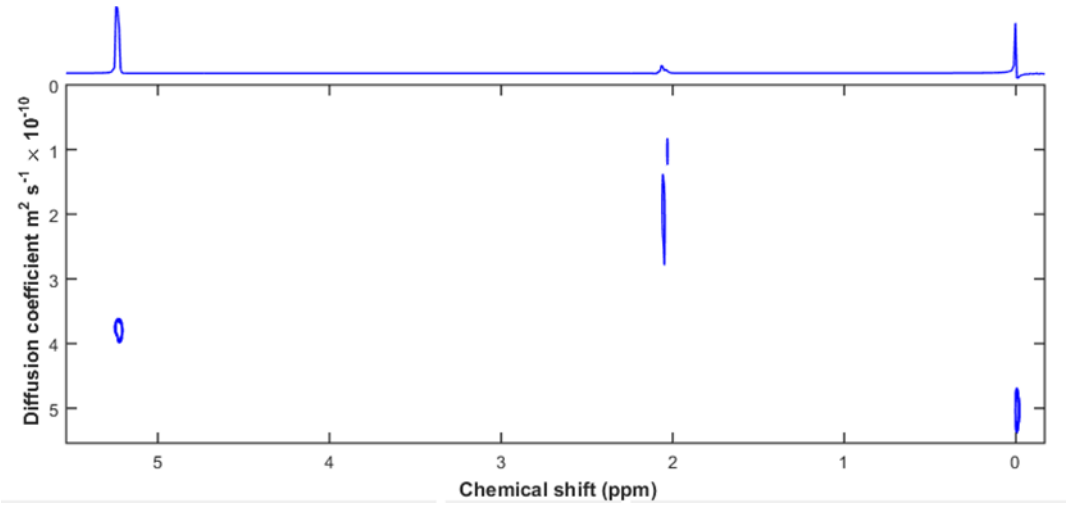
b) Region from 3.5 to 6 ppm



c) Region from 6 to 8.5 ppm



Supplementary Figure 2: DOSY experiment of Friesian cow milk. The spectrum show the autodiffusion coefficients of TSP, lactose and two different N-Acetyl groups.



Supplementary Table 1 – Comparison between Autochtonous and Freisian milk by using U-Test Mann-Whitney Analysis of variables important for the prediction model in PLS-DA.

a) 100-200 days of lactation

Autoctone	Mediana	25%	75%	Frisone	Mediana	25%	75%	p
Fuc	0,0297	0,0138	0,0491		0,0116	0,00535	0,0167	0,011
Ac	0,0995	0,0825	0,116		0,131	0,106	0,14	0,026
Cit	6,546	5,745	8,147		5,494	5,162	6,466	0,028
CAR+ALCAR	0,11	0,0925	0,126		0,0806	0,0707	0,0878	0,04
PCho	0,0937	0,0401	0,117		0,155	0,0591	0,268	*0,104*
Fum	0,0532	0,0372	0,0714		0,0306	0,0154	0,0561	0,043
Hipp	0,256	0,197	0,343		0,161	0,142	0,221	0,034

b) 200-300 days of lactation

Autoctone	Mediana	25%	75%	Frisone	Mediana	25%	75%	p
Met	0,11	0,0797	0,141		0,0728	0,0601	0,0899	0,006
CAR+ALCAR	0,116	0,0918	0,137		0,0743	0,057	0,0888	0,002
Ribosyl-nucl	0,00772	0,000883	0,0143		0,0157	0,0102	0,0289	0,039
CXP	0,0101	0,00226	0,0144		0,0206	0,00244	0,0349	*0,132*
Fum	0,0504	0,0337	0,0765		0,0316	0,0212	0,0423	0,004

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5.3
Milk microbiome characterization between healthy Holstein Friesian and Rendena cows

The data shown in this chapter refer to the accepted paper by PLOS ONE:

Paola Cremonesi & Camilla Ceccarani, Giulio Curone, Marco Severgnini, Claudia Pollera,Valerio Bronzo, Federica Riva, Maria Filippa Addis, Joel Filipe, Massimo Amadori, Erminio,Trevisi, Daniele Vigo, Paolo Moroni, Bianca Castiglioni. Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows.

Introduction

The evolution of molecular and "-omics" technologies made us aware of the varied and complex assortments of microbes that inhabit living animals and of the reciprocal interactions they entertain among themselves and with their hosts [1]. Among these, differently from "classical" methodologies, metagenomics enables the characterization of a microbial population in a culture-independent manner [2], providing a powerful means for identifying dominant and subdominant microbes and their dynamics in highly complex ecosystems.

Animals host on their skin, gut, oro-pharyngeal, urinary, and genital tracts a wide diversity of microbial communities that have evolved with them because of complex and mutualistic interactions, playing crucial roles in their biology and health [3]. Recently, the mammary gland, previously considered as a sterile organ, has also been included among these sites [1], although the extent and origin of microbial colonization is still under debate [4]. According to the current scientific literature, the milk microbiota composition depends, firstly, on the composition of microbial ecosystems in direct contact with the milk, the animal's teat canal and surface status, and the type of dairy equipment such as the milking machine. Secondly, it depends on various environmental microbial sources that are not directly in contact with the milk, such as bedding material, faeces, feed, drinking and washing water, stable and milking parlor air and, finally, the milker [5-6]. Several studies also support the hypothesis that the presence of bacteria in milk is not only the result of external colonization, because bacterial isolates present in the mammary gland are genotipically different from those found on skin within the same host and the same bacterial species [1]. Moreover, some authors described the ability of some microbes to move from intestinal lumen to the mammary gland throughout an entero-mammary pathway [1,7].

Most studies on the dairy ruminant milk microbiota have focused on how the microbial flora changes during food production and on its impact on milk quality, product maturation, flavor, taste, texture development and product

shelf life [8-9]. Other studies have investigated the impact of different dairy cattle diets on milk microbial communities [10], how the milk microbiota changes during mastitis or following antimicrobial treatment, and the effects on milk microbiota of different therapy conditions during the dry period [11-13]. The dry period and the early lactation period represent the most critical phases for udder health [14-15]. Indeed, during the peripartum period, dysregulations of the immune system can justify the onset of many metabolic and infectious diseases in dairy cows and could also have a role in the variability of the microbial mammary gland colonization [16]. The highest incidence of new intra-mammary infections (IMI) is usually recorded in highly productive, selected dairy breeds such as the Holstein Friesian (HF) in the first 2-3 weeks after calving [17], which, partly, accounts for the highest culling prevalence routinely observed in the first 2 months of lactation [18]. This is in contrast with the low prevalence of clinical mastitis in some autochthonous cattle breeds, including the Rendena breed (REN) [19-20]. The Rendena is an indigenous Italian dual-purpose alpine cattle breed with good aptitude to pasture and appreciable milk production (~ 5,000 kg of milk per lactation). Animals are mainly reared in Northeastern Italy, especially in low-output systems in which pasture represents the main source of feeding during the summer season. In a previous study, this autochthonous breed was suggested to have a higher resistance to disease in comparison to HF breed reared in the same conditions [20].

The aim of this study was to characterize and compare the milk microbiota of HF and REN cows reared in a single mixed-breed farm and under the same management conditions, to define bacterial group prevalence with a plausible effect on mammary gland health.

Materials and methods

Ethic statement, animals and sampling

This study complied with Italian laws on animal experimentation and ethics (Italian Health Ministry authorization n. 628/2016-PR). Sampling was carried out on 6 HF and 3 REN cows housed in a loose housing system during the dry period and, after parturition, in tie-stalls housing system in the same farm in Pavia (Italy), as previously described [20]. The lower number of sampled REN cows was dependent on their availability in the farm. All the cows were between 2 and 4 lactations, with an average of 3.6 for HF and 2.7 for REN. The cows were milked twice daily and were fed *ad libitum* with a total mixed ration without silage using alfalfa hay, straw and concentrated feed with mineral and vitamin supplementation. HF produced about 42% more milk than REN (average milk yield 5,366 kg *vs* 3,769 kg for HF and REN, respectively; p=0.0147), whereas milk fat and protein content (3.52% *vs* 3.37% and 3.02% *v*s 3.08% for HF and REN, respectively) were comparable between the two breeds. At the dry-off period, the mean somatic cell count (SCC) was 184,000 and 71,000 cell/ml for HF and REN, respectively, confirming that we selected only healthy cows. Therefore, no dry cow therapy was used, and all the cows remained healthy for all the periods of the study.

Sample collection

Quarter milk samples were collected from each animal at four different time points: dry-off (T1), 1 d after calving (T2), 7-10 d after calving (T3) and 30 d after calving (T4). Time point T2 corresponds to colostrum sampling. The first streams of foremilk were manually discarded, and approximately 10 ml of milk were collected aseptically, after teat ends cleaning, from each quarter into sterile vials. Samples were delivered to the laboratory at 4°C, immediately processed for bacteriological analysis and SCC, and frozen for metagenomics analysis.

Bacteriological analysis and SCC

For bacteriological analysis, 10 µl of milk were plated onto blood agar plates containing 5% defibrinated bovine blood, to define udder health, as previously described [20]. Plates were incubated aerobically at 37°C and evaluated after 24 and 48 hours. Bacteria were identified according to the guidelines of National Mastitis Council [21]. For each quarter, SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN, USA). Quarter samples showing SCC counts higher than 200,000 cell/ml or positive for relevant environmental pathogens were discarded. No contagious microorganisms were found.

DNA extraction, library preparation and sequencing

For each quarter, 5 ml of milk sample were centrifuged and analyzed as previously described in literature [22]. The choice of the extraction method was based on a previous research [23], considering its good sensitivity and the lack of influence by matrix-derived factors [22]. The method was shown, also, to be suitable for healthy milk samples with a low bacterial load [23] and produced good results in samples extracted from whole milk. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was stored at -20°C until use.

Bacterial DNA was amplified using the primers described in literature [24], which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µl volumes per sample. A total of 12.5 µl of Phusion High-Fidelity Master Mix 2× (ThermoFisher Scientific, Walthem, MA, USA) and 0.2 µl of each primer (100 µM) were added to 2 µl of genomic DNA (5 ng/µl). Blank controls (i.e.: no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific), as follows: samples were denatured at 98°C for 30 s, followed by 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a

final extension at 72°C for 7 min. Amplicons were cleaned-up with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (KapaBiosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2×300-base paired-end reads.

Microbiota profiling

The reads obtained were analyzed merging pairs using Pandaseq [25] and discarding low quality reads. Filtered reads were processed using the QIIME pipeline (v 1.8.0) [26], clustered into Operational Taxonomic Unit (OTUs) at 97% identity level and taxonomically assigned via RDP classifier [27] against the Greengenes database (release 13_8 http://greengenes.secondgenome.com). Alpha-diversity evaluations were performed using Chao1 and observed species metrics and rarefaction curves were employed to determine whether we had been able to capture most of the bacterial diversity. Statistical evaluation of differences in alpha-diversity was performed by a non-parametric Monte Carlo-based test, using 9999 random permutations. For beta-diversity, principal coordinates analysis (PCoA) was performed using weighted and unweighted UniFrac distances. "Adonis" function, which performs a partitioning of distance matrices among sources of variation, using a permutation test with pseudo-F ratios, of the R package "vegan" [28] was employed to determine statistical separation of the microbiota profiles.

Taxonomic classification of all the bacteria, down to the genus-level, was performed on counts of relative abundance. Species-level characterization was performed by BLAST-aligning all reads belonging to genus *Streptococcus* to a custom reference database made up collecting all available reference sequences in NIH-NCBI database (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/) within this genus and having a finishing status of "contigs", "scaffolds" or "complete genomes", for a total of 11420 strains belonging to 68 species.

Potential matches were filtered to retrieve an unequivocal classification for each read. A functional prediction of the bacterial metabolic pathways was performed using PICRUSt software (v 1.0.1) [29] and KEGG pathways database [30]. Differences in functional categories profiles between breeds were assessed using Bray-Curtis distance among samples and "adonis" permutation-based test on the experimental labels.

Raw sequencing reads were deposited in NCBI Short Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under accession number SRP120497. The full dataset (including data about the samples) has been saved as NCBI BioProject PRJNA414712.

Statistical analysis

Statistical comparisons were performed using MATLAB software (Natick, MA, USA). For evaluating differences in relative abundances of bacterial groups and functional categories, a Mann-Whitney U-test was performed, excluding a normal distribution of data at every level (Shapiro-Wilk test at 0.99 confidence). Correlation between SCC counts and relative abundances of microbial taxa was assessed through calculation of the Pearson's coefficient and of the p-values of the related linear model. Unless otherwise stated, p-values <0.05 were considered as significant.

Results

Out of 144 samples collected during the experimental period, 19 were discarded after bacteriological and SCC analyses. Only samples from healthy quarters with SCC lower than 200,000 cell/ml were analyzed to focus on physiological microbiome changes and avoid shifts in diversity due to suspected diseases. From the 125 milk quarter samples passing microbiological screening, 8 samples were, further, excluded, since their microbiota was almost exclusively constituted by only one (i.e.: *Escherichia* spp.) or few environmental microorganisms (i.e.: *Pseudomonas* and *Staphylococcus* spp.), representing more than 10% of the relative bacterial abundance (data not shown), bringing

the final number of quarter milk samples analyzed to 117, for a total of 74 and 43 samples for HF and REN, respectively (dropout rate of 22.9% and 10.5% for HF and REN, respectively). As a result, the milk microbiota structure of HF and REN cows was characterized by a total of 5,257,683 high-quality reads, with a mean of 44,937 ± 3,315 reads per milk sample at the different time points.

Comparison between breeds

The first aim of this study was to characterize and compare the general microbial profile of HF and REN healthy milk quarters (i.e.: 117 quarter milk samples selected as above). This was done by considering the samples deriving from all lactation time points and milk quarter data, separately for the two breeds.

As preliminary results, OTU rarefaction curves based on Chao1 and observed species metrics reached the plateau after about 35,000 reads, suggesting that the depth of coverage was sufficient to capture nearly the entire biological diversity within the samples. According to alpha-diversity results, the difference of biodiversity between the two breeds was statistically significant (p-value ≤ 0.01 for both metrics), showing a lower diversity in the microbial ecosystem of REN milk (Fig 1A-B).

Beta-diversity analysis, on both weighted and unweighted Unifrac distances, showed a pronounced and statistically significant (p-values < 0.001) separation among the breeds as shown in the PCoA graph (Fig 1C-D), revealing major differences in the principal constituents of the microbial community.

These data were, further, investigated in terms of relative abundance in bacterial distribution. In both HF and REN, most of the reads belonged to the phylum *Firmicutes*, typically the dominant one in dairy cow milk microbiota (Fig 2A). The mean relative abundance of *Firmicutes* in HF milk was about 66%, as opposed to 94% in REN; HF milk also contained *Proteobacteria* (~13%), *Bacteroidetes* (~8%) and *Actinobacteria* (~6%), which, on the other hand,

accounted for only about 1% each of the total relative abundance in REN milk. All these differences were highly significant ($p<0.001$).

At the family level (Fig 2B), the microbiota of the two breeds was characterized by significant differences in the average abundance of *Streptococcaceae* (HF 29.3%, REN 74.1%) and *Lactobacillaceae* (HF 6.9%, REN 14.0%). Significant differences were observed also for *Ruminococcaceae*, *Bradyrhizobiaceae, Aerococcaceae* and *Staphylococcaceae*, which were found almost exclusively in HF milk. At the genus level, both breeds were dominated by *Streptococcus*, although in very different proportions (average HF 27.5%, REN 68.6%); *Bradyrhizobium, Staphylococcus* and *Corynebacterium* were present almost only in HF milk, while *Lactobacillus* and *Pediococcus* were more present in REN milk. All these bacterial genera were diversely present in HF and REN milk (p-values < 0.05, Fig 3).

HF and REN milk samples did also show a different core OTU composition. Considering the OTUs present in 100% of samples, the two breeds shared only two genera in their core microbiota: *Lactobacillus* and *Streptococcus.* In fact, the HF core was composed, alphabetically, by the genera *Bradyrhizobium, Corynebacterium, Lactobacillus, Propionibacterium, SMB53, Staphylococcus, Streptococcus*; the REN core, on the other hand, was composed by the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus*. The species-level analysis of sequences within the *Streptococcus* genus revealed that the main species in both breeds was likely *Str. thermophilus*; a minor quantity of the environmental *Str. uberis* and *Str. dysgalactiae*, accounting for about 5-10% of the total relative abundance, was found only in a minority of HF samples (7 and 2 for *Str. uberis* and *Str. dysgalactiae*, respectively), as well as *Str. suis* in REN samples (present at about 0.6% of the total relative abundance in 20 REN samples) (S 1 Fig.).

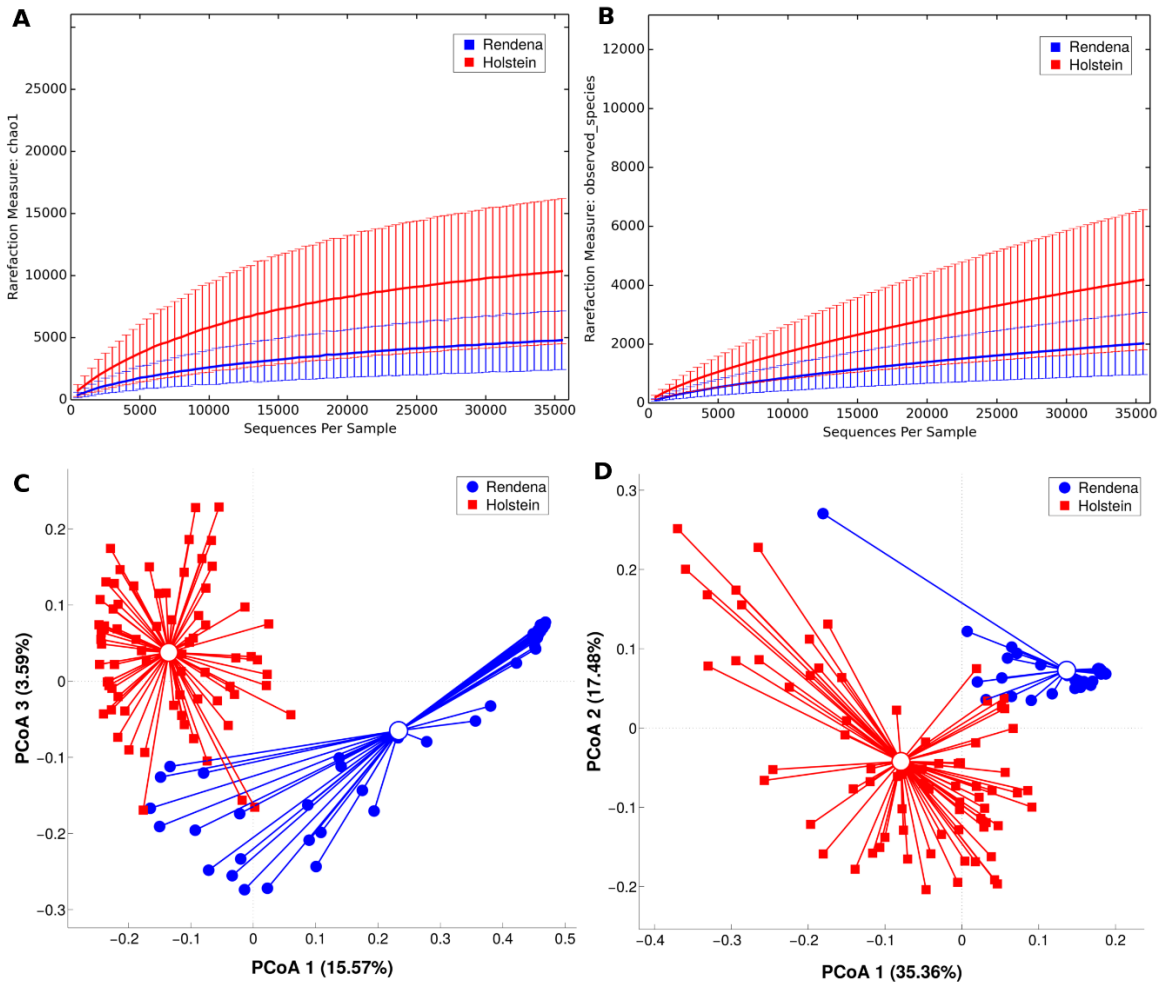

Figure 1. Alpha and beta-diversity among HF (red) and REN (blue). Rarefaction at 35,959 sequences per sample. Alpha-diversity average indexes (plus standard error bars) for phylogenetic diversity Chao1 **(A)** and observed species **(B)** are reported for HF and REN milk samples. Diversity among breeds is statistically significant in all the metrics (also with the Shannon index, not shown), p-value = 0.001. Beta-diversity analysis is represented by PCoA graphs of weighted UniFrac distance between HF and REN along the principal components **(C-D)**. Each dot represents a single quarter milk sample, while the centroids represent their average value; separation among the centroids is statistically significant (p-value < 0.001). Percent variance accounted for by the first, second and third principal component is shown along the axis.

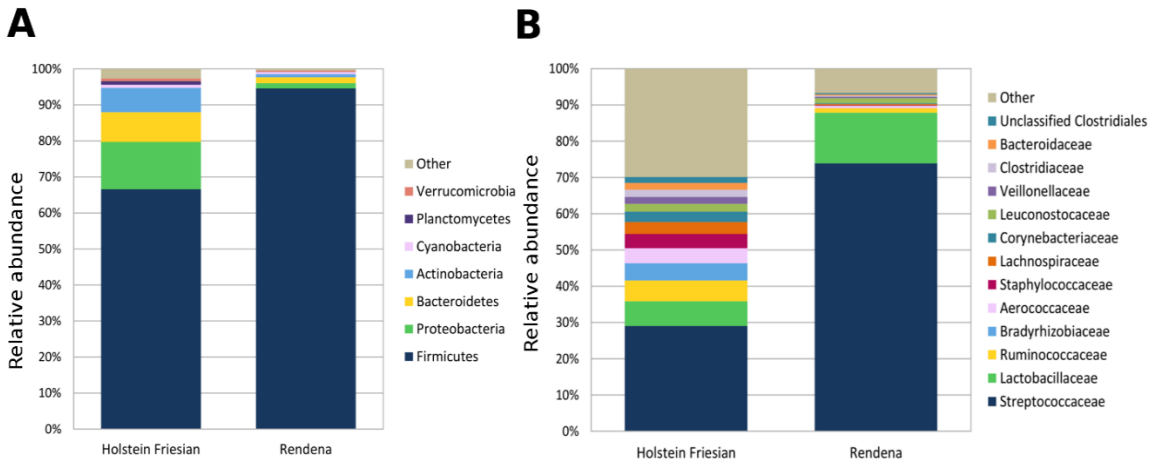

Figure 2. Distribution of the sequence relative abundances summarized at phylum (A) and family (B) levels. Relative proportions of bacterial taxonomic groups that were present in at least 1% relative abundance in quarter milk samples at a rarefaction depth of 35,959 sequences. All bacterial taxa present at less than 1% relative abundance were grouped into the "Other" classification.

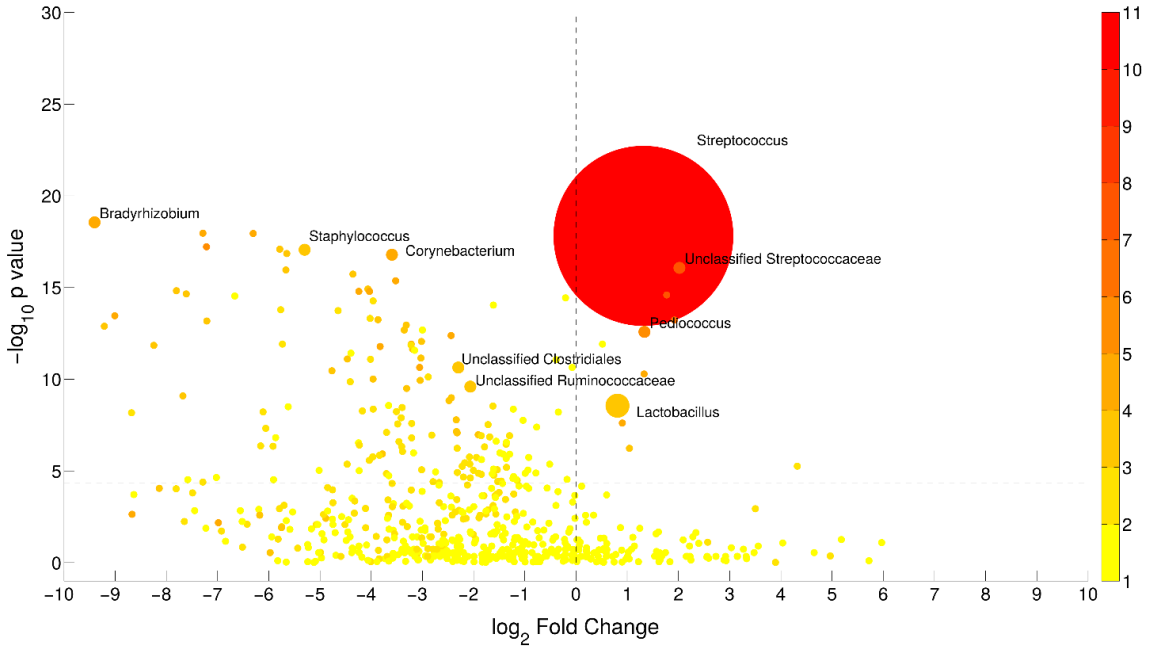

Figure 3. Bubble graph illustrating the groups significantly different between the two breeds (HF and REN) at genus level. X-axis reports the $\log_2$ ratio (REN/HF) of relative abundances; Y-axis depicts the $-\log_{10}$(p-value) of the two-sided Mann-Whitney U-test for comparing bacterial groups; bubble dimension is related to the average relative abundance of sequences; color code is according to Cohen's size effect. Bacterial groups namely indicated are the ones with relative abundance >1%, p-value <0.05 and $\log_2$(ratio)>1.5.

Comparison among time points

The second aim of this study was to assess the longitudinal changes occurring in the milk microbiota at the different time points (T1, T2, T3, T4) for both breeds. This was done by grouping the data from all quarters, separately for the two breeds.

According to our findings, the microbiota profile of the two breeds remained well separated at all time points, showing a high statistical significance ($p<0.001$) on both weighted and unweighted Unifrac distances (S2 Fig). Fig 4 reports the differences between each time point in HF milk through the PCoA distribution; apart from T2 and T3, which were not statistically different, all other time points in HF showed a significant separation ($p<0.05$) on both Unifrac distances. On the other hand, the REN milk microbiota resulted indistinctly clustered at all time points (S3 Fig). These results indicate that the microbial structure of HF milk changed profoundly throughout the calving period, while REN milk maintained a more stable microbiota composition.

We further investigated these differences by looking at the bacterial relative abundances for each time point per breed. At the genus level, the HF and REN milk microbiota composition varied in peculiar ways along time. In HF milk, an increase in *Streptococcus* (from 24.3% at T1 to 36.2% in T2), *Lactobacillus* (from 3.8% at T1 to 5.0% at T2) and *Bradyrhizobium* (from 0.8% at T1 to 4.3% at T2) was observed near the calving period, followed by a decrease at T4 back to dry-off values. In REN milk, on the other hand, *Streptococcus*, *Lactobacillus* and *Pediococcus* underwent a slight decrease right before and after calving but recovered at T4.

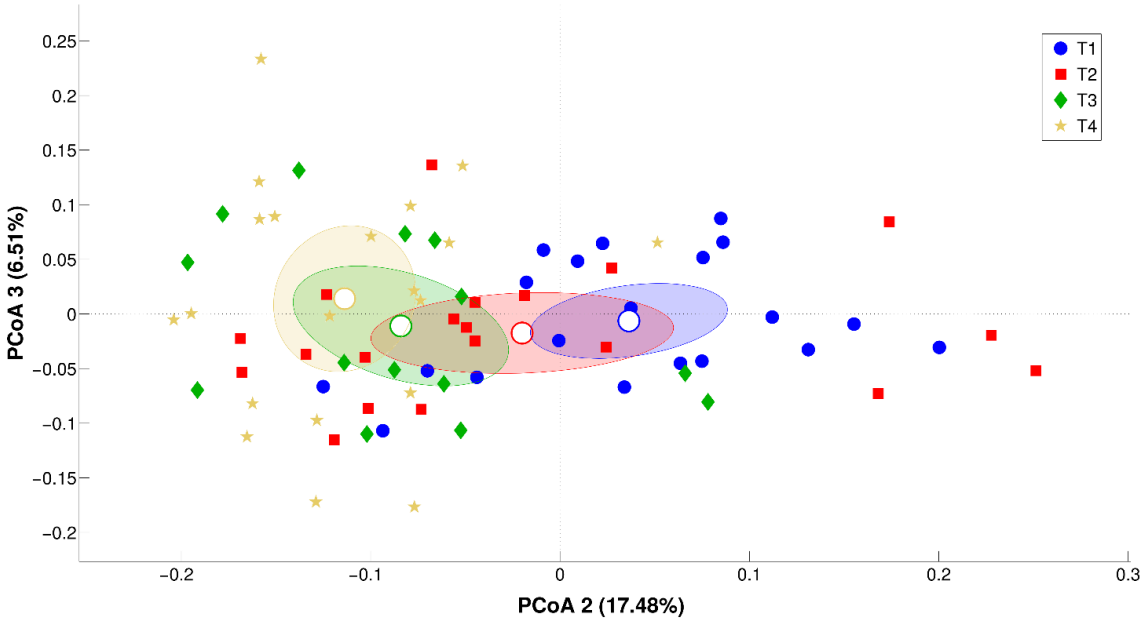

Figure 4. PCoA of weighted UniFrac distances representing the differences in milk microbiota structure among HF time points. Each dot represents a single quarter milk sample, while the centroid represents its average value. P-values are statistically significant ($p<0.02$) for all pairwise comparison, except T2 vs T3. Percent variance accounted for by the second and third principal component is shown along the axis.

Somatic Cell Count and taxonomic composition

By comparing the SCC for the selected, healthy, quarter milk samples, higher values were seen in both breeds at the calving time point T2. Table 1 reports the correlations between bacterial taxa and SCC at different phylogenetic levels (p-value of the linear model <0.01): notably, we found a weak positive correlation between SCC and many bacterial groups belonging to the *Proteobacteria* phylum, such as those within the families of *Enterobacteriaceae*, *Sphingomonadaceae*, *Xanthomonadaceae* and *Pseudomonadaceae*.

Table 1. Linear correlation coefficients between microbial relative abundances and SCC along the dataset. Only correlations with a p-value of the linear model <0.01 are reported.

		Avg. rel. ab (%)		Correlation
		REN	**HF**	**coefficient**
phylum	*Proteobacteria*	1.43	13.21	0.264
	Unclassified Bacteria	0.03	0.27	0.429
class	*Gammaproteobacteria*	0.77	3.41	0.335
	Solibacteres	0.00	0.33	0.265
	Proteobacteria (other)	0.03	0.28	0.360
	Unclassified Bacteria	0.03	0.27	0.429
order	*Enterobacteriales*	0.02	0.82	0.367
	Sphingomonadales	0.02	0.78	0.319
	Xanthomonadales	0.07	0.52	0.258
	Solibacterales	0.00	0.33	0.265
	Proteobacteria (other)	0.03	0.28	0.360
	Unclassified Bacteria	0.03	0.27	0.429
family	*Enterobacteriaceae*	0.02	0.82	0.367
	Sphingomonadaceae	0.02	0.77	0.321
	Pseudomonadaceae	0.09	0.43	0.266
	Xanthomonadaceae	0.07	0.40	0.288
	Proteobacteria (other)	0.03	0.28	0.360
	Unclassified Bacteria	0.03	0.27	0.429
genus	*Lactococcus*	1.30	0.52	0.250
	Escherichia	0.01	0.65	0.349
	Novosphingobium	0.01	0.48	0.270
	Unclassified Solibacteriales	0.00	0.32	0.278
	Proteobacteria (other)	0.03	0.28	0.360
	Unclassified Bacteria	0.03	0.27	0.429
	Pseudomonas	0.02	0.28	0.323

Predictive metabolic analysis

For each breed, 329 bacterial metabolic KEGG pathways at level 3, 41 at level 2, and 6909 KO genes were analyzed. The predictive metabolic pathways at each level showed pronounced and significant differences (p=0.001) in HF and REN milk concerning functional characterization (Fig 5A). Indeed, the two breeds had the same predicted functional composition, but in significantly different proportions: p-values were significant for the clear majority (*i.e.*: 272 out of 301, 90.4%) of the pathways, suggesting that different microbial metabolic functions might be present in the milk of the two breeds, contributing to their peculiar characterization. Metabolic pathways such as butanoate metabolism and lipopolysaccharide biosynthesis were more present in HF milk microbiota, whereas cellular pathways like purine and pyrimidine metabolism, along with DNA proteins for repair and recombination and ribosomal proteins, were more present in REN milk microbiota. The main level 3 KEGG pathways are shown in Fig 5B.

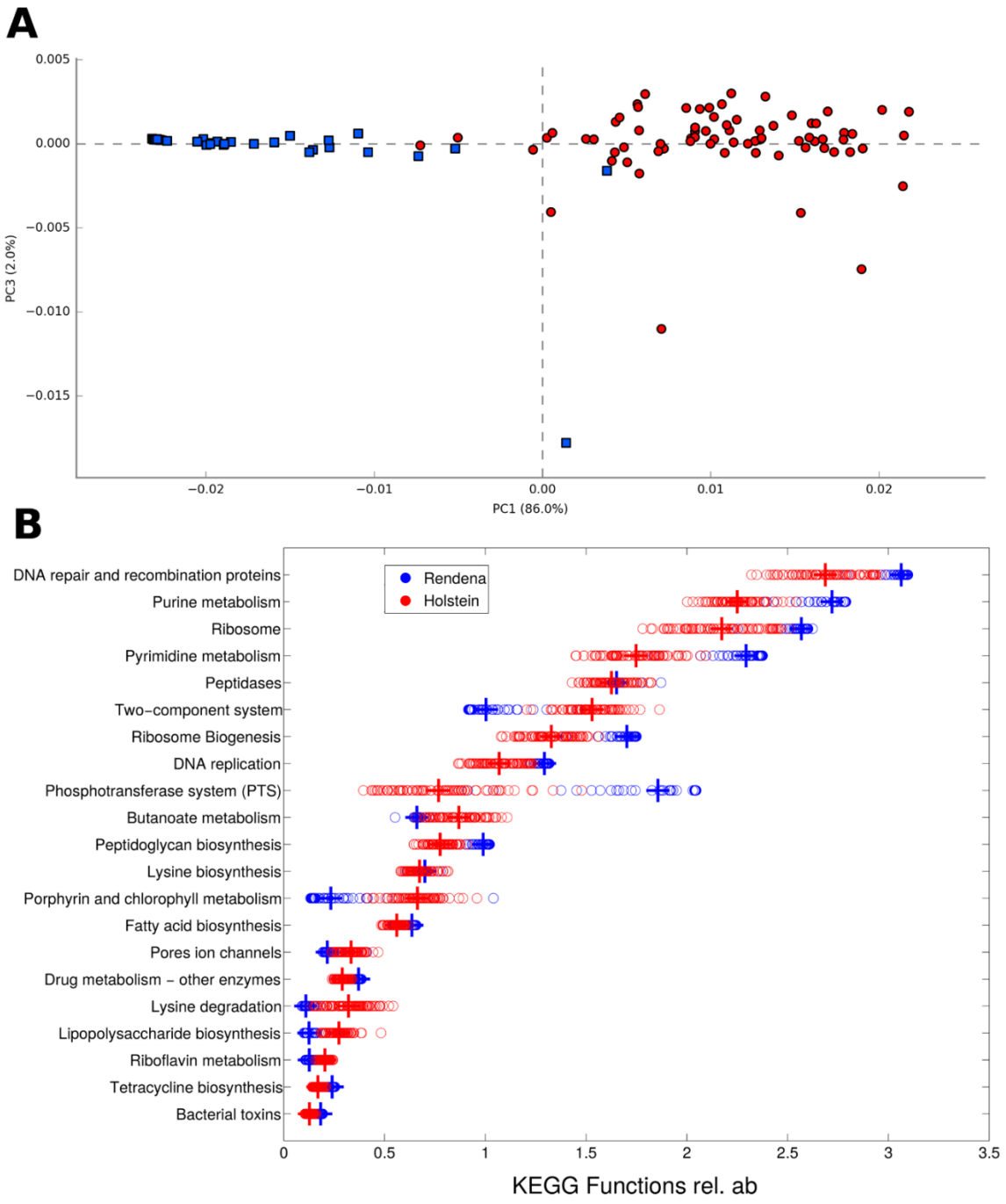

Figure 5. Functional comparison among HF and REN milk microbiota. (A) PCA of HF (red) and REN (blue) samples based on level 3 KEGG predicted pathways; the difference between breeds is highly significant (p=0.001). Each dot represents a single quarter milk sample. Percent variance accounted for by the first and third principal component is shown. **(B)** Dotplot showing the specific level 3 KEGG predicted pathways that are enriched in REN and HF milk quarter samples. Most abundant gene categories for each breed were sorted out and the ratio between their averages was calculated. Only the first 20 significantly different gene categories between cow breeds (p-value <0.05) are shown.

Discussion

The development of the so-called "-omics" technologies and the progresses in culture-independent techniques have provided evidence that milk is not sterile, but that it harbors a diverse and complex microbial community [1,31]. The selective pressure on HF cows based on production performances has led to their higher propensity to develop diseases in the transition period, including mastitis, and, perhaps, a different ability of the immune system to react against the environmental pressure [7, 16]. On the other hand, less selected breeds, such as REN, are typically characterized by a lower milk production but show a higher resistance to disease [20]. All this considered, to assess if structural differences in their microbial ecosystems might exist and if these might be related to mammary gland health, in this study we characterized the bovine mammary gland microbiota in healthy quarters of HF and REN in the transition period when cows are more prone to develop disease [20; 32]. In the study farm, all cows were kept under the same conditions, and, therefore, the influence of confounding factors such as diet, environment and animal management were minimal. This farming style, thus, created the ideal conditions for a study aimed to understand the reciprocal differences in the microbial composition of milk between the two breeds and during the transition period.

The milk microbiota has, previously, been found to vary among herds and geographical areas [33]. In our study, it was also shown to be significantly different both between breeds and during the calving period. Moreover, many more HF quarters compared to REN breed were found to be contaminated during the experimental period, highlighting both an easier destabilization of the mammary gland microbiota and a lower defensive ability in HF during the periparturient period.

In line with the results of Falentin and co-workers [34], the taxonomic profiles of both HF and REN milk were dominated by *Firmicutes*, followed by *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria*, but a significantly lower diversity was observed in the microbial profile of REN milk for all the time

points analyzed. At the genus level, only *Lactobacillus* and *Streptococcus* were shared between the two breeds, with *Streptococcus* being the most prevalent in both cases. Similar results were obtained during a study on milk samples derived from clinically healthy quarters [35].

The observed discrepancies between the two microbiota could actually bear on disease resistance in the mammary gland, in agreement with recent data about lactic acid bacteria [36]. Further investigations will be necessary to evaluate the real effect of some HF and REN bacteria on cow mammary gland diseases. In our study, the main species within the *Streptococcus* genus was *Str. thermophilus*, a lactic-acid bacterium widely used in the fermentation of dairy products (fermented milks, yogurt, different cheeses), which was present in both HF and REN milk, although in different proportions: it accounted for over 95% of the total *Streptococcus* abundance in REN, while it was less than 90% in HF. Species-level characterization, however, will need further and more precise investigations to be confirmed, considering the debate about the possibility of obtaining species level identification based on V3-V4 regions of 16S rRNA, and the known difficulties in discriminating species within certain genera [37]. Above all, the differences in the microbial profile coincided, temporally, with the beginning of lactation period, when metabolic and adaptation differences were observed between the two breeds. As previously reported [16; 20], it is worth underlining that HF showed both an increase in beta hydroxybutyrate (BOHB), responsible for immune functions depression, and more intense inflammatory phenomena. This situation can justify different responses even at a local level, as, for example, in the mammary gland [38].

The different relative abundance at which every bacterial group was present in the two breeds suggested that the proportion of genes encoding each function might be different, possibly reflecting different metabolic activities. The imputed relative abundances of KEGG pathways were used to predict bacterial metabolic functions encoded by the milk microbiota of the two breeds (as in [39-40]), showing profound and significant differences between HF and REN. As a matter of fact, it is intriguing that REN's milk major pathways seem to be more

related to cellular processes at several levels (such as DNA proteins, nucleotides, ribosomes, phosphotransferase) while HF's relate to nutrients and cofactors (such as butanoate, riboflavin and lipopolysaccharide metabolisms; porphyrin and chlorophyll metabolism, belonging to "metabolism of cofactors and vitamins" KEGG category) and to two-component signal transduction systems, which enable bacteria to sense, respond, and adapt to environment or intracellular state changes [41]. These functional differences among breeds might provide a clue for further investigations on the mammary gland health.

Based on previous findings and on the protective role of a balanced microbiota, breed-specific differences might have an influence on the resistance to infections in the mammary gland [1]. Interestingly, we found a positive correlation between increasing SCC and the relative abundance of bacterial opportunists, such as those belonging to the *Proteobacteria* phylum, which were found in higher amounts in HF milk microbiota, consistently with the higher incidence of mastitis and other transition-associated diseases in this breed [20]. In agreement with this, it is also worth noting that milk microbial profiles changed significantly along the transition period only in HF, while REN maintained a more stable microbiota composition.

Conclusions

In this study, the implementation of high-throughput technologies for milk analysis provided detailed insights into the milk microbial population of a cosmopolitan breed, HF, and of a local cattle breed, REN, along the transition period. Our results highlighted the existence of differences in terms of general microbial diversity, taxonomy, and predicted functional profiles: in addition to the influence on the final characteristics of dairy products obtained from milk of the two breeds, those differences might also have an impact on their mammary gland health for what concerns disease and pathogen resistance. Interestingly, these differences seem related with inflammo-metabolic changes occurred around calving, which suggest a possible relation among these responses and the mechanisms of resistance in the mammary gland. Further

studies carried out on a larger number of animals from both breeds will contribute to reinforce our findings in terms of inter-breed differences, as well as in terms of discrimination at and below the genus level.

Acknowledgments

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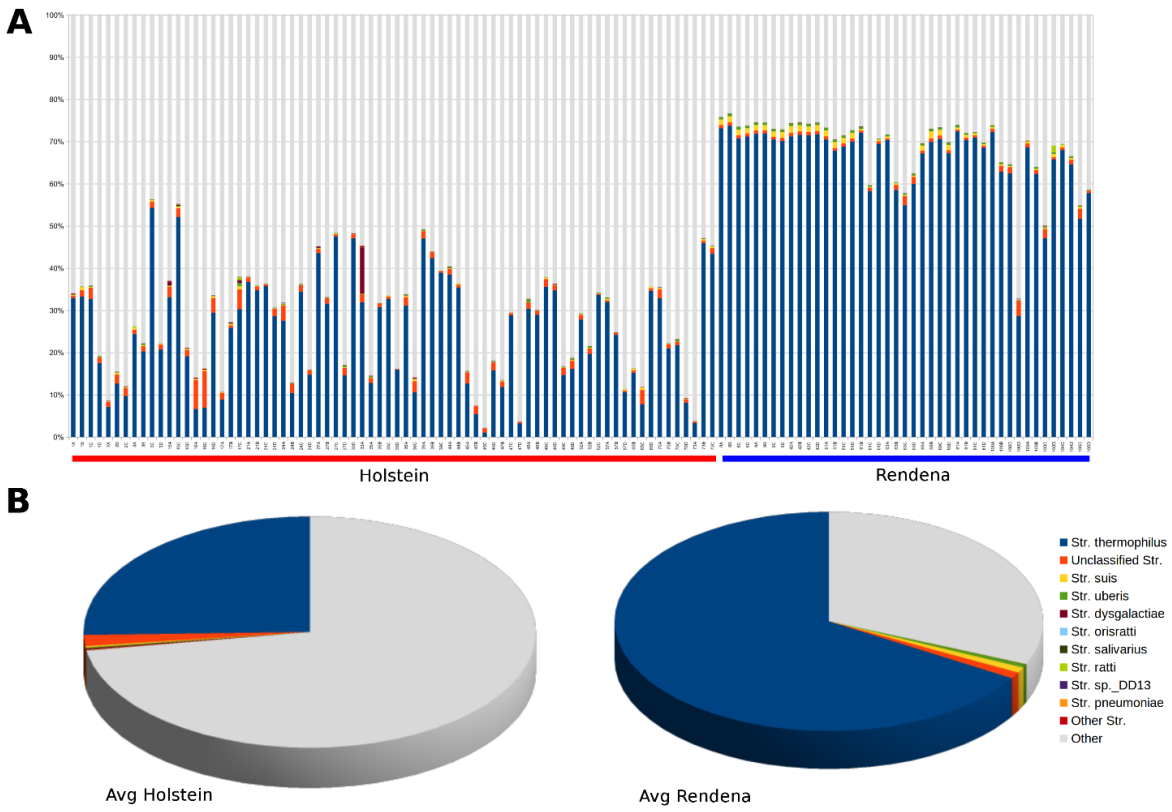
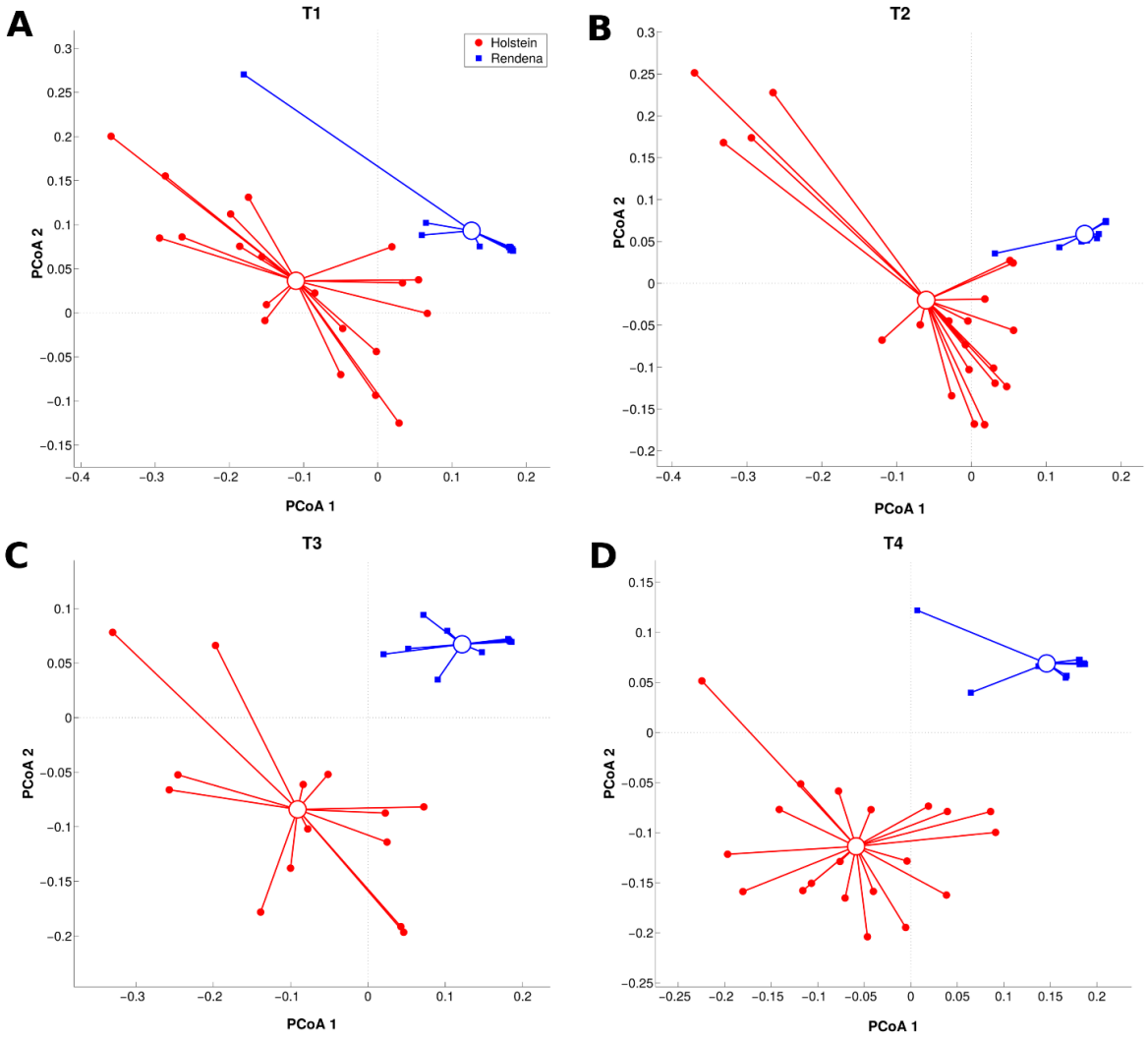
Supplementary materials

Figure S1.



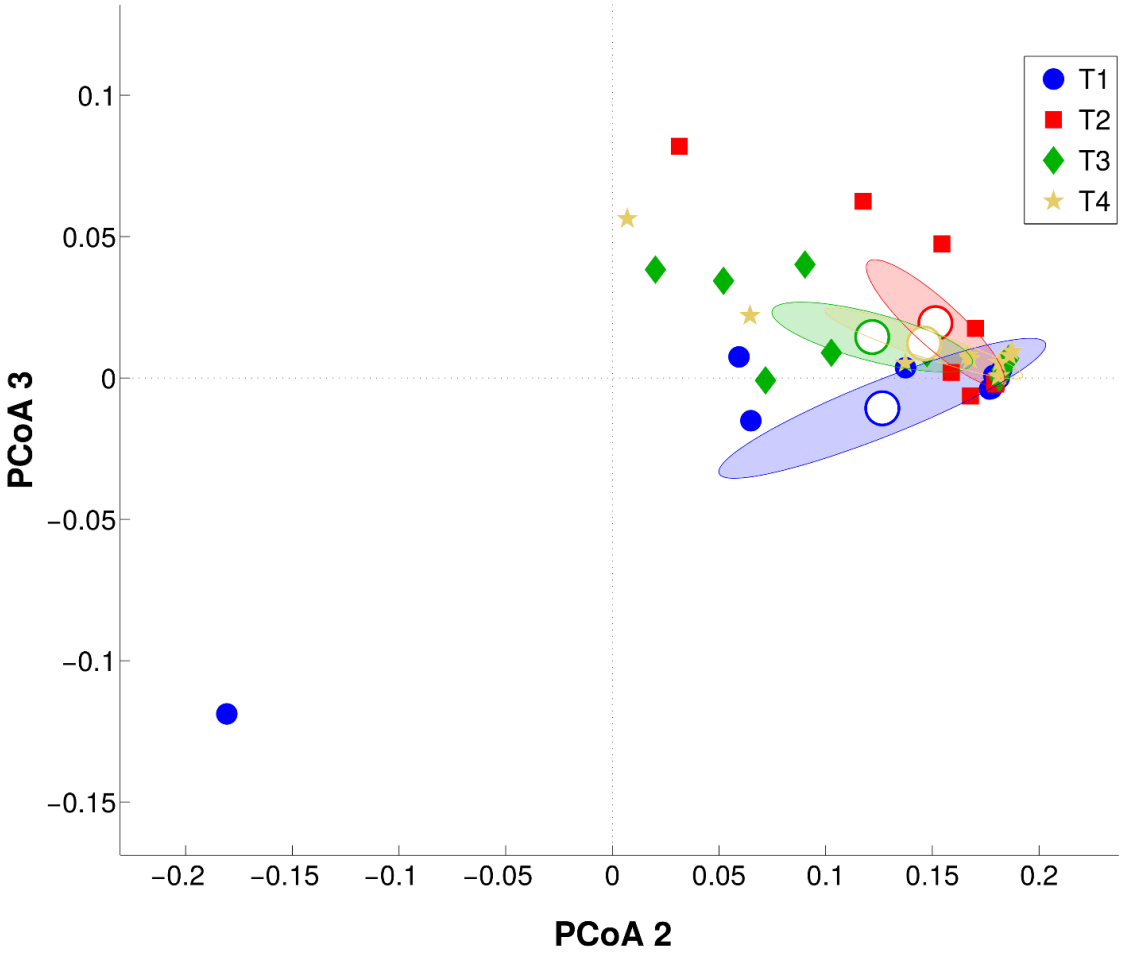
Species-level analysis of *Streptococcus*. The relative abundances of genus *Streptococcus* is shown for each quarter milk sample in the stacked bar plot **(A)** and in the pie charts **(B)**. The "Other" category (gray) represents all of the genera that do not belong to the genus *Streptococcus*; blue category show how *Str. thermophilus* is the main species among the *Streptococcus* genus.

Figure S2.



PCoA of weighted UniFrac distances represent the differences in milk microbiota structure between each time point for HF and REN. Average distance is statistically significant (p = 0.01) for T1 **(A)**, T2 **(B)**, T3 **(C)**, T4 **(D)** time points.

Figure S3.



PCoA of weighted UniFrac distances represent the differences in milk microbiota structure between REN time points. Each dot represents a single quarter milk sample, while the centroids represent their average value. P-values not statistically significant.
tatistically significant (p>0.05).

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Chapter 6

Ethological Biodiversity

6.1
Cattle Personality biodiversity in autochthonous Italian breeds: a pilot survey

The data shown in this chapter refer to the already published paper:
PASTORINO GQ, FAUSTINI M, VITALI F, MAZZOLA SM, CURONE G. WHAT ARE WE LOSING? ARE THE PERSONALITY TRAITS OF ITALIAN AUTOCHTHONOUS COWS DIFFERENT FROM THOSE OF COSMOPOLITAN BREEDS? JOURNAL OF ADVANCED VETERINARY AND ANIMAL RESEARCH. 2018; 5(3):315-323

Introduction

The 1960s saw a Livestock Management "revolution" in the meat and dairy industries; in about 20 years, the increase of meat consumption in developing countries was almost triple the increase in developed countries, and milk consumption saw more than double the increase that occurred in developed countries (Steinfeld, 2004). The increase in meat and milk consumption was fueled by urbanization, population growth, and income growth (Delgado et al., 2001). Between 1970 and 2006, the number of U.S. and Canadian dairy farms decreased by approximately 88% (Steinfeld, 2004) but remaining farms have considerably increased the size of their operations, in order to meet growing global demand for animal products (Robbins et al., 2016). Production systems became intensive, with larger farms housing animals in increasingly confined spaces (Fraser, 2008). In many regions of the world, these management changes threatened the existence of several autochthonous breeds, reducing the livestock biodiversity: The autochthonous cows were abandoned in favor of more productive cosmopolitan breeds.

At present, the numerical consistency of the autochthonous breeds considered in our study, bred solely in Italy, is exiguous. The most endangered breeds are Modenese and Varzese, with a total number of females raised in Italy are of 678 and 513, respectively (Associazione Italiana Allevatori, 2016). Even the distribution of animals on farms is quite characteristic: for Modenese and Rendena, the mean is around 23 and 35 females/farm, respectively, the mean female presence drops to about 7.8 heads/farm in Varzese breed (Figure 1A). The 2014–2016 annual trend for the number of farms rearing the three local breed considered is reported in Figure 1B. For the most endangered breeds (Modenese and Varzese), the trend is slight but positive, with 45 to 48 farms in the 2014–2016 range for Modenese and 41 to 49 farms for Varzese, in the same time span. Therefore, the presence of these cows on the territory is "atomized", and far to be considered an intensive type of breeding, with farmers in strict contact with their animals. The mono-aptitude selective criterion had also a negative impact on many aspects, affecting the reproductive performance and quality of products

(Schennink et al., 2007). When we compare the less selected and lower producing dairy breeds to Holstein Friesian dairy cows, it emerges that selective pressure to increase milk production has led to a higher propensity to disease, including mastitis (Curone et al., 2016). The negative side of the high production levels in dairy farms is the increase in culling rates, the reduction of life expectancy, the increased occurrence of diseases, and consequently, the greater use of veterinary drugs (Communod et al., 2010; Petrera et al., 2014; Curone et al., 2018). According to the technical report of the Italian Breeders Association, the average milk yield of Italian Holstein Friesian cows amounted to 9,884 kg in 2016, with average contents of 3.27% and 3.71% for fat and protein, respectively (Table 1). The impact of these performances on animal welfare and health has been considerable (Varotto et al., 2015). The genetic ability to increase milk production has been associated with a higher risk of metabolic and infectious diseases, as well as with reduced fertility; in North-Eastern USA, cows alive at 48 months of age decreased from 80% in 1957 to 13% in 2002 and the mean calving interval went from 13 to
15.5 months (Oltenacu and Broom, 2010). As a result, cattle were subjected to dramatic physiological and behavioral changes in their social and physical environments (Petrera et al., 2014); animals respond to such stimuli in species-specific ways, as well as individually. Modifications of normal behaviors are indicative of a substantial decline in cow welfare. Improving welfare is important, as good welfare is regarded by the public as indicative of sustainable systems and good product quality and may also be economically beneficial (Oltenacu and Broom, 2010).

Animal personality

Research into animal personality, defined as "individual differences in behavior that are thought to be stable across time and situations" (Freeman and Gosling, 2010) has grown over the last decade as its relevance to animal health and welfare has become more apparent (Gartner, 2013). In particular, personality has sometimes been used for aspects of captive management, including

decreasing stress, increasing positive health outcomes, successful breeding, and infant survival.

In studying personality traits in animal species, many ethologists have found five main domains similar to the human five-factor model (FFM) of personality (Gosling and John, 1999). The five domains in the FFM are commonly referred to as neuroticism (featuring anxiety, depression, a vulnerability to stress, and moodiness), agreeableness (featuring trust, cooperation, and a lack of aggression), extraversion (featuring sociableness, assertiveness, activity, and general positive emotions), openness (featuring intellect, imagination, creativity, and curiosity), and conscientiousness (featuring deliberation, self-discipline, dutifulness, and order) (Gosling and John, 1999).

Within cattle, there is no stated consensus as to how many personality traits may exist. The research tends to focus on those traits which have a clear relationship with welfare, such as fearfulness and sociableness, which may be related to the FFM domains of neuroticism, agreeableness, and extraversion. Van Reenen (2012) showed that within a herd of cows there is variation in the behaviors displayed by individuals and not all cows will display the same aspects of the species' behavioral repertoire to the same degree. These differences can often be seen during certain group activities: Cows must compete at a feeding area but not all cows will show the same levels of aggression to other cows (Gibbons et al., 2009). Similarly, cattle have the capacity to show fear in response to unexpected stimuli (Forkman et al., 2007) but the levels of fear displayed by individuals towards the same stimuli will vary, but this variation is consistent within the individual (Gibbons et al., 2009). Based on these considerations, MacKay et al. (2013) speaks of "aggression" and "fearfulness" as being personality traits in cattle. One of the main methods used to examine individual differences in animals is rating against a set of criteria (Highfill et al., 2010), whereby an animal's behavioral tendencies are scored against a set of traits or descriptors (Razal et al., 2016), by the people who know the individual animal best.

Previously, few studies on dog behavior and temperament have used questionnaire survey methods, using dog owners as the singer rater and primary source of information (Mirkó et al., 2012; Gartner, 2015). Using a questionnaire approach to collecting behavioral data on dogs is based on the assumption that the owner (or primary caregiver) of a dog usually knows more about its typical behavior than anyone else (Serpell and Hsu, 2001). Similarly, in the present study, given the very small size of the farm enrolled, the milkers had a very close relationship with the cows and therefore, their evaluation can be compared to that of the owner or primary caregiver of a pet. We used a personality questionnaire modified from Chadwick (2015) for this survey. Although it was primarily used in studies surveying felids, it has been shown to be a valid tool applicable to other taxa (Pastorino et al., 2017a). In the present study, we wanted to evaluate the expression of personality traits in cows of different breeds, managed with the same routine. The purpose of this work was to investigate how personality traits vary in a sample of cosmopolitan and endangered autochthonous dairy cows and if it is possible to identify breed-specific personality trends. In order to verify this hypothesis, we had to provide a preliminary evaluation of the personality dimensions obtained by our questionnaire and to investigate if personality trait assessment in cattle matches the FFM of personality.

Materials And Methods

Dairy farms:

The animals selected for the study were part of three different dairy farms where the management and feeding conditions were similar. All farms were very small and family-owned and conducted and adopted the tie-stall housing system during the lactation and a free loose system for the dry period (Broucek et al., 2017).

The first farm was located on a mountain (1,050 m a.s.l.), where the cows were fed with hay from permanent meadows (meadow established for over 200 years,

consisting of wild-grown herbaceous plants, without the use of chemical fertilizers and weed control). The herd was composed of 20 Varzese cows. The second farm was located in the lowland, where the cows were fed with hay from semi-permanent meadows, with a low energy and protein integration, supplemented with cornmeal and soybean meal. This farm had a mixed herd composed of 35 animals belonging to Holstein Friesian, Brown Swiss, and Italian Red Pied breeds. The third farm was also located in the lowland. Until the 1980s, this was a typical Lombardy farm with 100 Holstein Friesian in milking but in the 1990s the owner decided to focus on Italian endangered native breeds. He started by buying two senior (15 years) cows of Varzese breed, the unique autochthonous breed of Lombardy, and today, almost 30 years on, his farm is one of the greatest examples of farm animal biodiversity; the herd was composed of about 200 animals and included 15 of the 16 Italian cattle native breeds. The owner provided the traditional forage feed for his cows with minimal concentrates.

Animals

For this survey, a total of 40 female cows were employed. The subjects belonged to five breeds, whose brief description is summarized in Table 1. The cows were housed in three different farms, distributed as described in Table 1. On these subjects, 26 personality variables pertaining to the five-factor personality dimensions were determined. The composition of the five domains, in terms of variables, is reported in Suppl. Table 1.

Personality questionnaire

Milkers in the three dairy farms were asked to complete a personality questionnaire for each cow enrolled in the study. The questionnaire consisted of two parts. The first part intended to evaluate the experience of milkers with bovines, including how long each milker had worked with the cows and how often he/she had contact with them. Due to the very small size of the farms, only one

milker was present in each stable. As a consequence, each milker had a close relationship with every single cow.

In the second part of the questionnaire, the cows were scored against 26 descriptors, each focusing a different aspect of the animal's personality. For each descriptor, the milkers were asked to assign a score on a scale of 1 (descriptor was never exhibited) to 12 (descriptor was always exhibited) (Pastorino et al., 2017b, c). The close relationship between milkers and cows meant that questions like "friendly to you", "aggressive to you," and "fearful of you" were deleted from the questionnaire while the small size of the farms and the tie-stall housing system made the scoring of the descriptor "solitary" unreliable, rounding down the descriptors to a total of 26. As is the case for many questionnaire-based dog personality studies with owner-reported responses, inter- rater reliability was not measured because a single milker scored the animals (Wiener and Haskell, 2016).

For the analysis, the descriptors were further grouped into personality domains, using a similar approach of some previous studies that examined personality in bottlenose dolphins, brown bears, and sloth bears (Highfill and Kuczaj, 2007; Pastorino et al., 2017a). This approach utilized each of the five-factor personality dimensions: Extraversion, agreeableness, conscientious- ness, neuroticism, and openness to experience. Conscien- tiousness was removed, as it was found as a relevant trait only in primates (Gosling, 2008). The five-factor dimensions were chosen to provide a framework of possible personality characteristics and to encourage cross-species comparison (Highfill et al., 2010).

Statistical analysis

The considered variables were analyzed through descriptive statistics; for each variable, the minimum value, the maximum value, the range, the first, the third quartile, the median value, the mean, and the standard deviation were calculated. In order to evaluate differences between breeds, every breed was

analyzed for the aforementioned variables; the differences between breeds were calculated using the Kruskal-Wallis nonparametric test, calculating the differences between median values. The statistical significance was set at $P<0.05$. A multivariate multiple factor analysis (MFA) was applied to the variables. Each variable was assigned to the five-factor personality dimensions of competence (FFM). All statistical analyses were performed with the software XLstat for Windows platform. The statistical significance was set to $P<0.05$.

Results And Discussion

The statistical analyses for the four breeds and the results for the Kruskal-Wallis test are reported in the Suppl. Table 2. Eight variables out of 26 differ between breeds in median values. The interquartile interval and minimum-maximum interval for personality traits that have shown statistically significant differences between the breeds is reported in Figure 2.

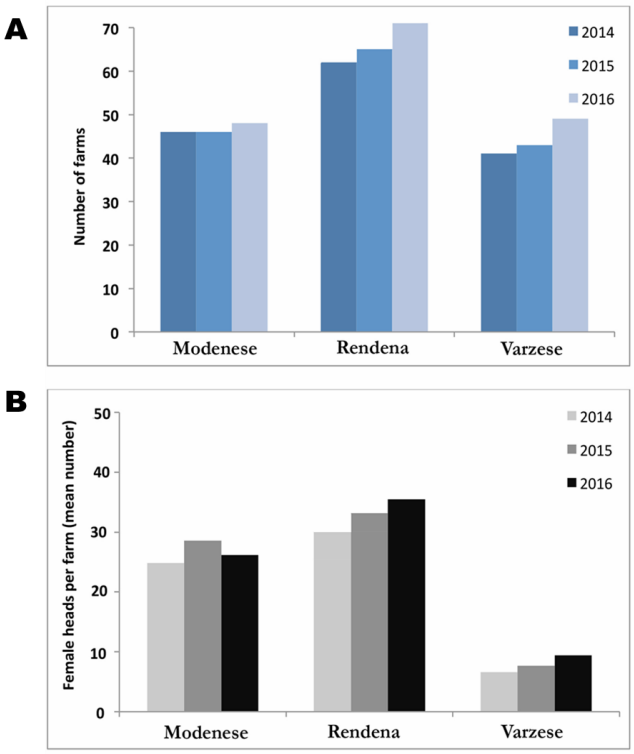

Figure 1. (A) Mean number of heads per farm (in Italy) rearing the local bovine breeds included in the study. While for *Modenese* and *Rendena,* the mean is around 23 and 35 females/farm,

respectively, the mean female presence drops to about 7.8 heads/farm in *Varzese* breed. (**B)** the 2014–2016 annual trend for the number of farms (in Italy) rearing the three local breeds considered. For all three autochthonous breeds considered, the trend is slightly positive with 45 to 48 farms in the 2014–2016 range for *Modenese*, 60 to 70 farms for *Rendena,* and 41 to 49 farms for *Varzese*, in the same time span.

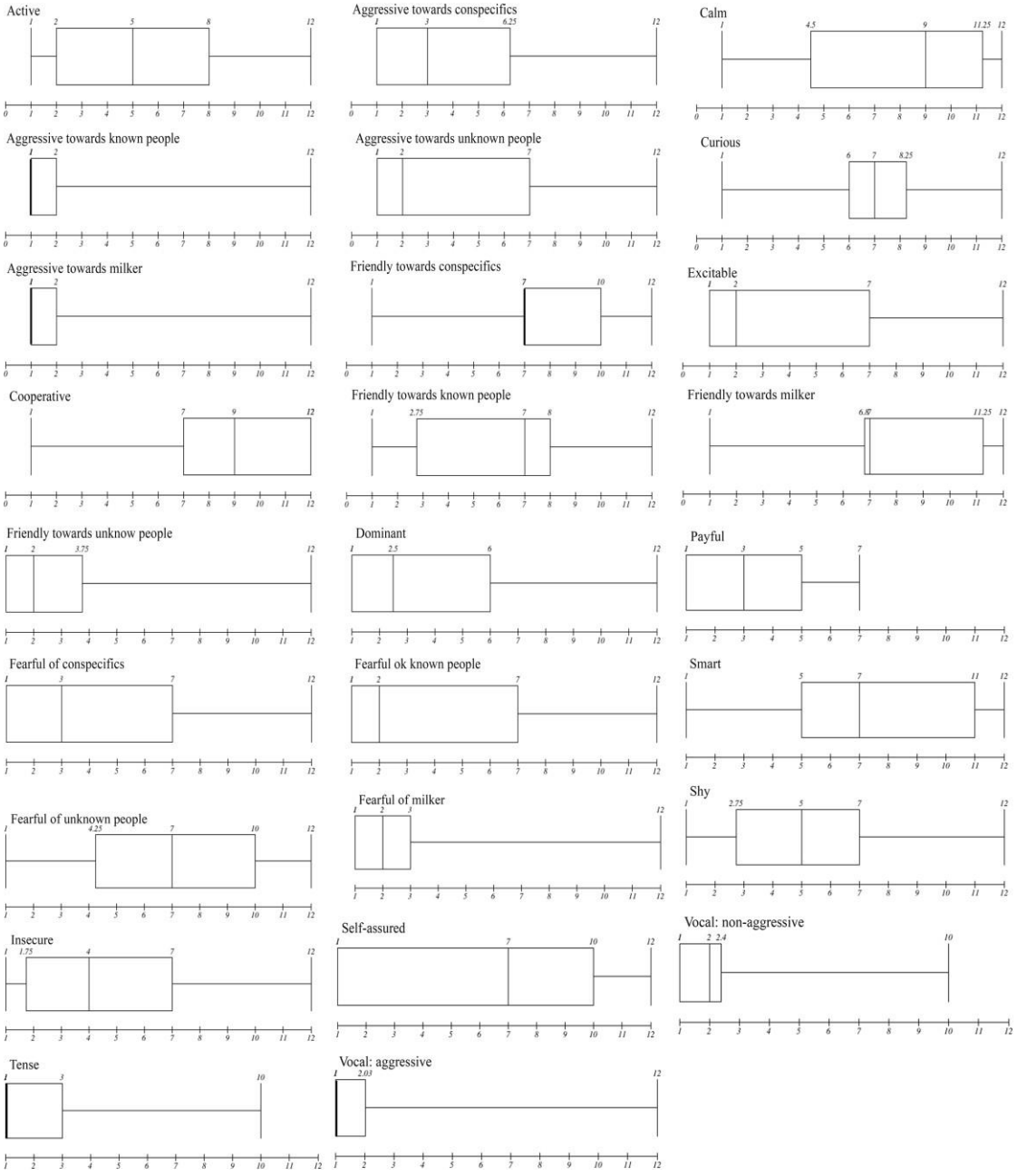

Figure 2. Box and whiskers plots for each personality trait analyzed. The box represents the interquartile interval and the whiskers the minimum–maximum interval.

Besides, the multivariate MFA of data was applied: It is a particular kind of principal component analysis, that involves several groups of variables instead of a single group (Escofier and Pages, 2008). In our case, two groups of variables were analyzed: The measured variables and the breed. The MFA gave, on the basis of the first five multivariate dimensions, the results reported in Suppl. Table 3. The table reports for each dimension (Dim.X) correlations of the quantitative and qualitative variables (e.g., the breed). For each multivariate dimension, quantitative variables can be negatively or positively correlated. The same is true for the breed. Henceforth, if we take into consideration the first dimension (Dim. 1), i.e., the dimension with the stronger variability, two breeds strongly distinguish themselves from the other breeds.

Table 1. Animals involved in the study: Number of subjects, distribution on farms, and main breed characteristics

Cattle breeds	Cows Enrolled	Farm	Heigh (cm)	Weight (kg)	Attitude	Mean Milk production	Days open (days)	Services per pregnancy (Average)
Modenese	8	#3	125–140	650	Milk, work, and meat	Kg/lactation: 4,792 (3.44% fat, 3.69% protein)	98	2
Rendena	6	#3	130	550	Milk, work, and meat	Kg/lactation: 4,596 (3.12% fat, 3.28% protein)	161	1.6
Varzese	5 4	#1 #3	135	450	Milk, work, and meat	Kg/lactation: 3,228 (3.35% fat, 3.54% protein)	99	1.4
Holstein Friesian	4 5	#2 #3	130–150	550–750	Milk	Kg/lactation: 9,884 (3.27% fat, 3.71% protein)	173	3
Brown Swiss	4 4	# 2 #3	135	550	Milk	Kg/lactation: 6,945 (3.55% fat, 4% protein; high% of k-casein, beta-casein, and beta-lacto-globulin)	182	2.4

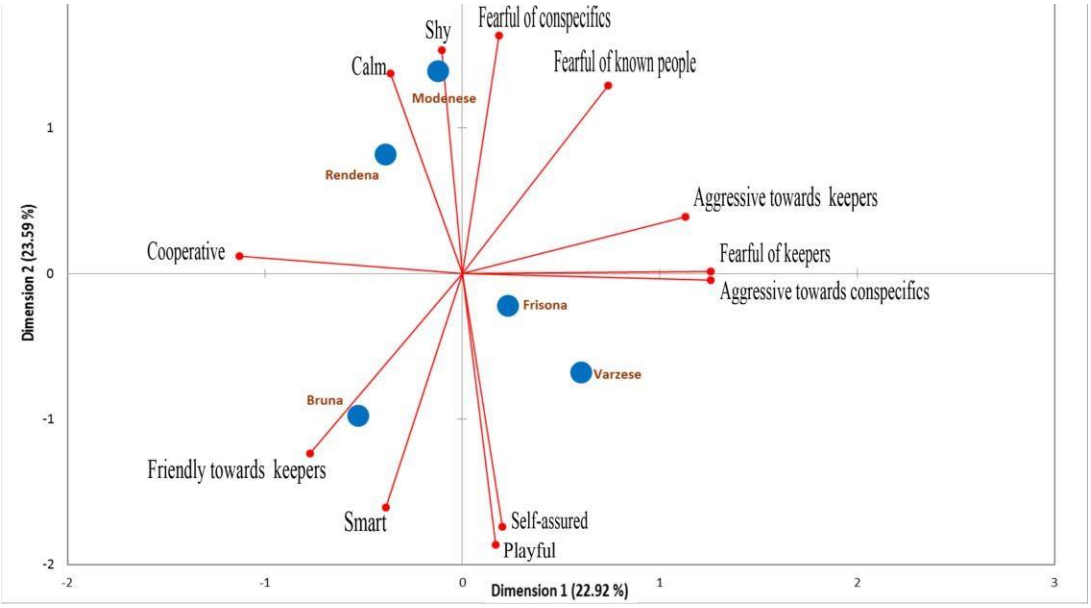

Figure 3. Proximity of the autochtonous breeds versus the cosmopolitan Holstein Friesian. Multiple factor analysis: First two dimensions. As shown in the figure, *Modenese* clusters as the shyest, *Rendena* is calmer then cooperative, *Brown Swiss* clearly friendly towards keepers (milkers) and smart while *Holstein Friesian* appears less defined compared to other breeds. *Varzese* stands in an area between self-assured and playful and aggressive towards conspecifics and fearful of keepers (milkers), mildly showing these traits.

The Rendena breed was positively correlated with insecurity, encompassing the four fearful traits and the four aggressive traits. Rendena cows also distinguish themselves through the active, excitable, and shy components. Conversely, Brown Swiss cows were positively correlated with the three friendly traits, the cooperative trait and playful trait from Dim.1, showing an opposite trend to the Rendena (Suppl. Table 3). The second dimension showed less variability; Varzese cows were characterized by curiosity, playfulness, and aggression towards conspecifics (Suppl. Table 3). Modenese featured traits for calmness, cooperation, and friendliness to unknown persons (Suppl. Table 3). The third dimension of MFA was positively related to the activity (Modenese) and negatively to shyness (Rendena). The Holstein Friesian breed showed the weakest correlations with the first five MFA dimensions (Suppl. Table 3) confirming itself as the less characterized breed.

The proximity of the autochtonous breeds versus Holstein Friesian breed to dimensions 1 and 2 is shown in Figure 3. The graph evidences the proximity of the autochtonous breeds versus the Holstein Friesian in relation to the dimensions measured. Applying the NEO Five-Factor Inventory (NEO-FFI), an inventory that was developed to measure five major dimensions of personality (Ashton, 2013), we propose the addition of dominance as the fifth domain instead of consciousness, as proposed for lions (Gartner, 2015). Rendena scored highly in extroversion, both positively and negatively, and scored positively in neuroticism and dominance. Brown Swiss scored positively in agreeableness, extroversion, and openness but negatively in neuroticism. Varzese positively correlates with extroversion, openness, and mainly positive with neuroticism. Modenese cows relate positively in agreeableness and negatively in neuroticism.

Discussion

In this study, an adapted adjective-based personality questionnaire was applied to measure personality traits in different breeds of cows, at three Italian small dairy farms. Given the close individual relationship with the cows, the farms' milking staff provided responses for the questionnaire; this is similar to what is described in dog personality studies, in which the questionnaires are answered by the owners of the pet dogs (Wiener and Haskell, 2016). Many years of research have proven that data gathered by means of questionnaires can be accurate, reliable, and consistent in evaluating individual animals for various behavioral traits (e.g., Gosling, 2008; Hudson et al., 2015; Lush and Ijichi, 2018). However, to date, little has been published about personality in cows or cow breeds. In human personality research, the so-called FFM has been found to be one of the most useful organizing structures attempting to depict some aspects of personality (Mirkó et al., 2012). In a review, Gosling and John (1999) applied this model to characterize animal personality, where they compared personality structures in 12 species including dogs. Gosling (2008) further examined whether canine personality dimensions represent analogs of the human FFM factors.

The analysis of breed-specific personality profiles could be revealing because it is not clear how genetic and environmental factors may have contributed to behavioral variations among breeds (Mirkó et al., 2012), though many authors agree to consider differences in animal personality to be the result of adaptive evolutionary processes (Dall et al., 2004; Wolf, 2007; Réale et al., 2010). As shown in dogs, the differences in an individual's behavior within a breed may exceed variations among breeds. Therefore, an individual-based analysis might be useful in order to unveil whether a given behavioral trait differentiates not only an individual cow but also a greater number of cows belonging to a particular breed (Mirkó et al., 2012).

The data suggest that the Holstein Friesian was the least reactive of the breeds studied. This could be related to the fact that this breed has been selected for intensive husbandry regimes in dairy farms and these personality traits allow the cows to better adapt to more intensive management systems. The Rendena was the shyest breed, but also the most dominant and the most aggressive towards unknown people. The Varzese breed was more "curious" than other breeds of this study We suggest that these traits may be connected to the attitude and to the traditional husbandry regimes these breeds have been selected for. Most of these traditional breeds had a triple purpose: Dairy, beef, and work. They were selected to work in small farms, in small numbers, interacting with few humans for different tasks. Today, they survive in reduced numbers, on small-scale local farms, that have changed little of the classic husbandry regimes; this is vastly different compared to the intensive farming that the Holstein Friesians were subject to.

A major limitation of this study is the small number of animals considered, which is itself limited by the poor consistency of the population of autochthonous breeds. Another limitation of the study is the impossibility of measuring inter-rater reliability since only one milker was available on each farm.

We had two main aims. The first aim was to provide a preliminary evaluation of the personality dimensions obtained, in cows, by our questionnaire.

Secondly, we wanted to compare some Italian autochthonous breeds with the cosmopolitan one. Our choice fell upon the comparison of the Varzese, the Modenese, the Rendena, the Brown Swiss, and the Holstein Friesian because the genetic selection at the base of the typical attitudes has induced different characteristics, and as a consequence, detectable differences in personality can be expected between the populations.

Conclusion

The data presented in this study suggests that the evaluations obtained through the analysis of the questionnaires are able to highlight the presence of differences in the personality traits of the different bovine breeds, while certain personality traits are shared in each of the five dairy breeds considered. We hope that this pilot study will lead to further research on cattle personality, widening the sample of screened individuals and breed types, in order to preserve rare breeds personality characteristics along with their genetic, morphological, and productive distinctiveness.

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Chapter 7

General discussion

7.1 General discussion

In the last decades, the issue of the loss of biodiversity has become increasingly important, as it is pointed out by the huge efforts, in order to reduce this process, put in place by the main sovra-national agencies, in particular Food and Agriculture Organization, United Nations General Assembly and European Union. In this context, the preservation and rational use of autochthonous cattle breeds plays a key role in the maintenance of the biodiversity.

Recently, especially in the agricultural and livestock sector, biodiversity is no longer seen only as a resource that must be saved but also as an opportunity to face the future challenges, which are well identified by the report "World Agriculture: towards 2030/2050" of FAO. In this report, it is expected that world population will reach nine billion people, mainly due to an increase of the birthrate in the developing countries. As a result, the 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 different allocation 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, designating the cereals and vegetable proteins availability to human consumption. Furthermore, it should not be overlooked that climate changes will continue to advance, modifying the environments in which plants grow, increasing the marginal areas not suitable for growing cereals. This fact will put farmers and breeders facing new challenges. In the end, it should not be forgotten the huge problem of the antibiotics resistant pathogens, which will lead to a limitation of the antimicrobial molecules consumption, especially in the livestock.

Within this background, the characteristics which are attributed to the Italian autochthonous cattle breeds, such as rusticity, frugality, good food conversion into milk and meat of elevated quality, good fertility and good resistance and resilience against the diseases, make these animals a possible excellent choice for facing future challenges of the livestock sector.

Little is known about the physiological mechanisms underlying these characteristics, therefore we tried to shed some light on some of these using the comparative evaluation method, studying animals belonging to different breeds (cosmopolitan and autochthonous) reared in the same farm or similar conditions. A considerable limitation of this work was the small number of animals considered, which is itself limited by the poor consistency of the population of autochthonous breeds.

To understand the possible physiological mechanisms behind the rusticity, good fertility and disease resistance, we decided to focus our efforts on the study of the transition period in two farms with mixed herds, one farm with a herd composed by Holstein and Modenese, a second one with Holstein and Rendena. The transition period is defined as the time frame from 3 weeks prior to calving till 3 weeks after calving and is critically important to health, production, and profitability of dairy cows, because in this period is localized the highest incidence of diseases and reproductive problems. The results achieved by comparative evaluations in both cases showed, especially during the post-partum period, differences in the metabolic pathways in the high producer breed Holstein when compared with autochthonous breeds. The higher milk production of the Holstein requires a high energy demand, and this causes in these animals a more severe mobilization of the body reserves, especially from the adipose tissue (higher levels of NEFA and ketone bodies). It is well known and confirmed also in our results, that higher levels of lipomobilization are linked with greater systemic and local (in the mammary gland and uterus) inflammatory condition and alteration of the activity of the immune system. The lower metabolic stress of autochthonous cows, as well as the lower inflammation and oxidative stress, could be some of the phenomena underlying their better disease resistance and reproductive efficiency.

In order to expand our "picture" on the physiology of autochthonous breeds, we decided to move our attention on one of the different products of these breeds, the milk. Milk is the most complete food in nature; it's the phenotypical result of several correlated physiological processes, or rather, reproduction, pregnancy,

calving, metabolism and of course the functionality of the mammary gland. Moreover, we must not forget the contribution of the genetic component.

We started our analyses using the classic approach of the veterinary physiology, studying the milk components. The results achieved by comparative evaluations of Varzese, Cabannina, Valdostana and Holstein breeds show how different breeds in similar conditions can produce completely different "milks". A number of differences between breeds have been found, in particular local breeds showed an higher percentages of UFA, MUFA, PUFA, and a higher UFA/SFA ratio, as well as lower desaturase indices (related to C14, C16 and C18) and atherogenic index, when compared to Holstein. These preliminary results give us first informations about how a different genetic background can influence the functionality of the mammary cells and can start to explain why different milks have different technological properties.

In order to have a better and new characterization of the properties and components of the different milks, we decided to move our analyses one step forward, applying the omics techniques, in particular metabolomics and metagenomics.

Appling the metabolomics techniques, we have been able to distinguish, inside the same farm, the milks derived from the Holstein and autochthonous breeds. Holstein milk showed different levels of some metabolites, especially higher levels of choline and acetate and lower levels of citrate and carnitine. Milk with these characteristics are associated with a greater rapidity of coagulation and bigger casein clots. The absence of these conditions can help to explain the different coagulation properties and the smaller dimension of the casein clot typical of the milk of the Italian autochthonous cows.

The application of the metagenomics techniques on the milk gave us the opportunity to study the physiology of the "forgotten organ", the microbiota. We found significant differences in the general composition of the milk microbiota during the transition period of two breeds, Rendena and Holstein, despite they belong to the same farm and have in common the same management, environment and nutrition. In particular, the microbiota biodiversity of Rendena

milk resulted clearly lower than the one of Holstein milk. Furthermore, the Holstein milk showed significant changes in the microbial composition along the transition period, while Rendena one maintained a more stable microbiota. These findings can influence the final characteristics of dairy products obtained from milk of the two breeds and those differences might also have an impact on the mammary gland health, regarding the disease and pathogen resistance.

Of course, the results here presented about the different milks are just a starting point, other analysis, like a complete proteins profile, study of the dimension and surface composition of the milk fat globules, minerals concentration and structural study of the casein clot, are required to obtain a better characterization of the milks biodiversity.

The last physiological field on which we decided to be focused on has been the ethological one, in order to understand how much different genetic backgrounds and breeding techniques-tradition have influenced the personality and the adaptation to interaction with humans of the different breeds. We did a comparison of two cosmopolitan breeds (Holstein Friesian and Brown Swiss) and three endangered Italian autochthonous breeds (Varzese, Rendena, and Modenese). We found the presence of differences in the personality traits of the different bovine breeds, in particular, the Modenese resulted as the shyest, Rendena is calmer then cooperative, Brown Swiss clearly friendly towards keepers (milkers) and smart while Holstein Friesian appears less defined compared to other breeds. Varzese stands in an area between self-assured and playful and aggressive towards conspecifics and fearful of keepers (milkers), mildly showing these traits.

In conclusion, with this PhD thesis we tried to characterize and define, using a multidisciplinary approach, the some physiological mechanisms at the base of the interesting trades of some Italian autochthonous cattle breeds. Our results can contribute to the re-evaluation of bovine local breeds and their products, remembering that these animals could have a fundamental role to help the agricultural-livestock sector to face the future challenges.

7.2 Future perspectives

This PhD thesis represents a preliminary study that needs further investigations in order to better understand the physiology of Italian autochthonous cattle breeds and their possible future role in the Italian agro-economy. Based on the results presented in this thesis some possible further developments are:

- Better evaluation of the physiological regulatory mechanisms during the transition period, increasing the number of animals and breeds. In particular, it is important to put more efforts on the study of how the different genetic backgrounds of the breeds have influenced the regulation of the energy balance (lipids/proteins mobilization and insulin levels) during the first part of the lactation.
- Deeper characterization of the colostrum production and composition in the different breeds, with particular attention on the different molecules and hormones related with the growth and immunity development of the calves.
- Studying the reproductive physiology of the different breeds, based on the evaluation of the reproductive hormones (progesterone, estrogens and gonadotropins) using different substrates (blood, urine, feces and hairs). Besides, it is necessary a better monitoring of the reproductive performances, using the most common indices (insemination rate (IR), conception rate (CR), pregnancy rate (PR), and culling rate (CR)).
- Better evaluation of the technological characteristics of the different "milks", studying the 3D structure /dimension of the milk clots and the titratable acidity and pH of the "milks".
- Extending the study of the microbiome population on different breeds and not only in the milk but also on other body districts.
- Investigating the economic value of the agriculture based on the use of native breeds, in order to describe the economic benefits and disadvantages to the use of these animals.