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METAGENOMIC APPROACH TO RUMEN, INTESTINE AND MILK IN RELATION TO THE ENVIRONMENT AND QUALITY OF PRODUCTS: THE SUMMER ALPINE TRANSHUMANCE AS CASE STUDY

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“You’ll never do a whole lot unless you are brave enough to try”

Dolly Parton

CONTENTS

ABSTRACT

Transhumance is an ancient practice of pastoralism that consists of the seasonal migration of herds and shepherds in the Mediterranean and the Alps, usually in the mountainous regions there is a vertical transhumance of livestock as there is a change in altitude. It begins with the ascent to high altitude, between late May and mid-June, and ends with the moving back to the valley floor or plains in mid-September.

This herding practice shapes the relationships between people, animals, and ecosystems; it has played a key role in proper landscape management, biodiversity conservation, soil protection, and maintenance of traditions. Transhumant herders have an in-depth knowledge of the environment, ecological balance and climate change, as well as the types of handicrafts and food production that result from them; in fact, it is one of the most efficient farming methods, while coping with lower average temperature, shorter growing season, greater soil slopes, lower soil fertility and the need for longer working time. By itself, mountain animal husbandry is defined as multifunctional and multidisciplinary, it is able in addition to integrating the environmental aspect also the economic aspect that reflects on tourism and social culture of the place, still ensuring the maintenance and vitality of the mountain.

The analysis of the inter-relationships among environment, pastures, animals, and food obtained is fundamental for improving our knowledge on this complex mountain farming system. Metagenomic is a recent approach, with an increasing interest, used to study the complexity of microbial populations in different sectors. Metagenomics is combined with culture-dependent methods for providing a better characterization and understanding of the microbial communities in a lot of biological samples. In dairy cows, metagenomics has been used in several studies on the microbiota of rumen, and intestinal content, and of milk and cheese. Often these studies are sectorial, analysing only one of the matrices and without identifying the relationships between the matrix studied and the others or the quality traits of food products.

Therefore, the present dissertation as a general objective aims to study the metagenomics of rumen and intestinal contents on one side and metagenomics and quality of milk produced on the other side in relation to transhumance to highland pasture (and return to lowlands) as a case study. Specifically, the first two contributions (Juribello project) aim to study in detail the ruminal microbiota, the first, and the milk microbiota, the second sampled contemporarily on the same cows. In these two groups of cows from the same lowland farm were compared: a group that was moved to highland summer pastures and again to lowland, and a second (control) group that remains in indoor housing for the entire duration of the trial (June to October with monthly samplings). In the first contribution, the complex relationships among the end products of rumen fermentation, the predicted methane production, the microbiological count and the rumen metagenomics traits were investigated. While in the second contribution, the possible relationships between milk microbiota and milk quality and technological properties were studied. Special focus was reserved to bacterial taxa related to specific activities, such as cheese-making, health maintenance, milk spoilage and pathogenesis.

The third and fourth contributions (Vezzena Project), on the other hand, concerns the comparison of milk from 26 cows from 4 herds during summer highland pasture and then later during indoor housing in valley floor farms. In the third contribution, individual milk metagenomics was associated with milk composition and quality, udder health, milk B vitamin content and microbiological counts. Finally, the fourth and last contribution aimed at evaluating the relationship between intestinal metagenomics and milk quality and metagenomics on the same herds and cows during the summer Alpine transhumance, and after returning to the lowland permanent valley farm.

In all the 4 contributions, the microbiological, chemical and technological traits analyzed were studied one at a time, but also all together with a multivariate approach. Firstly, the heat-maps of the correlations among the analysed traits were obtained and discussed. But the large number of traits considered in each of the 4 contributions suggested the need of identifying few independent latent explanatory factors responsible of the complex relationships among the many traits analysed. Overall, the results of the thesis offer interesting point of view on the evolution of the microbiome of dairy cattle in

mountain areas, starting from the practice of summer transhumance, the different changes of pastures, animals' physiology and behaviour and getting to the final products such as milk and cheese, passing through the microbial evolutions of rumen an intestinal contents. The use of this innovative approach that compares livestock data with microbiology has highlighted even more the various possible connections and interactions between the surrounding farming environment, animals and consequently the final products. The various future perspectives merit in-depth analysis and interpretations of new possible interconnections between the microorganisms (bacteria) and the other animal compartment (rumen content, intestinal content and milk) considered within this thesis and microorganisms (yeasts) and products (cheese) not included here (but under analysis).

RIASSUNTO

La transumanza è un'antica pratica del pastoralismo che consiste nella migrazione stagionale di greggi e pastori, mandrie e mandriani nel Mediterraneo e nelle Alpi. Nelle regioni montane si effettua una transumanza verticale del bestiame, in quanto si ha un cambiamento di quota. Inizia con la salita in quota per la monticazione, tra la fine di maggio e metà di giugno, e termina con la demonticazione, cioè la ridiscesa nel fondovalle o pianura verso la metà di settembre. Questa pratica di allevamento modella le relazioni tra persone, animali ed ecosistemi; ha avuto un ruolo fondamentale per la corretta gestione del paesaggio, la conservazione della biodiversità, la protezione del suolo, il mantenimento delle tradizioni. I pastori transumanti hanno una conoscenza approfondita dell'ambiente, dell'equilibrio ecologico e dei cambiamenti climatici, oltre alle tipologie di artigianato e alle produzioni alimentari che ne derivano, si tratta infatti di uno dei metodi di allevamento più sostenibili ed efficienti, facendo comunque fronte alla temperatura media più bassa, stagione di crescita più breve, maggiori pendenze del suolo, minore fertilità del suolo e la necessità di un orario di lavoro più lungo. Di per sé la zootecnia montana si definisce multifunzionale e multidisciplinare, è in grado in integrare oltre l'aspetto ambientale anche quello economico che si riflette su turismo e cultura sociale del luogo, assicurando ancora il mantenimento e vitalità alla montagna.

L'analisi delle interrelazioni fra ambiente, pascoli, animali e alimenti ottenuti è fondamentale per migliorare le conoscenze su questo complesso sistema zootecnico montano. La metagenomica è un recente approccio con un interesse sempre più crescente, viene usata per studiare la complessità della composizione microbica in settori differenti. La metagenomica combinata con i metodi di coltura-dipendenti fornisce una migliore caratterizzazione e comprensione delle comunità microbiche in diversi campioni biologici. Nel caso dell'allevamento delle vacche da latte la metagenomica è stata impiegata in numerosi studi sul microbiota del contenuto ruminale o intestinale, del latte e del formaggio. Spesso questi studi sono settoriali, analizzando solo una delle matrici prima ricordate e senza approfondire le relazioni fra la matrice studiata e altre matrici e/o la qualità degli alimenti prodotti.

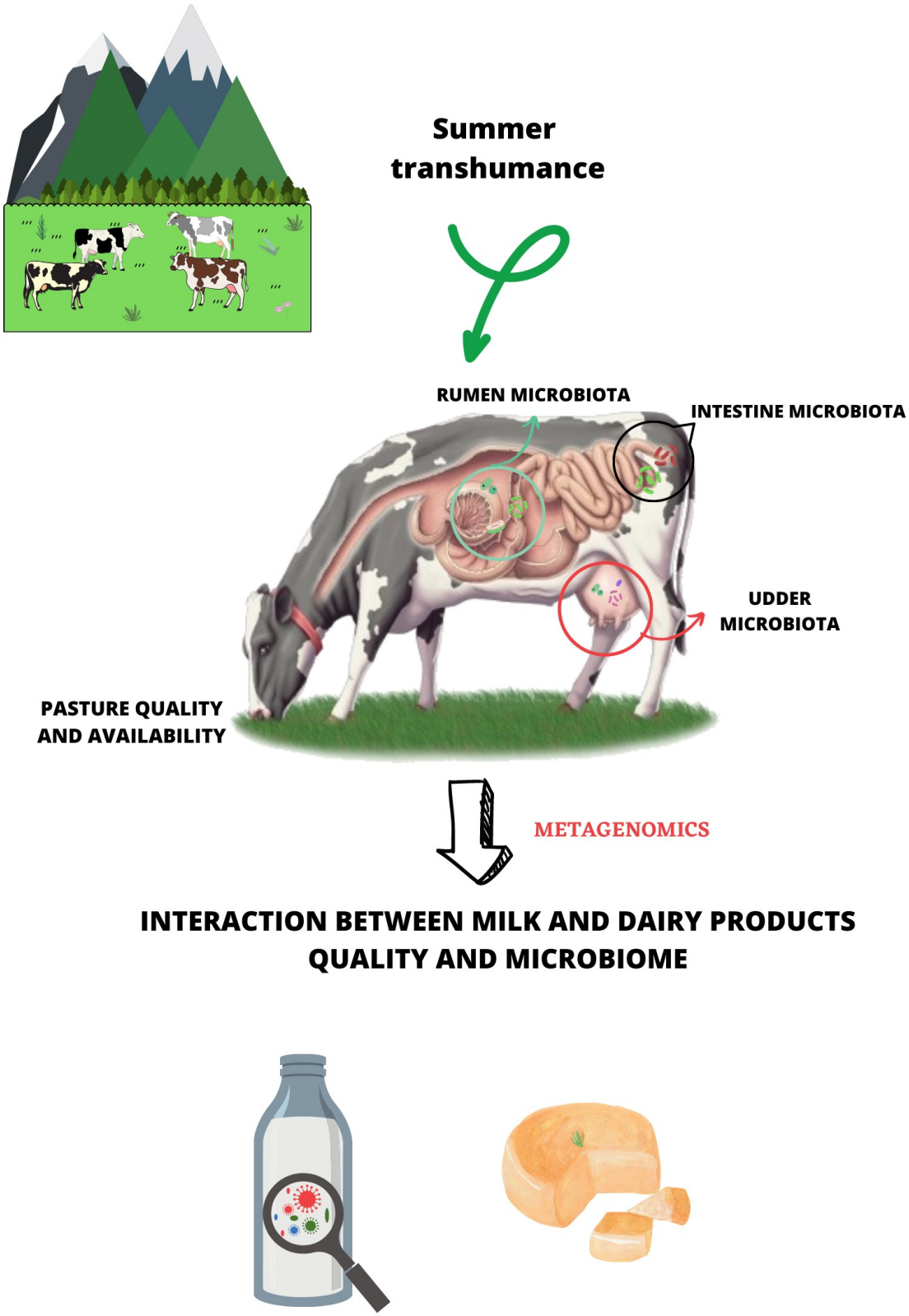
Pertanto, la presente tesi di dottorato come obiettivo generale mira a studiare la metagenomica del contenuto ruminale e intestinale da un lato e del della metagenomica e caratteristiche qualitative del latte dall'altro, in relazione alla monticazione e demonticazione come caso studio. Nello specifico i primi due contributi (Progetto Juribello) hanno come obiettivo lo studio dettagliato del microbiota ruminale, il primo, e il microbiota del latte, il secondo, da campioni prelevati contemporaneamente sulle stesse bovine. In questi due studi si mettono a confronto due gruppi di vacche della stessa stalla: uno che attua la transumanza estiva in pascoli di alta quota, e l'altro (come gruppo di controllo) che rimane in stabulazione nell'azienda di fondovalle per tutta la durata della prova (da giugno a ottobre con campionamenti mensili). Nel primo contributo si sono andate a ricercare le possibili relazioni tra microbiota ruminale con VFA ruminali e produzione di metano. Il secondo contributo è volto invece allo studio in dettaglio del microbiota del latte con le sue possibili relazioni con la qualità del latte e le proprietà tecnologiche, con particolare focus ai taxa batterici legati a varie attività specifiche, come caseificazione, mantenimento della salute, deterioramento del latte e patogenesi, oltre sempre a tenere in considerazione anche gli effetti della transumanza estiva.

Il terzo e il quarto contributo invece (Progetto Vezzena) riguardano il confronto del latte di 26 vacche provenienti da 4 aziende durante la transumanza estiva e poi successivamente durante la stabulazione in stalla di fondovalle. Nel terzo contributo si è andati a studiare in dettaglio la metagenomica individuale del latte associata alla composizione e qualità del latte, salute della mammella, contenuto di vitamine B nel latte e conte microbiologiche. Infine, il quarto e ultimo contributo riguarda le complesse relazioni tra la metagenomica intestinale e la qualità e metagenomica del latte durante la transumanza estiva e dopo il ritorno all'allevamento di fondovalle in pianura.

In tutti i 4 contributi, le variabili microbiologiche, chimiche e tecnologiche analizzate sono state studiate una ad una, ma anche tutte assieme con approcci multivariati. Prima di tutto sono state ottenute e discusse le heat-maps delle correlazioni fra tutte le variabili. Ma il grande numero di variabili analizzate in ciascuno dei 4 contributi ha suggerito la necessità di identificare pochi fattori latenti indipendenti in grado di spiegare la maggior parte delle complesse interrelazioni fra le variabili analizzate.

Complessivamente i risultati della tesi offrono un interessante punto di vista sul microbioma dei bovini da latte allevati nelle zone montane, a partire dalla pratica della transumanza estiva, dai diversi cambiamenti dei pascoli, la fisiologia e il comportamento degli animali fino ai prodotti finali come latte e formaggio. L'utilizzo di questo approccio innovativo che mette a confronto i dati zootecnici con la microbiologia ha evidenziato ancor di più le varie possibili connessioni e interazioni tra l'ambiente di allevamento circostante (pascolo di alta quota, o allevamento di fondo valle), gli animali e di conseguenza i prodotti lattiero-caseari che ne derivano. Le varie prospettive future meritano un'analisi approfondita sulle nuove interconnessioni tra i microorganismi (batteri) e i comparti (rumine, intestino, latte) riportati in questa tesi e gli altri microrganismi (lieviti) e comparti (formaggio) non considerati in questa tesi, ma previsti nel progetto di ricerca e in corso di studio.

GRAPHICAL ABSTRACT



GENERAL INTRODUCTION

The summer transhumance practice and its role in mountain areas

In the past, in mountain regions of many European countries, livestock farming and human activities were of great importance for the rural economy, maintaining the cultural heritage, the preservation and protection of the landscape, biodiversity, natural habits and conservation of local traditional dairy products (Mack et al., 2013; Sturaro et al., 2013a). The ancient human use of mountain slopes for hay meadows has contributed greatly to the colourful appearance of the European Alps in early summer. Transhumance is an age-old practice of pastoralism to cope with ecological variability and take advantage of the seasonal availability of pasture and water resources (Brottem et al., 2014); nowadays, it still plays a crucial role in the dairy farming system, landscape, and benefits ecosystem conservation, although a drastic reduction has been observed in recent decades (Leroy et al., 2018).

The word transhumance derives from the Latin words *trans*, which means "across", and *humus*, which means "soil" or "land". In fact, this practice is based on the vertical movement of livestock from winter lowland permanent farms to summer alpine pastures where temporary highland farms are located. Highland temporary farms are the constructions used seasonally as shelter for animals during the transhumance summer period and are also known as *Malga* in North Italy, *Alm* in Germany, Swiss and Austria, and *Alpage* in France. They are usually smaller than the modern barns located in the permanent farms in the valleys and lowlands, since topographical and climatic constraints have limited the opportunities for intensification. Usually, these temporary farms are also equipped with milking-room and dairy facilities, including a small ripening room, for the production and the early ripening of farmhouse cheese, providing an appreciable added value, nutritional and renowned quality, to products and systems that have to deal with difficult conditions and low productivity.

Farming in mountain areas has several limitations and difficulties to face. The local climate, the limited growing season for culture, steep slopes, and less fertile soils lead to the need for additional efforts and complex machinery, resulting in lower productivity and much longer times compared to an intensive

farming system. During the summer grazing season, mountain farmers moved the animals and hay making from one pasture to another, or meadow to meadow, in order to follow the natural renewal of grasses spreading upwards during spring and summer, and to make full advantage of the available territory by chasing the vegetation gradient. Due to the great distances and changes in altitude, mountain communities commonly occupied a succession of settlement sites, moving from one exploitation area to another.

Currently, the rapid changes experienced by agrarian practises (intensification in the valley, mechanization and abandonment of mountain areas), led in the last decades to the progressive abandonment of farming in marginal areas, and consequently, also the highland pastures (from 600 to 2.500 m a.s.l), resulting in the reappearance of trees on pastures that had been used for centuries (MacDonald et al., 2000; Strijker, 2005) causing alteration in the composition, structure and function of mountain ecosystems.

The shift to intensive livestock systems has affected the livestock sector, creating several alarms about environmental and dairy sustainability in mountainous areas, including the abandonment of pastures and alpine farms. For many years, the reduction of grazing in highland pastures has been associated with soil degradation, reforestation, loss of biodiversity and loss of aesthetic quality of the landscape (Streifeneder et al., 2007; Ramanzin et al., 2009; Sturaro et al., 2009). For these reasons, nowadays mountain farming is strongly subsidized for the preservation of landscapes, accessibility of tourists and recreational environments, control of woods advance, maintenance of local traditions, and last but not least the conservation of local flora and fauna biodiversity (Kianicka et al., 2010; Battaglini et al., 2014; Fuerst-Waltl et al., 2019). Moreover, traditional products with the long-standing historical culture of these communities and the new opportunities for tourists can be strengthening the local economy. Transhumant farmers have an in-depth knowledge of the environment, ecological balance and climate change as it is one of the most sustainable and efficient farming methods, as well as possessing excellent skills in handicrafts, food productions (MacDonald et al., 2000). Every farming practice is also shaped by the environment, topography and climate, and this seasonal practice is a particularly complex agricultural and social phenomenon in which people and animals exploit, through movement, the seasonality of different

environments. Maintaining transhumance helps contribute to a sense of local identity in an ever-globalizing world.

Several studies have been carried out, focused on the investigation of positive effects of mountain transhumance: the benefit on the biodiversity of grasslands in particular when compared with of abandonment or intensity of grazing (Parolo et al., 2011), the positive mitigating effect on the environmental impact of farming (Penati et al., 2011; Guerci et al., 2014), the positive effects on animal health and welfare (Mattiello et al., 2005; Corazzin et al., 2010; Comin et al., 2011), and the positive influence on the bacterial sensorial and nutraceutical properties of milk (Martin et al., 2005; Gorlier et al., 2012; Carafa et al.,2020).

The metagenomic approach

Metagenomics is a recent addition to the molecular toolbox and is the simplest and most unbiased way to test the dynamic of microbial populations. It is very powerful approach to study the complexity of environmental microbiota, and some of the main ways in which this method can be applied are: comprehensive analysis of microbial diversity, functional analysis of microbial communities, discovery of new microorganisms and genes, identification of microbial interactions and monitoring of environmental changes.

Microbial studies, including microbiology and microbial ecology, are highly influenced by culture-dependent and culture-independent approaches. Briefly, culture-dependent methods involve the isolation and characterization of microorganisms based on their ability to grow on specific culture media under controlled laboratory conditions, but this have some limitations due to their dependence on laboratory conditions, and additionally not all microorganisms can be cultured in the laboratory. While the culture-independent methods, such as Next-Generation Sequencing (NGS), are complementary to culture-dependent methods; they provide a more comprehensive view on the microbial community, including the uncuclturable microorganisms and their functional potential. However, these methods also have limitations, such as the inability to determine the viability of microorganisms, the possibility of

contamination, and the lack of information about the specific growth requirements of individual microorganisms. A combination of both culture-dependent and culture-independent technologies may provide a better characterization and a more complete understanding of the microbial communities, than that obtained when using each approach independently.

This analysis encompasses a wide range of biological ecosystems: it is possible to characterise the microbial communities in a lot of biological samples that were difficult to investigate with classical microbial analyses cause their symbiotic nature (rumen fluid or fecal samples; Dowd et al., 2008); obtaining new relevant information on the different microbial taxa present in these samples. In the context of milk, metagenomics can be particularly useful for several reasons. Milk is a complex and dynamic ecosystem that contains a diverse range of microorganism, including bacteria, yeast, and fungi, which can impact milk quality and safety. By applying metagenomic analysis to milk samples, researchers can obtain information on the diversity and functional potential of the milk microbiome, including the possible presence of potentially pathogenic microorganisms.

Therefore, a key advantage of this approach is that it offers a potential means of investigating the genomic properties of the large proportion of bacteria, archea and viruses that are suitable for standard culture techniques. Consequently, metagenomics is of great importance to the understanding of microbial ecology, shedding new light on both phylogenetic and functional perspectives.

The effects of transhumance on animals and final products

Alpine pasture is an important feed resource, that is often considered only for some negative aspects such as: low productivity, limited nutritional value, high fibre content and seasonal variations (Leiber et al., 2006; Zendri et al., 2016). Alpine pasture has also a lot of positive aspects: they are unique in terms of plant biodiversity, generally consisting in complex plant communities, mainly herbs with smaller presence of legumes and forages. Soil properties, altitude, climatic and edaphic factors affect their botanical composition.

Ruminants, through a symbiotic relationship with their rumen microbiota, rely completely on their rumen for fermentation of ingested plant material into essential nutrients and energy (Mizrahi, 2013; Matthews et al., 2019). Rumen is the largest compartment in the gastrointestinal tract (GIT) of bovines: it could be compared to a pre-gastric anaerobic fermentation chamber where is working an ecosystem, known as the rumen microbiota consisting of various microbes including bacteria (95% of the total), protozoa and fungi (Mizrahi, 2013). Rumen internal environment is portioned into different sacs by reticulo-ruminal fold in which the ingested food enters the rumen. The contribution of rumen microbiota varies with the host; the choice and development of gut microflora hence is a collaborative play of host genetics as well as environment.

Various studies have shown that microbial colonization of the rumen occurs as soon as the animal comes in contact with the outer world, even weeks before the rumen actually become functional (Fonty et al., 1987; Jami et al., 2013). The bacteria population, the most abundant ruminal microorganisms (Sirohi et al., 2012), is affected by the type of the diet by the host ruminants (Henderson et al., 2015), and it can be grouped accordingly to the type of substrate fermented, such as cellulose, hemicellulose, starch, sugars, intermediate acids, proteins, and lipids and produce methane. Over the last two decades, knowledge about the rumen microbial ecosystem has evolved and changed considerably with the advent of molecular techniques such as PCR and DNA-fingerprint methods (Dohrmann et al., 2004; Kim et al., 2011). The recent studies by sequencing bacterial 16S rRNA genes showed that there is a group of core bacteria in the rumen, with *Firmicutes* and *Bacterioidetes* the most predominant bacterial phyla (Liu et al., 2016; Mayorga et al., 2016; Sun et al., 2019). At the genus level, *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* were the most abundant bacteria (Henderson et al., 2015).

Changes and manipulations in the rumen bacterial community, such as diet supplementation, can significantly affect the health and productivity of ruminants, and more sustainable and efficient livestock (Lourenco et al., 2020). The adaptability of rumen microbiota is a key feature of ruminant physiology and survival strategy (Russell and Rychlik, 2001; McCann et al., 2014).

Numerous studies have reported that the rumen bacterial community is influenced by various factors, such as species, diet, age, health conditions, feed additives, season and geographical location. Nakano et al., (2013) showed that rumen microbiota needs 3-4 weeks to adapt to a pasture based ration when no gradual adaptation to the new nutritional situation is granted. Furthermore, De Menezes et al., (2011) demonstrated in a cross-over design, with two weeks of diet adaptation, that the rumen bacterial and archea community of TMR and pasture fed dairy differs significantly. In both *Prevotellaceae* were more prevalent on pasture, and a possible key role of this bacterial family in reducing methane production and in transitioning cows to a pasture-based ration was suggested (De Menezes et al., 2011; Nakano et al., 2013; McCann et al., 2014). Depending on the microbiota composition, the feeding nutrients input are transformed in an output product (milk and meat protein for humans) in a more or less efficient manner. As soon as the feed particles arrive in the rumen, they are colonized by different microorganisms within minutes (Martin et al. 1993; Edwards et al., 2007). Microorganisms can digest feedstuff, such as carbohydrates, protein and fiber obtaining energy, volatile fatty acids, vitamins and microbial protein that play important roles in the host metabolism (Flint and Bayer, 2008; Beaudet et al., 2016; Snelling and Wallace, 2017). Fermentation of carbohydrates, structural and non-structural, plant fiber and starch the most important, in the rumen leads to the formation of volatile fatty acids (VFA), carbon dioxine (CO_2), hydrogen (H_2), and microbial biomass. In detail, VFA, mainly acetate, propionate and butyrate, can provide up to 70-80% of the animal's energy requirements (Bergman, 1990; Flint and Bayer, 2008).

Diet and methane-producing bacteria influence the proportion of VFA, over these also depend largely on pH. Methanogenic bacteria are the second most abundant class of microorganisms, belonging to the kingdom archea (up to 99% of all archea) (Moissl-Eichinger et al., 2018). They are responsible for regulating the overall fermentation that takes place in the rumen, and contributing ~40% off global agriculture's greenhouse gas emissions (GHGE) through the production of methane. This production, as known as methanogenesis, not only negatively impacts on the environment, but it is also an energy loss to the host animal, which ultimately directly impacts farm profitability.

To remove the carbon dioxide and methane from the rumen, the cow regularly eructates these gases (Mizrahi, 2013). During the degradation of ingested feed particles, some members of the rumen community produce hydrogen (H_2), as the end product of fermentation. Therefore, methanogenesis acts homeostatically, in that it results in the expulsion of excess hydrogen by promoting the continuation of fermentations. While methanogens directly produce methane, various other bacteria, fungi and protozoa collude to produce a variety of fermentation end products, some of which act to supply the methanogens with substrate for methanogenesis, such as CO_2 and H_2. The methanogenesis process is energy inefficient, and has the capability to divert an estimated 6-12% of the animal's gross dietary energy intake away from productive aspects of the animal's performance, i.e., muscle growth or milk production.

The protozoal genera present in the rumen are influenced by feeding practices, and are in higher numbers when high-digestibility diets are fed, they also appear to be a stabilizing factor for fermentation end products. Fresh plants on pasture phenolic compound tannins could affect rumen protozoa due to its toxicity (McMahon et al., 2000; Vasta et al., 2010). While the protozoa are an integral part of the microbial population and have a marked effect on the fermentation, their benefit to the ruminant is still controversial. The anaerobic fungi are the most recently recognized group of rumen microbes. When animals are fed a high forage diet, rumen fungi may contribute up to 8% of the microbial mass. While it is still unclear whether these fungi are functionally significant, they have been shown to degrade cellulose and xylans, indicating some role in fiber digestion.

Another critical rumen function is microbial protein synthesis by rumen microbes, which can use ammonia nitrogen as a source of nitrogen. Ammonia is obtained through microbial degradation of dietary protein and dietary non-protein nitrogen, from hydrolysis of recycled urea to the rumen, and from degradation of microbial crude protein. While it disappears from the rumen in different ways, such as incorporation of the nitrogen by microbes, absorption through the rumen wall, and flushing to the omasum. The ruminant animals rely upon microbial crude protein synthesized in the rumen and dietary protein which escapes digestion in the rumen for its supply of amino acids (Pfeffer and Hristov, 2005). The amino acids are absorbed and utilized in the small intestine. Most of them are used in the synthesis

of body proteins, such as muscle and milk protein. Further, also urea can be actively transported from the blood to the lumen (urea recycling, depending of the dietary N content), supplying the rumen microorganisms with N, thereby increasing the microbial protein synthesis and allowing to augment diet low in N (Pfeffer and Hristov, 2005).

In addition to microbial protein, studies of rumen microbial vitamins have been conducted for decades and rumen fermentation could synthesize B group vitamins, including thiamine, riboflavin, niacin, vitamin B_6, folates, and vitamin B_{12} for mature ruminants (Beaudet et al., 2016; Castagnino et al., 2016; Seck et al., 2017). Seck et al., (2017) suggested that the differences in feed ingredients and nutrient composition may play an important role in the difference in vitamins production. The vitamin B_{12} producers in the rumen were members of the genera *Anaerovibrio*, *Mitsuokella*, and *Selenomonas* within the *Firmicutes* (Seshadri et al., 2018).

Rumen microbes rapidly and extensively modify dietary lipids. Hydrolysis in the rumen proceeds rapidly after ingestion. Following the breakdown of lipids, the microbes are responsible for biohydrogenation, or the addition of hydrogen to fatty acids with double bonds.

After considering the ruminal and fecal microbiome, it is important to pay attention to the bacterial community of the cows's teat skin, which is highly dependent on the rearing environment. Microorganisms colonize the teat through, for example, contact between skin and bedding material, a factor that depends on the type of animal housing and the feeding conditions of the animals (Zdanowicz et al., 2004; Verdier-Metz et al., 2012). Another source of variation could also be due according to the characteristics of pastures that create more or less favourable conditions (relative humidity, temperature, and ultraviolet radiation) for colonization of the phyllospere by bacteria, yeasts, and filamentous fungi. In addition, hygienic practice during milking (washing of milking equipment, care of teats before and after milking) are also further sources of microbiome variation. Frétin et al., (2018) have highlighted how the grazing system is a potential secondary source of microbial diversity in cheese, and how it is a key factor in the assembly of the teat skin microbiota.

The microbial composition of milk is highly dependent on the composition of the microbiota of the various reservoirs that are directly in contact with the milk, including teat skin and the milking equipment. The milk microbiome has always need attention. The theory about the evolutionary benefits of a microbiota in milk is, in brief and freely interpreted, as follow: The gut microbiota is essential for health and wellbeing of mammals, facilitating uptake of nutrients and providing the host with vitamins, etc. consequently, establishing the gut microbiota is a highly important event. Milk is produced in order to provide the offspring with nutrients during the first period in life. A bacterial community present in milk would, thus, inoculate the intestines of the offspring during the critical phase of gut microbiota establishment and be beneficial for the offspring (Martín et al., 2004).

Over the environment and farming in mountainous areas, summer grazing, would appears, over recent years, to have a beneficial aspect for animal welfare and health (Corazzin et al., 2010) due to the change in physical environment and diet. Some point of views on important issues regarding the effects of transhumance on nutritional status and on milk production and milk quality, which is often processed into high-value products, have been analysed in several previous studies (Leiber et al., 2006; Romanzin et al., 2013; Sturaro et al., 2013b; Farruggia et al., 2014; Zendri., 2016). While the knowledge on comparing cow's kept on lowland farms with those temporarily moved to highland pasture during the summer are limited (Saha et al., 2019; Carafa et al., 2020). In addition, several other changes occur, such as physiological, increased energy expenditure and body fat mobilisation associated with daily grazing activity, losing body conditions, ruminal ecosystem alterations, new interactions with other animals if mixed herds, and new behaviour adaptations to the surrounding environment (Berry et al., 2001; Leiber et al., 2004). All these conditions cause a state of nutritional imbalance in lactating cows, which have some bearing on milk production, milk composition, cheese-making properties, fertility and health. Therefore, pasture supplementation with some concentrates is generally advised for lactating animals. Milk and dairy products are an excellent source of different components of most diets. Dairy products from animal alpine grass feed-based are believed to be healthier due to their favourable fatty acid profile and are positively perceived by consumers for their taste, health, wholesomeness and animal welfare

characteristics. It is well known that dairy cows fed on pasture-based diets, characterised by high botanical biodiversity, produce milk with distinctive organoleptic and nutritional qualities, e.g. it is richer in unsaturated fatty acids than cows fed on total mixed rations, or on hay-based diets or concentrated cereal-based feeds. Several studies have shown that these fatty acids (FA) are beneficial for human health, and the effect of grazing on the FA composition of milk, cheese and rumen is highly variable and depends on many factors such as the amount of grass intake, the botanical and chemical composition and vegetative stage of the pasture, and of course the amount of feed in the diet. In recent years, the influence of the feeding system on composition has received much attention, in particular for polyunsaturated fatty acids (PUFA), which are beneficial to health because they are able to reduce the risk of cardiovascular disease and, in animal models, conjugated linoleic acids (CLA) have been shown to be involved in anticarcinogenic, immunomodulatory and antidiabetic activities (Dewhurst et al., 2006).

In dairy production, a small variation in milk composition can be attributed, directly or indirectly, to several aspects. Some of these factors include breed, individual, stage of lactation, diet, animal health, herd management, variation in the feeding system and the impact of seasonal changes end environmental conditions. Alpine grazing generally results in a milk yield decline as a consequence of moving to and staying on the pastures (Leiber et al., 2006; Zendri et al., 2016). Similarly, the nutritional aspects and chemical composition of milk produced in Alpine areas have been well studied. Milk fat is typically lower for cows fed high-quality pasture as compared to cows receiving stored forages. The lower protein level in milk produced in mountain pastures could be due to low energy supply and hypoxia, which are characteristic of high mountain pastures (Leiber et al., 2006).

Diverse studies have been conducted to try understand better the relationship between alpine pastures and milk quality during summer grazing period. The nature of the mountain and alpine pasture ingested by ruminants is the main factor of variation in the organoleptic and quality properties of dairy products, over the added value of the milk obtained, destined mostly for the production of high-value cheeses (Bovolenta et al., 2009; Bergamaschi et al., 2016). Actually, the obtained cheese has a more

intense odour, flavour, and colour, compared to that obtained from the milk of cows fed hay and silage rations (Coulon et al., 2004).

For a long time, there has been agreement on the idea that milk is produced sterile and becomes contaminated at sampling (Rainard, 2017). In recent years this idea has been challenged when sequencing techniques have been used to study the bacterial composition in milk (Oikonomou et al., 2012; Kuehn et al., 2013).

Milk microbiota has been shown to be influenced by many factors including region, season, cowshed environment, and hygienic management of milking (Latorre et al., 2010; Kable et al., 2016; Doyle et al., 2017; Seon Kim et al., 2017).

Overall changes in pasture nutritive value that occur between seasons, with spring pasture having a greater nutritive value as compared with summer/autumn pasture (Delagarde et al., 2000; Brink et al., 2007) and a potentially greater energy deficit in the summer months compared to spring. Delagarde et al., (2000) observed a decrease in crude protein content (45%) from the top to the bottom 5 cm of the ryegrass sward. In contrast, NDF, lignin and non-structural carbohydrate tend to increase from the top to the bottom of the pasture sward (Delagarde et al., 2000; Brink et al., 2007; Griggs et al., 2007; Nave et al., 2014).

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AIMS

Over the past few years, there has been a growing interest in summer transhumance of livestock on the one hand, and the complex living entities defined as microbial communities in different anatomical sites, from the most obvious to the less obvious, on the other.

Given these interests, the research conducted during my PhD studies and presented in this thesis aimed to provide new insights and extending the knowledge on the interaction of summer Alpine transhumance and the complex and dynamic microbial communities. Ultimately, this research could have important implication for improving the sustainability and efficiency of the dairy industry.

In detail, the study focused on the evolution of the microbiome starting from pasture, moving through rumen, feces and the ending to final products such as milk, taking into consideration the possible positive effects on animals and humans.

Specific aims were:

- To study the individual end products of rumen fermentation of Brown Swiss cows affected by the summer highland pasture, compared with a control group kept in a lowland permanent farm during the trial in order to find possible relationship with predicted methane, microbiological counts, and rumen metagenomics traits (Chapter 1).
- To compare the effects of transhumance to highland summer pasture of Brown Swiss with a control group kept in lowland farm conditions towards milk metagenomics and cheese-making properties (Chapter 2).
- To assess the milk microbiota variation during and after transhumance to highland summer pasture of Simmental cows, and to characterize milk composition, quality traits, and the milk B-vitamins content. The study involved four summer farms located at Passo Vezzena (Trento), and four lowland permanent farms in Trentino province. The data were collected individually from a total 26 cows during and after transhumance (Chapter 3).

- To analyze the relationship between intestinal metagenomics and milk quality and metagenomics of 4 herds (5-7 cows/each) during the summer Alpine transhumance, and after returning to the lowland permanent valley farm (Chapter 4).

CHAPTER I

Rumen fluid fermentation and metagenomic traits as affected by indoor farming and summer highland grazing

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INTERPRETIVE SUMMARY

Rumen fluid fermentation and metagenomic traits as affected by indoor farming and summer highland grazing

By Secchi et al., page 000. This research deals with the study of rumen microbiota, rumen fermentation traits and their interactions in dairy cows. Two groups of cows of the same herds, one maintained indoor from June to October, the other moved (July to September) to Alpine pastures were monthly sampled for the analysis of 10 fermentation traits of the rumen fluid and for microbiological analyses. Six microbial counts and the relative abundancies of 21 rumen bacterial taxa characterized the rumen microbiota during the experiment. Almost all traits were affected by the summer transhumance to Alpine pasture, but the carryover effect after returning to indoor farming were modest. The complexity of the relationships among the traits studied allowed to obtain 5 latent factors explaining rumen fermentation traits, 8 latent factors explaining the rumen microbiota, and 10 latent factors when the two datasets were merged. Six of these latent factors included contemporarily traits from the two datasets demonstrating the strong relationships between rumen microbiota and rumen fermentation activity.

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ABSTRACT

The rumen microbiome consists in an extremely biodiverse environment, which includes bacteria, archea, fungi and protozoa. The rumen microbial populations contribute to the health and productivity of ruminants, and shift when dairy cow changes diet. To this end, we investigated the complex relationships between rumen metagenomics and rumen fermentation pattern as affected by indoor farming and summer highland grazing sampling monthly (5 months) the rumen fluid produced by 12 Brown Swiss cows divided in two groups: the first remained in a lowland indoor farming condition from June to October; the second was moved to highland pastures from July to September, and then returned to lowland farm. After DNA extraction and Illumina Miseq sequencing, a total of 60 rumen fluid samples were also processed by means of an open source pipeline called Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2; https://qiime2.org); microbiological analysis were also performed. Moreover, the rumen fluid was analyzed for quantifying the ammonia nitrogen and the volatile fatty acids concentration and to estimate the methane production.

Six microbial counts and the relative abundancies of 21 rumen bacterial taxa characterized the rumen microbiota during the experiment. Almost all traits were affected by the summer transhumance to Alpine pasture, but the carryover effect after returning to indoor farming were modest. The complexity of the relationships among the traits studied allowed to obtain 5 latent factors explaining rumen fermentation traits, 8 latent factors explaining the rumen microbiota, and 10 latent factors when the two datasets were merged. Six of these latter latent factors included contemporarily traits from the two datasets demonstrating the strong relationships between rumen microbiota and rumen fermentation activity.

This study provides a broad picture of the microbial populations in the rumen liquid of dairy cows reared in the permanent farm or in a summer temporary farm, and their co-occurrence implicates specific relationship between different microbial domains in response to dietary and environmental changes.

Keywords: rumen fluid microbiota, volatile fatty acids, rumen ammonia, enteric methane emission, summer Alpine transhumance.

INTRODUCTION

The rumen resident microbial population and its potential roles have been the focus of extensive research in recent years and have contributed significantly to the understanding the ruminant nutrition. Ruminants can feed on plant material rich in cellulose and hemicellulose thanks to the action of the microbial community living in symbiosis in the rumen. Bacteria account for more than 95% of the total number of microorganisms, including anaerobic fungi, protozoa, and methanogenic archea, and they are able to degrade plant lignocellulosic matter, contributing greatly to the health status and energy requirements of the host (Mizrahi, 2013; Kuma et al., 2015).

Although they have flexible metabolic capacities, ruminal microorganism populations are influenced by various external factors, such as diet composition and physical characteristics of feed, feeding frequency, age, geographical location and ruminant-host interaction, all of which affect the maintenance, performance and productivity of animals (Denman and McSweeney, 2006; Sirohi et al., 2012; Mizrahi and Jami, 2018; Zeineldin et al., 2018).

During summer transhumance cows experience an extreme change of diet end environment, they have a higher energy requirement due for the physical activity considerably increases when grazing in the mountains. These factors could cause a nutritional imbalance and influence the rumen microbial community structures, as well as the production, quality and composition of milk (Leiber et al., 2006; De Menezes et al., 2011; Zendri et al., 2016a) and dairy products (Bergamaschi et al., 2016 ; Bittante et al., 2022).

Exploring the microbial composition and interactions occurring in the rumen is fundamental for driving rumen function towards enhancing animal health and productivity. Many metagenomics studies have opened new frontiers on investigating the biodiversity of rumen, the relation between dietary shifts, microbial composition and methane emissions, have been performed recently (Kumar et al., 2015; Denman et al., 2018; Delgado et al., 2019).

Methanogenic bacteria (up to 99% of all Archea) are the second most abundant class of microorganisms (Moissl-Eichinger et al., 2018) and responsible for regulating the overall fermentation

that takes place in the rumen, contributing about 40 % of global agriculture's greenhouse gas (GHGE) emissions through methane production (FAO, 2020). This production, known as methanogenesis, has both a negative impact on the environment, as well as representing a loss of energy for the host animal, directly impacting livestock profitability and being largely affected by breed of cows and farming systems (Bittante et al., 2018; Martínez-Marín at al., 2023). While methanogens produce CH_4 directly, various other bacteria, fungi and protozoa collaborate to produce a variety of fermentation end-products, some of which act to provide the methanogens with the substrate for methanogenesis, such as CO_2 and H_2; these gases are regularly erupted by cows (Mizrahi, 2013).

In the rumen, volatile fatty acids are produced by fermentation of organic matter. The predominant VFA are acetic, propionic and butyric acids, with isobutyric, valeric, iso-valeric, 2-mehylbutyric and others generally present in relative small amounts. The concentration and the relative proportions of VFA were related to the nature of the feed (Bergman, 1990). VFA are produced in large amounts through ruminal fermentation and are of paramount importance in that they provide greater than 70% on the ruminant's energy supply (Bergman, 1990; Flint and Bayer, 2008).

Therefore, the general aim of this research was to study the effects of farming systems, and particularly the extreme conditions of summer Alpine pasture vs indoor feeding, on the fermentation end products concentration in the rumen fluid and the composition of rumen microbiota and the possible relationships between rumen fermentation and rumen metagenomics traits.

MATERIALS AND METHODS

The experimental design and rumen fluid sampling

This work is part of a larger project (Juribello project) on the effect of transhumance of cows to summer highland pastures on their productivity and on the chemical, technological and microbiological characteristics of milk produced. All the previous details about the environmental conditions and the methods used are available in three prior studies: the first dealing with cow's body condition, milk production and composition, and cheese-making efficiency (Saha et al., 2019a), the second reporting some preliminary data on milk microbial counts (Carafa et al., 2020), and third on milk metagenomics analysis (Secchi et al., 2023).

The research was carried out in a mountain area in Trentino province (North-eastern Italian Alps), and was based on the selection of two farms: the first was a modern lowland permanent farm (**PF**) (Malè, Trento, Italy, 737 m above sea level) where lactating cows are kept indoor with loose housing, total mixed diet and milking parlor; and a summer temporary farm (Malga Juribello) in the highland Alpine pasture (**ALP**) (within the "Paneveggio – Pale di San Martino" Natural Park, Passo Rolle, Trento, Italy, 1860 m above sea level) where cows are maintained at pasture days and night free to graze, with the milking and the supplementation with a compound feed (3 to 6 kg/d according to milk production) in a milking parlor placed in an old barn.

Briefly, 12 Brown Swiss cows from PF were selected at the end of May; at the beginning of June they were randomly divided into two groups (6 cows each) reared in the same conditions; at the beginning of July one of the two groups was moved to the temporary farm in the ALP highland pastures (**HIGH** group) for three months, while the other group remained indoor in the lowland permanent farm (**LOW** group) all the times; at the end of September the HIGH group return back to the PF together with LOW group in the same pen till the end of experiment, at end of October. Each cow was sampled monthly (from mid-June to mid-October).

The sampling of June represented the initial conditions of the two groups: being reared together, the expectation was of non-significant differences for all traits. The samplings of July, August and

September represented the effects of the two farming conditions: indoor keeping in the lowland vs pasture in the highlands. The last sampling of the two groups reared together in October represented the evaluation of possible carry-over effects of summer pasture on the indoor rearing.

A total of 60 individual rumen fluid samples (12 cows × 5 months) were collected by using a flexible stomach tube. Each sample was divided into different aliquots: about 10 g of the rumen fluid were placed in 15 mL tubes, immediately frozen in liquid nitrogen, and stored at -80°C in the Research and Innovation Centre, Food Quality and Nutrition Department of the Fondazione Edmund Mach (San Michele all' Adige, Trento, Italy) until microbiological analysis; two aliquots with 4 mL of rumen fluid added with 1 mL of metaphosforic acid (25%, w/v) needed for blocking the fermentation, and stored at -20 °C in the 'LaChi' laboratory, University of Padova (Legnaro, Italy) until fermentation end product analyses. All the experimental procedure involving animals were approved by the "Ethical Committee for the Care and Use of Experimental Animals" of University of Padova (CEASA) and were completed under veterinary control.

Analyses of Ammonia and VFAs concentration in the rumen samples and prediction of methane production

The ammonia nitrogen ($N\text{-}NH_3$) content in the buffered rumen fluid was determined using a commercial ammonia assay procedure (Megazyme, K – AMIAR 02/20, Wicklow, Ireland). The ammonia concentration (mg/L) was calculated using the formula in the ammonia assay procedure of Megazyme, and the results were converted in mmol/L.

To determine individual VFAs, the acidified samples were filtered through a 0.22-µm whatman syringe filter. The VFA concentrations in the filtered samples were determined using a Jasco high-performance liquid chromatography (HPLC) equipped with a PU-2080 pump, a model RI-2031 refraction index detector, a model AS-2055 autosampler, and a model CO-2060 column oven. The chromatographic separation was performed with an Aminex HPX 87H column (300 mm × 77.8 mm, Biorad), the flow-rate

was set at 0.6 ml/min, the volume of the sample injected was 20 μL, and the column temperature was maintained at 65°C. At the end data were interpreted using the ChromNAV software (version 2.0, Jasco).

For the prediction of methane (CH_4), we used the equation proposed by Ramin and Huhtanen, (2012), predicted according to VFA stoichiometry equations by Wolin, (1960). The authors developed an in vitro method for prediction of methane production in the rumen of cows using the kinetic parameters from an automated in vitro gas production (GP) system in s 2-compartment rumen model. This approach takes rumen dynamics into account and may have advantages compared with single time point batch culture systems. The equation used was:

Predicted methane (mL)= 22.4 × (0.5 × AA – 0.25 × PA + 0.50 × BA – 0.25 × VA)

where AA, PA, BA, VA are the production (mmol) of acetic acid, propionic acid, butyric acid and valeric acid, and 22.4 correspond a gas volume (mL/mmol gas). For the present work we decided to remove the gas volume because we used *in vivo* sample while *in vitro* samples as used by the authors.

Microbiological counts and isolation

Ruminal fluid samples were homogenized, decimally diluted in sterile peptone water and plated onto the following agar media: a bicarbonate-buffered mineral medium supplemented with vitamins (BBM), incubated in anaerobic conditions for 2 weeks at 37 °C, was used to cultivate rumen bacteria (Kenters et al., 2011); de Man, Rogosa and Sharpe (MRS) agar acidified to pH 5.5 with 5 M lactic acid, incubated in anaerobic conditions for 48 h at 37 °C; MRS agar with 0.05% (w/v) L-cysteine (MRS-cys), incubated in anaerobic conditions for 48 h at 37 °C; M17 agar for 48 h, incubated at 30 °C in aerobic conditions; sodium lactate and yeast extract lactate (YELA) incubated at 37 °C for one week in anaerobic conditions for counting propionibacteria; Wilkins Chalgren (WC) incubated at 37 °C for 48 h in anaerobic conditions for the total count of anaerobic bacteria; Wallerstein Laboratory Nutrient Agar (WL) supplemented with chloramphenicol, incubated for 5 days at 30 °C. All culture media were purchased from Oxoid (Milan, Italy).

Ten or more colonies were randomly isolated from BBM, MRS-cys, WC, YELA, and purified by subsequent culturing. Cell morphology was determined by microscopy, Gram and catalase tests were performed. Only Gram-positive, brown and catalase-positive colonies were isolated from YELA, which are reported to belong to *Propionibacterium* genus (Thierry and Madec, 1995); whereas, Gram-positive and catalase-negative bacteria were isolated from the other media. Pure cultures were stored at -80 °C in glycerol (20%, v/v).

DNA extraction and genotypic identification of the rumen indigenous bacteria

The bacterial DNA was isolated using the InstaGene™ Matrix (Bio-Rad Laboratories S.r.l., Segrate, Italy). Quick-gDNA™ MicroPrep (Zymo Research, Italy).

A fragment of the 16S rRNA gene was amplified using the primers 27F (50-GAGAGTTTGATCCTGGCTCAG) and 1495R (50-CTACGGCTACCTTGTTACGA) (Grifoni et al., 1995). The PCR products were purified using the Exo-SAP-IT™kit (USB Co., Cleveland, OH), and sequenced in an ABI PRISM 3100 sequencer (Applied Biosystems, Italy), using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI, USA). All amplifications were run in a T100™ ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA).

Total DNA extraction and preparation of the MiSeq library from rumen samples

For total genomic DNA extraction, 2 mL of ruminal fluid were centrifuged at 4,000 g for 10 min at +4 °C, and the supernatant was discarded. Genomic DNA was extracted from the pellet using the the QIamp® PowerFecal® DNA Kit (Qiagen, Milano, Italy), with an additional bead beating step incorporated into the protocol. All DNA samples were purified by DNeasy PowerClean Pro Cleanup Kit (Qiagen, Milan, Italy), and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific, USA).

The primers 515F (5′- GTGCCAGCMGCCGCGGTAA-3′) and 909R (5′- CCCCGYCAATTCMTTTRAGT-3′) targeting for the 16S rRNA gene, were used to amplify bacterial and archaeal V4-V5 region (Xue et al., 2016); the primers ITS1F (5′-GTTTCCGTAGGTGAACCTGC -3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC -3′) were used to amplify the ITS1-5.8S region of yeasts and fungi (Gardes and Bruns, 1993). Unique barcodes were attached before the forward primers to facilitate the differentiation of samples, and the Agencourt AMPure kit (Beckman coulter) was used for cleaning the amplicons and preventing preferential sequencing of smallest amplicons. The concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following quantitation, the cleaned amplicons were mixed and combined in equimolar ratios. Amplicon library preparation, quality and quantification of pooled libraries and high throughput sequencing by Illumina technology were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy).

Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2; https://qiime2.org; Bolyen et al., 2019). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013). Alpha and beta diversity metrics were calculated using the core-diversity plugin within QIIME2 and emperor (Vázquez-Baeza et al., 2013). Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the

Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables.

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA528228

Statistical Analysis

The microbiological counts data were expressed in log CFU/mL, while the metagenomic relative abundancies were $\log_{10}$ transformed. Two samples were excluded from the statistical analysis because of incomplete data.

Mixed model analysis of variance

The concentration of end products of rumen fermentation, the predicted methane production, the rumen microbiological counts, and the $\log_{10}$-transformed relative abundancies obtained from the rumen metagenomic analysis were analyzed with a linear mixed model (RStudio version 1.4.1106), which include the fixed effects of the month × group interaction (10 levels: 5 months, from June to October; and 2 groups of cows, HIGH and LOW), and the random effect of cow within group. We used a function to estimate R^2_{GLMM} statistic, *r.squaredGLMM*, included in the *MuMln* package for the R statistical software, we consider the conditional R-squared because concern variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013).

To measure the effect of season and lactation in progress in the control group kept indoor in the PF during the 5 month, polynomial contrasts were estimated between the 5 least-square means of the months within the LOW group to determine the response curve of each trait (linear, quadratic, and cubic components). Contrasts between the HIGH and LOW groups were estimated separately within each month

to test for the following: the homogeneity of groups in the same environment (indoor) at the beginning of the trial (June); the effect of transhumance to highland pasture during the summer months (July, August, and September) compared with the control indoor group; and the carryover effect of summer pasture on the HIGH group after returning to indoor conditions on the PF (October).

Correlation analysis and latent explanatory factors analysis

Three datasets were created:

a) Rumen fermentation (**rf-dataset**): including all the chemical analyses carried out in the cow's rumen fluid samples and the calculated (C2+C4)/C3 ratio and CH_4 production (10 traits);
b) Rumen metagenomics (**rm-dataset**): including the relative abundancies data of the 21 bacterial taxa identified in cow's rumen fluid samples;
c) Rumen fermentation and metagenomics (**rfm-dataset**): merging the two sub-datasets of the chemical characteristics of rumen fluid and the relative abundancies of the bacterial taxa identified in cow's rumen samples (31 traits);

Correlations were calculated within the rf-dataset and within the rm-dataset, and also between the rf-dataset and the rm-dataset.

Given of the complexity and high number of the relationships among all the traits of the three datasets, we used a multivariate factor analysis (**FA**) to summarize the interrelated measured traits in a small number of unmeasured latent independent explanatory variables (factors). Factor analysis was performed separately on each dataset obtaining a variable number of latent explanatory factors named respectively FN-rf, FN-rm, and FN-rfm, where N is the progressive number of factors (N: 1 to a max of 8) within FA. First, we performed Kaiser–Meyer–Olkin and Bartlett's tests, which showed that the traits were suitable for FA. The FA was carried out with Varimax rotation in the R environment (R Core Team, 2016) using the psych package (available at CRAN, the Comprehensive R Archive Network, version 2.2.9; https://cran.r-project.org/web/packages/psych/index.html) in 3 steps: (1) extraction of factors such

that the minimum number of uncorrelated latent factors explained the greatest proportion of common variance; (2) factor rotation until each factor was defined by a few variables with high loadings; and (3) biological interpretation of the factors based on the strength of the loadings of the variables. The eigenvalues of the factors and the communalities of the variables after rotation were also determined. Three FA were performed with eight latent explanatory factors each. The scores of each samples for each factor were analysed using the same linear mixed model used for metagenomics.

RESULTS AND DISCUSSION

Rumen fluid fermentation traits

Table 1 shows the descriptive statistics for the rumen fluid traits, including the prediction of methane production. The proportion of different VFA found on average on the four herds practicing the summer transhumance is different from those usually found on the rumen fluid of cows maintained indoor in intensive farming systems (Saha et al., 2019b). The acetic acid is much higher and the propionic, butyric and valeric (both in the n- and iso forms) acids are much lower. This testimony the lower quantity of concentrates supplemented to cows of the mountain transhumant farming system respect to the lowland indoor intensive system.

The analysis of the major sources of variation of rumen fermentation traits, illustrated in Figure 1, showed that the combination of month of the year (which reflect also different climatic conditions and the advancing of lactation and pregnancy stages) and group of cow (HIGH vs LOW) represents the greatest source of variation for ammonia N and n-valeric acid (≈ 60%), and an important source for all the other VFA proportions or VFA-derived traits (≈25% to 40%). The permanent effect of individual cow is much less important, varying from almost a null value for the n- and iso-valeric acids to ≈ 15% in the case of isobutyric acid. The remaining, very important for all traits, source of variation is the residual one.

The fixed combined effect of Month × Group of cows was significant for all the traits listed in Table 1. The disaggregation of the pattern of traits from June to October in LOW group (maintained constantly indoor) was opposite for acetic and for propionic acids (see Table 2). The contrasts between HIGH and LOW groups of cows within month of sampling was generally not significant (with two exceptions) in June, when no difference was expected because were kept together indoor in the same pen, with the same diet. In July, August and September, when HIGH group was on ALP highland pastures and LOW group was still indoor, all traits listed in Table 2 presented a significant contrast in at least one of the three sampling months. In October only 4 of the 10 composition traits of the rumen fluid presented significant differences between the two groups.

The differences observed during summer are congruent with the greater content of fiber of the diet of grazing HIGH group (higher acetic acid) and with the greater content of starch (higher propionic acid) in the diet of indoor LOW group of cows (Saha et al., 2019a). It worth noting that the composition of grass of pasture was changing during summer with the increasing maturity of forages. The differences observed in October could be, at least in part, interpreted as residual effects of the different farming systems of the two groups of cows during the previous summer transhumance.

Correlations between rumen fluid fermentation traits and their latent explanatory factors

The 45 Pearson correlation coefficients observed among the 10 rumen fluid chemical composition traits are represented as heat-map plot in Figure 2. Ammonia N presents moderate positive correlations with total VFA concentration n-valeric acid and predicted methane production and a negative correlation with isobutyric acid. Increased ammonia N and VFA concentration in rumen fluid are both indicators of greater availability of fermentable nutrients in the rumen. The increase of the proportion of n-valeric acid, like the increase of ammonia N concentration in rumen fluids are indicators of greater fermentable proteins availability. Lastly the increased production of methane is congruent with the increased fermentation activity in the rumen (see the very large correlation coefficient between total VFA and methane production in Figure 2).

Regarding the relative molar proportions of VFA, it could be seen that propionic acid is negatively correlated with all the other VFA (except n-valeric acid), and particularly with the acetic acid. As expected the ratio (C2+C4)/C3 is positively correlated with the acids at the numerator (and also with the isovaleric acid) and negatively with the denominator (and also with the n-valeric acid). These 45 different, and sometime strong, correlations justify the attempt to combine them at the research of fewer independent latent explanatory factors.

The Table 3 summarizes the results obtained from FA on rf-dataset. Five latent factors (F1-rf to F5-rf) explained 81.6% of total variance-covariance matrix of the 10 traits considered. The first factor (F1-rf) represents 28.1% of total variance and is based on three traits with large loadings: acetic acid molar

proportion (positively), propionic acid (negatively), and the ratio (C2+C4)/C3 (positively), and also on a fourth trait (isovaleric acid molar proportion) with a moderate positive loading. Due to its composition we named this factor "VFA ratio" and could be considered a qualitative latent factor expressing the major type of fermentations in the rumen. The scores of this latent factor are affected by the combined effects of Month × Group and their 10 least squares means (LSM) are depicted in Figure 3. It can be seen that the baseline of LOW group of cow was quadratic with the minima values in summer. Values of the HIGH group of cow were numerically greater respect to those of the LOW group, but without reaching the statistically significance, during the summer transhumance.

The second latent factor (F2-rf) was strongly based on total VFA content of rumen fluid and on the derived predicted methane production, and, moderately, on ammonia N (Table 3). This factor represents 20.1% of total variance and could be considered a quantitative descriptor of rumen fermentation that we named "Methane". The combined effect of Month × Group of cows is much more important for this factor than for the previous one, but the disaggregation of the major factors sees, this time, a non-significant variation of the baseline of LOW group June to October, and, on the contrary, significant strong differences (in favor of LOW vs HIGH group) during the first three months, and a tendency to become similar at the end of the experimental period (October).

The third factor (F3-rm) is based on the two isoforms of the butyric (strongly) and valeric (moderately) acids (Table 3), thus named "iso-VFA". F3-rm represented 12.1% of total variance, was significantly affected by Month × Group combined effects, and showed no trend in the baseline and a significant contrast in favor of HIGH group of cows only in September sampling month (Figure 3).

The fourth (F4-rf) and fifth (F5-rf) factors are strongly and positively related, respectively, to n-butyric acid (and moderately negatively to acetic acid also) and to n-valeric acid (and moderately to ammonia N too), so that we named them "n-C4" and "n-C5", respectively (Table 3). They represented a similar proportion of total variance (11.0% and 10.3%), and were both affected by the combined effect of Month × Group of cows. Both F4-rf and F5-rf showed a similar cubic reference baseline for LOW group of cows (with the minimum score in July and the maximum in September) and an opposite effect of

summer transhumance. In fact, the HIGH group of cow showed a significantly higher score of F4-rf "n-C4" in July and a lower score for F5-rf "n-C5" in September. The meaning of these patterns is not easily interpretable.

In summary, the average scores of three latent factors out of five (VFA-ratio, n-C4, and n-C5) showed a variation from June to October in the LOW group of cows maintained constantly indoor on a TMD, and all factors, excluded VFA-ratio, showed significant differences during summer between HIGH group of cows grazing the Alpine pasture and LOW group of cows indoor.

Based on our knowledge, during transhumance, cows experience an extreme change in diet, environment, and physical activity that increases significantly. These various factors could cause nutritional imbalance and affect not only the quality and composition of milk and dairy products, but also the microbial composition of the rumen (Leiber et al., 2006; Henderson et al., 2015; Zendri et al., 2016b). In fact, it could be seen (Table 1) that the month × group interaction exerted a significant effect in all the trait considered. During the three summer months of grazing on pastures, we observed a decreasing concentration of nitrogen ammonia and methane in the rumen fluid (Table 4). The concentration of the ammonia nitrogen ($N\text{-}NH_3$) has been used as a qualitative reference to understand the adequacy of the rumen environment according to the microbial activity on fibrous carbohydrates (Dermann 2009). $N\text{-}NH_3$ is the preferred nitrogen source for growth of fibrolytic microorganisms (Russell, 2002). One of the factor that influence the low concentration of ammonia in the ruminant is the amount of feed protein entering the rumen. High dietary protein content with high degradability will result in an increased rumen fluid ammonia concentration. (Souza et al., 2013). The average nitrogen ammonia was in the range of that found by Souza et al., (2013) estimated by the catalyzed indophenol colorimetric reaction (CICR) and Kjeldahl distillation (KD) method.

The volatile fatty acids (VFA) are produced within the rumen by fermentation of organic matter, these can have an important effect on production and product composition in ruminants. The relative proportions in which VFA are produced, are influenced by a number of factors, including substrate

composition, substrate availability and rate of depolymerization, and microbial species present. Interaction between these factors hamper conclusions with respect to the effect of one single factor.

The influence of methane production in the rumen is shown by the increased levels of propionate produced when methanogenic bacteria are inhibited (Demeyer and Van Nevel, 1979). Fermentation of structural carbohydrates, compared to fermentation of starch, yielded high amount of acetic and low amounts of propionic acid.

Production of volatile fatty acids in ruminal fluid, including acetate, propionate and total butyrate (n-butyrate and iso-butyrate) have been related to methane production using stoichiometric equations (Moss et al., 2000). Changes in (acetate+butyrate)/propionate have also been associated with changes in methane production in vivo (Moss et al., 2000; Danielsson et al., 2012).

Microorganisms in the rumen degrade nutrients to produce volatile fatty acids and synthesize microbial protein as an energy and protein supply for the ruminant, respectively. However, this fermentation process has energy (losses of methane) and protein (losses of ammonia N) inefficiencies that may limit production performance and contribute to release of pollutant to the environment.

Methane, which is a major greenhouse gas produced during ruminal fermentation, has a significant ratio in the adverse economic and ecological impact of global climate change. However, O'Callaghan et al., (2016) showed that the fatty acid profile of milks from pasture-based and TMR feeding systems are markedly different, and that the fatty acid profile combined with multivariate analysis could be exploited to verify milk products from grass-fed system.

Rumen microbiota

Rumen microbiota was studied using two different methodological approaches: the classical methods leading to microbial counts referred to selective media and the metagenomics approach based on DNA analysis. The descriptive statistics of microbial counts included in Table 4 represent then quantitative data referred to some of the most important groups of live bacteria found in rumen fluid,

whereas those of the relative abundancies represent the proportions of the quantity of different bacterial taxa found in rumen fluid DNA, i.e. a qualitative data because the sum is constant.

The analyses of the relative importance of the major sources of rumen microbiota variation are represented in Figure 4 for bacterial counts and in Figure 5 for metagenomic relative abundancies of bacteria taxa found in rumen fluid. The combined effects of Month × Group of cows represent an important source of variation (≈ 35% to 50% of total variance) for all bacterial counts except BBM (rumen medium), whereas the permanent differences among different cows within groups was much less important being almost null in the case of BBM (Rumen medium) and YELA (putative *Propionibacteria*) counts and arriving to a maximum of ≈ 15% in the case of the other bacterial counts. The residual variance represents about half of total variance for all bacterial counts with the only exception of BBM (Rumen medium) where it reaches almost 80% of total variance.

The proportions among the most important sources of variation of the relative abundancies of individual bacterial taxa are, as expected, much more variable than for bacterial counts (Figure 5). The proportion of variance explained by the combined effects of Month × Group of cows was very scarce in the case of Other *Bacteroidetes* and *Gammaproteobacteria*, whereas represent about 30% to 80% of the total for all other bacterial taxa found in rumen samples. The permanent differences among different cows within group was almost null for 8 out of 21 bacterial taxa found in rumen fluid. The largest proportion of cows variability (≈ 20% of total) was observed for Other *Bacteroidetes*, i.e. the taxon with the lowest incidence of Month and Group. Also residual variance was extremely different in different taxa ranging from about 20% to 85%.

The great importance of the combined effects of Month and Group of cows is confirmed by their statistical significance achieved by all bacterial counts and individual bacterial taxa relative abundancy (Table 4), with the only exception of Other *Bacteroidetes* and *Gammaproteobacteria* taxa. The disaggregation of the two combined effects showed (Table 5) that 3 out of 6 rumen bacterial counts and 13 out of 21 rumen bacterial taxa were characterized by significant seasonal patterns of the baseline values showed by the cows of the LOW group (constantly maintained indoor). The bacterial trends identified

belong to 6 different patterns: linear increase or decrease, quadratic with a maximum or a minimum peak, and cubic with a maximum followed by a minimum or vice versa.

Regarding the comparison between the two groups of cows within each month of sampling, all bacterial counts and the bacterial taxa relative abundances (with the only exception of the two taxa with non-significant effect of the combined Month × Group) showed a significant contrast in at least one of the 5 months sampled (Table 5). In June none of the bacterial counts and only one taxon (*Lachnospiraceae*) out of 21 taxa presented a significant difference. This is congruent with the assumption of initial statistical homogeneity of the two groups of cows (in June both groups were reared indoor in the same pen with the same diet). During the three months of the summer transhumance about half of the bacterial traits (14, 14 and 15 out of 27 traits in July, August and September, respectively) presented significant a difference in each sampling month. In total, beyond the two previously cited taxa, only one more had no significant contrast during summer (*Spirochaetes*), and 4 taxa presented significant contrasts every month (*Fibrobacter*, *Lachnospiraceae*, *Lentispherae*, and *Succinovibrionaceae*), the remaining having one or two significant differences (Table 5).

In the case of microbial counts, all significant contrasts were positive, meaning greater counts in rumen samples of the HIGH than in the LOW group of cows. In the case of the taxa identified through metagenomics approach, as expected, the contrast of the relative abundance for the two group of cows were both positive or negative.

Lastly, the number of significant contrasts in October rumen samples (when all the cows were again together indoor) was 7 (one count and 6 bacterial taxa). This value is about half respect to the months of summer transhumance, but is not supporting the null hypothesis. This means that in October there is still a carryover effect of the previous transhumance on some microbial trait. It worth noting that on the same cows, on milk samples taken the same dates of rumen fluid samples, we found only two significant contrasts out of 46 microbial traits (Secchi et al., 2023) and only one out of 20 milk quality traits (Saha et al., 2019a).

Ruminal microbes are a major sources of other nutrients for the ruminant (Hussein et al., 1995). Major chemical components of ruminal microorganisms are nitrogen, carbohydrates, lipids and ash (Strom and Øskov, 1984). The content of organic matter, nitrogen and amino acids in mixed rumen bacteria increase by decreasing the level of forage in the diet (Martin, 1994). Ruminal microbial growth depends on their capability to degrade the ferment feed ingredients.

Prevotella has been associated with propionic acid production and this genus is known to play a pivotal role in the degradation and utilization of non-cellulosolitic plant polysaccharides, proteins, starches and xylans (Strobel, 1992; Accetto and Avguštin, 2019).

Correlations between rumen microbiological traits and their latent explanatory factors

The 210 Pearson correlation coefficients observed among the relative abundancies of the 21 bacterial taxa identified and quantified in rumen fluid samples are represented as heat-map plot in Figure 6. It is possible to see that there are many complex interrelationships (positive and negative) among bacterial taxa so that a clear understanding of all is not possible. The need for a simplification in this case is much more essential than in the case of the rumen fluid chemical traits. So we carried on a FA to search for relatively few independent latent explanatory factors explaining the major part of the variance/covariance of the matrix of rumen microbiota dataset. We decided to analyze only data obtained from metagenomics approach, excluding the bacterial counts, to put together only homogeneous data.

In total we obtained 8 latent factors from the rumen metagenomic dataset (F1-rm to F8-rm), representing 72% of the overall variability. The loading of the factors and communality coefficients of the 21 microbial traits are listed in Table 6. Only the Other *Bacteroidetes* taxon was not included in any latent factor and need to be considered an independent microbiological trait of rumen fluid. Two other taxa, involved in a latent factor, showed a communality coefficient slightly lower than 0.50 (*Erysipelotrichaceae*, and *Gammaproteobacteria*), all the other taxa could be well represented by the latent factors.

An interpretation of the 8 latent factor requires probably further research. Anyway, it could be seen from Table 7 that the first (F1-rm) represents almost one fifth of total variance, other 5 factors (F2-rm to F6-rm) represent slightly less than one tenth of total variance each, and only the last two factors (F7-rm and F8-rm) represents only 5.8% and 2.7% of variance respectively. Both these latter factors were two-trait factors, whereas the other factors are much complex, including 4 to 9 microbiological taxa each (Table 6). The F8-rm is also the only one not presenting a significant combined effect of Month × Group of cows (Table 7).

The disaggregation of the combined Month × Group effect depicted, in Figure 7, make clear that 3 out of 8 latent factors are characterized by a significant variation of the baseline from June to October of the LOW group of cows (maintained constantly indoor). It should be remembered that this variation is not merely e seasonal variation, but it includes the effect of advancing lactation stage and pregnancy condition of cows.

The effect of farming system seems much more evident than seasonal variation. All latent factors, excluding the F8-rm, presented some significant contrast between HIGH and LOW group of cows during the three months of the summer transhumance. Only one contrast was significant in June (F5-rm) and two contrasts in October (F5-rm, and F7-rm). These results substantially confirm those previously seen on milk microbiota in the same cows and dates (Secchi et al., 2023).

Correlations between rumen fluid fermentation traits and rumen microbiological traits, and their latent explanatory factors

The 210 Pearson correlation coefficients observed between the 10 rumen fluid chemical composition traits and the relative abundances of the 21 rumen bacterial taxa are represented as heat-map plot in Figure 8. Also in this case the number, complexity and variability of correlations obtained required to search for fewer independent latent factors, with the objective of identify possible relationships between the rumen microbiota and the fermentation pattern of rumen fluid.

Combining the two datasets of 10 rumen fermentation traits and 21 relative abundances of rumen bacterial taxa we extract 10 independent explanatory factors (F1-rfm to F10-rfm) explaining 73 % of total variance/covariance matrix. The loadings of the 10 latent factors and the communality coefficients of the 31 traits included in the FA are listed in Table 8. It is very interesting to note that 6 out of 10 latent factors are "mixed" factors, i.e. they are based contemporary on rumen fermentation traits and rumen metagenomic taxa. This confirm, as expected, the strong interrelations between microbiota and rumen fermentation. One latent factor (F4-rfm) is based only on two fermentation traits (total VFA concentration and predicted methane production and substantially mimic the F2-rf "Methane" factor obtained analyzing rumen fermentation traits alone. Three other latent factors (F5-rfm, F6-rfm, and F7-rfm), on the contrary, were based only on microbiological traits, substantially mimicking the latent factors F5-rm, F4-rm, and F3-rm, respectively.

The first "joint" latent factor (F1-rfm) reflect the combination of F1-rf "VFA ratio" with 9 bacterial taxa, the most important of which are the opposite effects of Other *Bacteroidales* (loading 0.621) and *Succinivibrionaceae* (-0.764). It explains 16.4% of total variance, it is affected modestly by the combined effects of Month × Group of cows (Table 9) because the only significant contrast between HIGH and LOW groups of cows was recorded in July samples, in favor of HIGH cows (Figure 9).

The second joint latent factor (F2-rfm) includes the ammonia N concentration of rumen fluid (with a negative loading) as the only fermentation trait, together with 7 microbial taxa (Table 8): the *Archea, Ruminococcaceae* and *Succinivibrionaceae* with negative loadings, and the Other *Bacteroidales*, *Fibrobactere*, *Lentispherae* and *Tenericutes* with positive loadings. F2-rfm represents 9.7% of total variance, has the strongest combined effect of Month × Group of cows (Table 9) and, in fact, is characterized by a linear growing baseline of the LOW group of cows, and by much higher scores in HIGH than LOW group of cows during summer transhumance in July and August samplings (Figure 9). Apparently, this latent factor could be considered a good indicator of the effect of farming system.

The third joint latent factor (F3-rfm) combines the F3-rf "Iso-VFA" latent factor of rumen fermentation traits, with the addition of a moderate negative loading for ammonia N concentration, and 7

rumen bacterial taxa, 4 of which representing the F6-rm latent factor of rumen microbiota with the addition of moderate positive loadings of *Ruminococcaceae*, *Clostridia*, and *Verrucomicrobia* (Table 8). It represents 9.5% of total variance, is affected by the combined effect of Month × Group of cows (Table 9), but present only one significant contrast, in favor of the HIGH group of cows, in September sampling (Figure 9).

The other three joint latent factors (F8-rfm, F9-rfm, and F10-rfm) are based 3, 2, and 2 traits (Table 8), representing only 5.1%, 3.6% and 3.3% of total variance (Table 9), respectively. F8-rfm is strongly based on n-butyric acid, and, with moderate negative loadings, on acetic acid and *Elusimicrobia*. F9-rfm is strongly based on n-valeric acid and, also in this case, on a moderate negative loading of *Elusimicrobia*. Lastly, F10-rfm is based on *Alphaproteobacteria* and, less strongly, on isovaleric acid. All these three latter latent factors present a seasonal baseline pattern from June to October in LOW group of cows maintained indoor and one or two significant contrasts between the two groups (Figure 9).

CONCLUSIONS

Ten fermentation traits, six microbial counts and the relative abundancies of 21 rumen bacterial taxa characterized the rumen microbiota and fermentation during the 5 months experiment. Almost all traits were affected by the summer transhumance to Alpine pasture, but the carryover effect after returning to indoor farming were modest. The complexity of the relationships among the traits studied allowed to obtain 5 latent factors explaining rumen fermentation traits, 8 latent factors explaining the rumen microbiota, and 10 latent factors explaining both rumen fermentation and microbiota, when the two datasets were merged. Six of these latter latent factors included contemporarily traits from the two datasets demonstrating the strong relationships between rumen microbiota and rumen fermentation activity.

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TABLES AND FIGURES

Table 1. Rumen fermentation traits: descriptive statistics, significance of the Month × Group interaction, conditional determination coefficient (R^2c) and root mean square error (RMSE) of the ammonia nitrogen, volatile fatty acids (VFA), and predicted methane production.

Traits	Samples N	Descriptive statistics: Mean	±SD	Month × Group, *F-value*	R^2c	RMSE
Ammonia N, mmol/L	58	4.74	2.41	10.4 ***	0.704	1.36
Total VFA, mmol/L	58	96.8	20.1	5.4 ***	0.547	14.1
Volatile fatty acids, mol%:						
Acetic Acid	57	67.59	2.72	4.8 ***	0.438	2.14
Propionic Acid	57	18.83	2.63	2.9 *	0.351	2.24
Iso-butyric Acid	58	0.70	0.20	3.0 **	0.434	0.16
N-butyric Acid	57	10.72	1.26	4.2 ***	0.455	0.98
Iso-valeric Acid	58	0.84	0.18	2.8 *	0.307	0.16
N-valeric Acid	57	1.01	0.23	9.4 ***	0.607	0.15
Ratio (C2+C4):C3	57	4.28	0.65	2.9 **	0.337	0.57
Methane production, mmol/mol of VFA	58	32.90	6.92	4.0 **	0.458	5.35

*P<0.05; **P<0.01; ***P<0.001;
C2: Acetic acid; C3: Propionic Acid; C4: Butyric acid;

Table 2. Rumen fermentation traits: significance, order and shape of the patterns observed for ammonia nitrogen, volatile fatty acids (VFA) and predicted methane production of rumen fluid samples collected during the experiment on cows maintained always indoor, and difference of cows moved to summer highland pastures respect to indoor cows before (June), during (July, August, and September) and after (October) transhumance.

Traits	Pattern on indoor cows:		Difference of transhumant respect to indoor cows:				
	P-value	Order : shape	June	July	August	September	October
Ammonia N, mmol/L	>0.05	-	0.50	-4.56***	-1.80	-5.65***	1.14
Total VFA, mmol/L	>0.05	-	-29.90**	-35.00***	-27.80**	-23.80**	-7.60
Volatile fatty acids, mol%:							
Acetic Acid	0.019	Q: down-up	2.40	1.10	1.80	3.90**	2.50*
Propionic Acid	0.028	Q: up-down	-1.70	-3.80**	-0.50	-3.00*	-0.60
Isobutyric Acid	>0.05	-	-0.02	0.17	-0.10	0.33**	0.15
n-Butyric Acid	<0.001	C: down-up-down	-0.11	2.57***	-1.16	-0.95	-1.40*
Isovaleric Acid	0.002	Q: down-up	-0.06	0.01	0.10	0.23*	-0.26**
n-Valeric Acid	<0.001	C: down-up-down	-0.02	-0.13	-0.18	-0.63***	-0.20*
Ratio (C2+C4):C3	0.038	Q: down-up	0.51	0.87*	0.10	0.69*	0.24
Methane production, mmol/mol of VFA	>0.05	-	-10.60**	-9.30**	-9.40**	-5.50	-1.80

*P<0.05; **P<0.01; ***P<0.001;
C2: Acetic acid; C3: Propionic Acid; C4: Butyric acid;
Q: up-down = zenithal quadratic pattern rising to a maximum during summer and then decreasing;
Q: down-up = nadir quadratic pattern decreasing to a minimum during summer and then increasing;
C: down-up-down = cubic pattern decreasing to a minimum in July, rising in September and then decreasing again.

Table 3. Rumen fermentation traits: designation, loadings, explained variance (in % of total variance), conditional determination coefficient (R^2c) and root mean square error (RMSE) of the scores of the latent explanatory factors of the rumen fermentation traits (FN-rf) ($\chi^2 = 410.71$; 5 degrees of freedom; $P < 0.001$).

Items	F1-rf "VFA ratio"	F2-rf "Methane"	F3-rf "iso-VFA"	F4-rf "n-C4"	F5-rf "n-C5"	Communality
Loadings:						
N-NH_3, mmol/L		0.384			0.324	0.351
Total VFA, mmol/L		0.954*				>0.900
Acetic acid, mol%	0.888*			-0.317		>0.900
Propionic acid, mol%	-0.938*					>0.900
Isobutyric acid, mol%			0.916*			>0.900
n-butyric acid, mol%				0.931*		>0.900
Isovaleric acid, mol%	0.339		0.420			0.324
n-valeric acid, mol%					0.882*	>0.900
Ratio (C2+C4):C3	0.939*					>0.900
Methane production, mmol/mol of VFA		0.961*				>0.900
Explained variance :						
individual	28.1%	20.1%	12.1%	11.0%	10.3%	-
cumulative	28.1%	48.2%	60.3%	71.3%	81.6%	-
Statistical analysis of the scores:						
Group × Month, *F-value*	2.85 *	4.46 ***	2.33 *	3.88 **	5.73 ***	-
R^2c	0.32	0.51	0.37	0.45	0.49	-
RMSE	0.80	0.71	0.80	0.74	0.61	-

* High loading, >0.50;
*P<0.05; **P<0.01; ***P<0.001;
C2: Acetic acid; C3: Propionic Acid; C4: Butyric acid;
R^2c = conditional R^2.

Table 4. Rumen microbiota: descriptive statistics, significance of the Month × Group interaction, conditional determination coefficient (R^2c) and root mean square error (RMSE) of the rumen microbiological counts (expressed as log CFU/mL), and of the log10 relative abundancy of the rumen bacterial taxa.

Traits	Samples N	Descriptive statistics:		Month × Group *F-value*	R^2c	RMSE
		Mean	±SD			
Rumen bacterial counts:						
WC: anaerobic TB	58	6.463	0.635	6.0 ***	0.535	0.451
M17: mesophilic lactococci	58	5.778	0.595	4.3 ***	0.513	0.436
MRS: mesophilic lactobacilli	58	6.146	0.638	3.9 **	0.486	0.483
MRS-*cys:* bifidobacteria	58	6.443	0.803	5.7 ***	0.588	0.54
YELA: putative propionibacteria	56	4.381	0.761	6.5 ***	0.522	0.548
BBM: Rumen medium	57	5.573	0.696	2.4 *	0.278	0.629
Rumen metagenomics:						
Archea	57	0.240	0.101	9.8 ***	0.633	0.063
Actinobacteria	58	0.043	0.035	4.8 ***	0.505	0.026
Prevotella	58	1.605	0.057	3.8 **	0.377	0.047
Other *Bacteroidales*	57	1.378	0.101	5.1 ***	0.532	0.072
Other *Bacteroidetes*	58	0.014	0.014	1.4	0.324	0.013
Elusimicrobia	58	0.035	0.028	4.2 ***	0.434	0.021
Fibrobacter	57	0.311	0.221	21.2 ***	0.777	0.106
Lachnospiraceae	58	0.667	0.141	12.6 ***	0.690	0.081
Ruminococcaceae	58	0.921	0.138	6.3 ***	0.503	0.101
Veillonellaceae	58	0.356	0.179	3.2 **	0.391	0.147
Clostridia	58	0.756	0.088	3.6 **	0.381	0.073
Erysipelotrichaceae	56	0.112	0.069	3.2 **	0.352	0.059
Lentisphaerae	56	0.055	0.061	14.9 ***	0.790	0.029
Planctomycetes	58	0.059	0.039	4.2 ***	0.465	0.030
Alphaproteobacteria	57	0.060	0.036	3.4 **	0.377	0.030
Deltaproteobacteria	57	0.097	0.067	10.8 ***	0.640	0.041
Succinivibrionaceae	58	0.576	0.381	4.3 ***	0.531	0.273
Gammaproteobacteria	58	0.677	0.293	2.1	0.252	0.270
Spirochaetes	58	0.634	0.146	3.7 **	0.375	0.121
Tenericutes	58	0.381	0.109	9.2 ***	0.599	0.072
Verrucomicrobria	58	0.042	0.032	5.8 ***	0.023	0.526

*P<0.05; **P<0.01; ***P<0.001;

R^2c = conditional R^2.
Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.*

Table 5. Rumen microbiota: significance, order and shape of the patterns observed for microbial count (expressed as log CFU/mL) and relative abundance of bacterial taxa on rumen fluid samples collected during the experiment on cows maintained always indoor, and difference of cows moved to summer highland pastures respect to indoor cows before (June), during (July, August, and September) and after (October) transhumance.

	Pattern on indoor cows:		Difference of transhumant respect to indoor cows:				
	P-value	Order:shape	June	July	August	September	October
Microbiological count:							
WC: anaerobic TB	>0.05	-	-0.23	1.24***	0.38	0.69*	0.25
M17: mesophilic lactococci	>0.05	-	0.20	0.48	0.86**	0.27	0.00
MRS: mesophilic lactobacilli	0.002	C: down-up-down	-0.48	1.09**	0.52	0.08	0.60
MRS-cys: bifidobacteria	0.019	Q: up-down	-0.32	0.99*	0.26	0.37	0.34
YELA: putative propionibacteria	0.023	C: up-down-up	-0.04	-0.17	-0.02	0.76*	0.07
BBM: rumen medium	>0.05	-	0.14	1.04**	0.03	0.64	0.77*
Rumen fluid bacteria:							
Archea	>0.05	-	-0.06	0.01	-0.20***	0.00	0.02
Actinobacteria	0.015	C: down-up- down	-0.01	0.06***	-0.01	0.01	0.02
Prevotella	>0.05	-	-0.01	0.02	-0.06*	-0.11***	-0.01
Other Bacteroidales	0.002	L:up	0.05	0.10*	0.12*	0.15**	0.02
Other Bacteroidetes	>0.05	-	0.00	0.00	-0.01	0.00	-0.01
Elusimicrobia	0.029	C: up-down-up	0.02	-0.01	0.02	0.03*	0.04**
Fibrobacter	>0.05	-	0.07	0.48***	0.58***	0.32***	0.08
Lachnospiraceae	0.042	Q: down-up	-0.13*	0.19***	0.20***	0.18***	0.12*
Ruminococcaceae	0.024	L: down	0.01	-0.11	-0.15*	0.12*	-0.10
Veillonellaceae	0.034	C: down-up-down	-0.11	0.26**	0.28**	0.00	0.07
Clostridia	0.047	L: down	-0.07	0.09*	-0.02	0.14**	0.12**
Erysipelotrichaceae	>0.05	-	-0.03	0.10**	0.13**	0.04	0.06
Lentisphaerae	>0.05	-	0.02	0.06**	0.12***	0.17***	0.05*
Planctomycetes	0.020	L: up	-0.01	0.00	-0.04*	0.04*	0.01
Alphaproteobacteria	0.003	Q: down-up	0.02	0.00	0.07***	0.02	0.00
Deltaproteobacteria	>0.05	-	-0.01	0.02	-0.03	0.17***	-0.04
Succinivibrionaceae	0.010	Q: up-down	0.09	-0.68***	-0.56**	-0.52*	-0.27
Gammaproteobacteria	0.017	Q: down-up	-0.17	-0.07	-0.09	-0.03	0.01
Spirochaetes	>0.05	-	0.05	0.01	-0.07	-0.08	-0.15*
Tenericutes	<0.001	L: up	0.09	0.17***	0.18***	0.10*	0.05
Verrucomicrobria	0.015	L: up	0.00	0.00	0.00	0.05**	0.03*

*P<0.05; **P<0.01; ***P<0.001;

L: up = linear pattern growing from June to October;
L: down = linear pattern decreasing from June to October
Q: up-down = zenithal quadratic pattern rising to a maximum during summer and then decreasing;
Q: down-up = nadir quadratic pattern decreasing to a minimum during summer and then increasing;
C: up-down-up = cubic pattern rising to a maximum in July, decreasing to a minimum in September and then increasing again;
C: down-up-down = cubic pattern decreasing to a minimum in July, rising in September and then decreasing again.
Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.*

Table 6. Rumen microbiota: loadings of the latent explanatory factors of relative abundance of the rumen bacterial taxa. ($\chi^2 = 97.76$; 70 degrees of freedom; P=0.016)

	F1-rm	F2-rm	F3-rm	F4-rm	F5-rm	F6-rm	F7-rm	F8-rm	Commu-nality
Archea		0.88*							0.79
Actinobacteria					0.67*				0.51
Prevotella	-0.35					-0.81*			>0.90
Other *Bacteroidales*	0.84*					0.33			>0.90
Other *Bacteroidetes*									0.14
Elusimicrobia							0.91*		>0.90
Fibrobacter	0.72*		0.47						>0.90
Lachnospiraceae			0.42		0.84*				>0.90
Ruminococcaceae		0.76*							0.73
Veillonellaceae			0.72*						0.73
Clostridia		0.31		0.37	0.47			0.41	0.77
Erysipelotrichaceae			0.51*						0.45
Lentisphaerae	0.64*			0.35		0.43			0.89
Planctomycetes	0.30	0.34		0.54*	-0.39				0.66
Alphaproteobacteria	0.54						0.31	-0.41	0.62
Deltaproteobacteria				0.45		0.66*			0.78
Succinivibrionaceae	-0.90*								>0.90
Gammaproteobacteria			-0.62*						0.43
Spirochaetes				-0.79*					0.71
Tenericutes	0.64*	-0.34							0.72
Verrucomicrobria	0.58*			0.49					0.74

* High loading, >0.50;
Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.*

Table 7. Rumen metagenomics: descriptive statistics, significance levels of the Month × Group interaction, and root mean square error (RMSE) of the latent explanatory factors of the rumen bacterial taxa.

	Explained variance:		Month × Group F-value	R^2c	RMSE
	By each factor	Cumulative			
F1-rm	18.9%	18.9%	5.0***	0.543	0.685
F2-rm	9.5%	28.4%	8.8***	0.616	0.581
F3-rm	9.2%	37.6%	4.3***	0.485	0.688
F4-rm	9.1%	46.7%	4.3**	0.393	0.762
F5-rm	8.8%	55.5%	8.0***	0.592	0.620
F6-rm	8.0%	63.5%	6.2***	0.518	0.683
F7-rm	5.8%	69.3%	3.2**	0.361	0.800
F8-rm	2.7%	72.0%	1.7	0.269	0.747

*P<0.05; **P<0.01; ***P<0.001;
R^2c = conditional R^2.

Table 8. Rumen fermentation traits and metagenomics: loadings of the latent explanatory factors and communality coefficients of rumen traits included in the factor analysis (χ^2 = 614.05; 200 degrees of freedom; P<0.001).

Items	F1-rfm	F2-rfm	F3-rfm	F4-rfm	F5-rfm	F6-rfm	F7-rfm	F8-rfm	F9-rfm	F10-rfm	Communality
Rumen fermentation traits:											
N-NH_3, mmol/L		-0.442	-0.440								0.622
Total VFA, mmol/L				0.920*							>0.900
Acetic acid, %mol	0.864*							-0.365			>0.900
Propionic acid, %mol	-0.948*										>0.900
Isobutyric acid, %mol			0.629*								0.534
N-butyric acid, %mol								0.923*			>0.900
Isovaleric acid, %mol	0.323		0.452							0.414	0.551
N-valeric acid, %mol									0.838*		>0.900
Ratio (C2+C4):C3	0.935*										>0.900
CH_4 production, mmol/mol of VFA				0.929*							>0.900
Rumen metagenomic taxa :											
Archea	0.367	-0.487									0.484
Actinobacteria					0.662*						0.509
Prevotella			-0.788*								>0.900
Other Bacteroidales	0.621*	0.432	0.449								>0.900
Other Bacteroidetes											0.149
Elusimicrobia								-0.426	-0.342		0.433
Fibrobactere		0.745*					0.312				>0.900
Lachnospiraceae					0.847*		0.374				>0.900
Ruminococcaceae	0.306	-0.521*	0.304								0.606
Veillonellaceae							0.820*				0.874
Clostridia	0.346		0.312		0.464	0.332					0.708
Erysipelotrichaceae					0.301		0.447				0.412
Lentisphaerae		0.519*	0.452			0.356					0.856
Planctomycetes	0.400				-0.488	0.374					0.713

Alphaproteobacteria	0.385					0.639*	0.746
Deltaproteobacteria			0.633*	0.411			0.646
Succinivibrionaceae	-0.764*	-0.425					0.894
Gammaproteobacteria	0.329				-0.336		0.318
Spirochaetes				-0.893*			>0.900
Tenericutes		0.775*					0.729
Verrucomicrobria	0.465		0.348	0.395			0.748

* High loading, >0.50;

Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio*.

Table 9. Rumen fermentation traits and metagenomics: descriptive statistics, significance levels of the Month × Group interaction, and root mean square error (RMSE) of the latent explanatory factors.

	Explained variance:		Group × Month F-value	R^2c	RMSE
	Individual	Cumulative			
F1-rfm	16.4%	16.4%	2.87*	0.33	0.80
F2-rfm	9.7%	26.1%	10.76***	0.63	0.59
F3-rfm	9.5%	35.6%	6.79***	0.52	0.68
F4-rfm	7.1%	42.7%	3.08**	0.43	0.77
F5-rfm	7.0%	49.7%	6.87***	0.56	0.65
F6-rfm	6.0%	55.7%	3.39**	0.35	0.80
F7-rfm	5.3%	61.0%	1.83	0.37	0.79
F8-rfm	5.1%	66.1%	3.95**	0.47	0.73
F9-rfm	3.6%	69.7%	4.15***	0.42	0.65
F10-rfm	3.3%	73.0%	2.29*	0.27	0.77

*P<0.05; **P<0.01; ***P<0.001;
R^2c = conditional R^2.

Figure 1. Rumen fermentation traits: major sources of variation (expressed as % of total variance) of each trait: month × group combined effects (dark blue), individual cow within group (red), and residual variability (light blue).

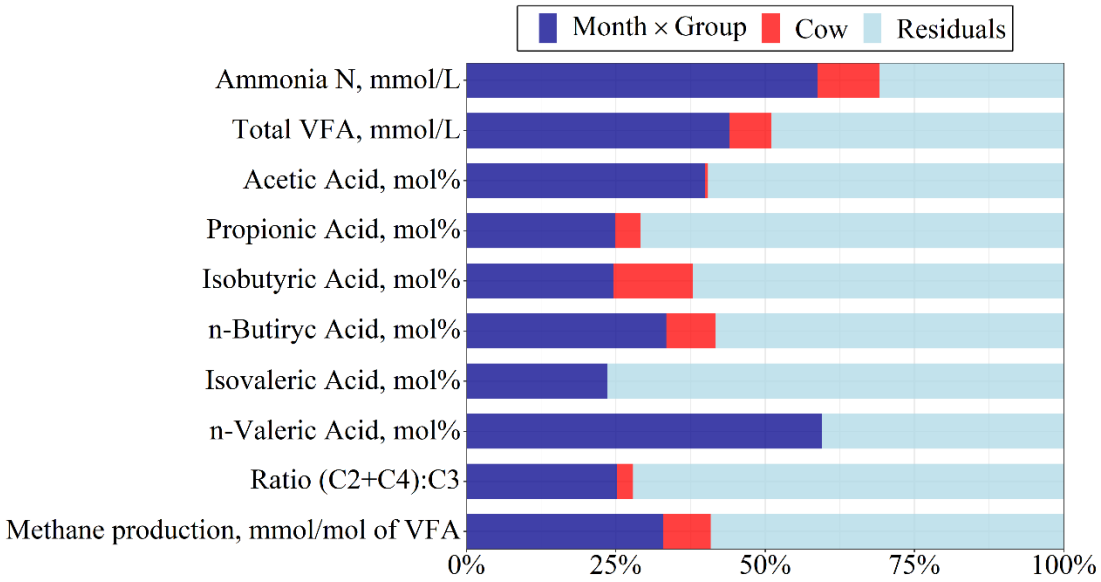
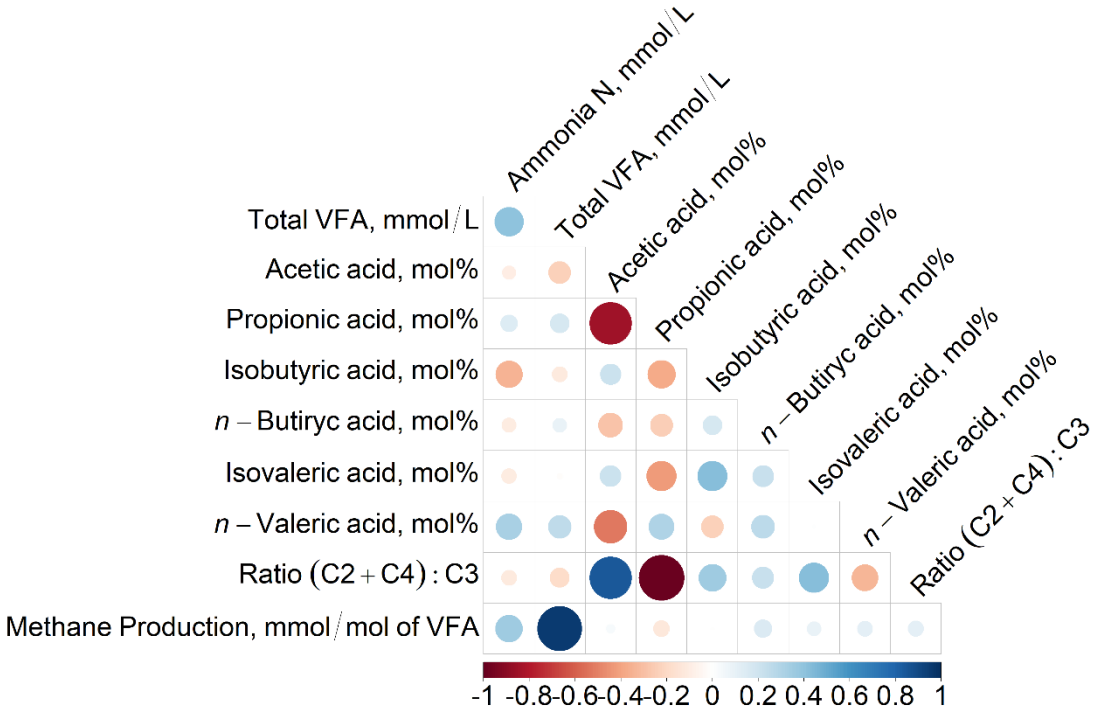


Figure 2. Rumen fermentation traits: Heat-map plot of correlations among rumen traits.



C2: Acetic acid; C3: Propionic Acid; C4: Butyric acid.

Figure 3. Rumen fermentation traits: patterns of the scores of the latent explanatory factors of rumen fermentation traits (F1-rf to F5-rf) during the experimental period. Blue circles represent LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent SE of estimates. Lines and curves represent significant linear, quadratic, or cubic patterns, with their R2 values, for the cows kept solely indoors. Asterisks indicate the significance levels of the differences between the 2 groups in each month (*P < 0.05; **P < 0.01; ***P < 0.001).

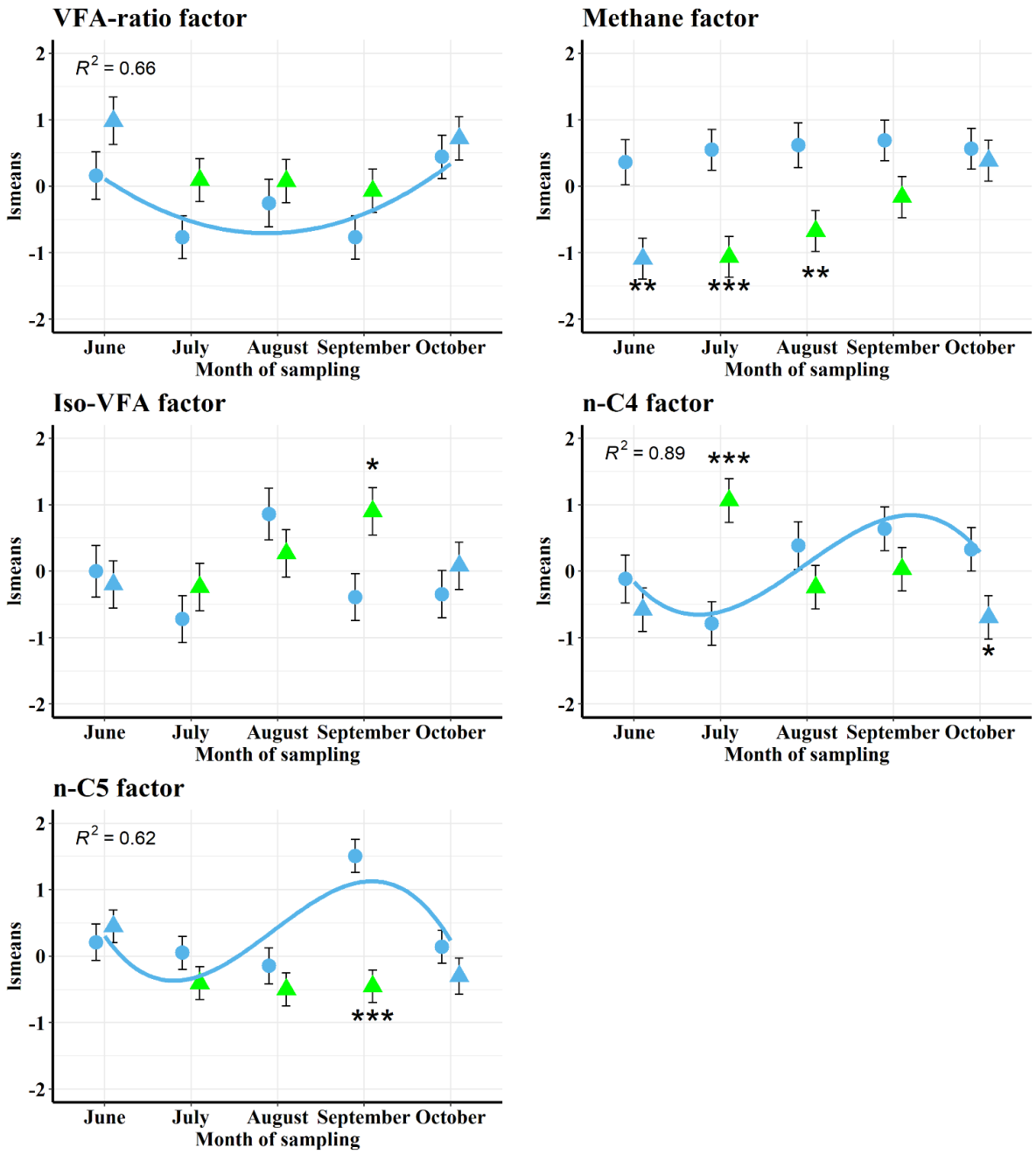


Figure 4. Rumen microbiota: major sources of variation (expressed as percentage of total variance) of each microbial count: month × group combined effects (dark blue), individual cow within group (red), and residual variability (light blue).

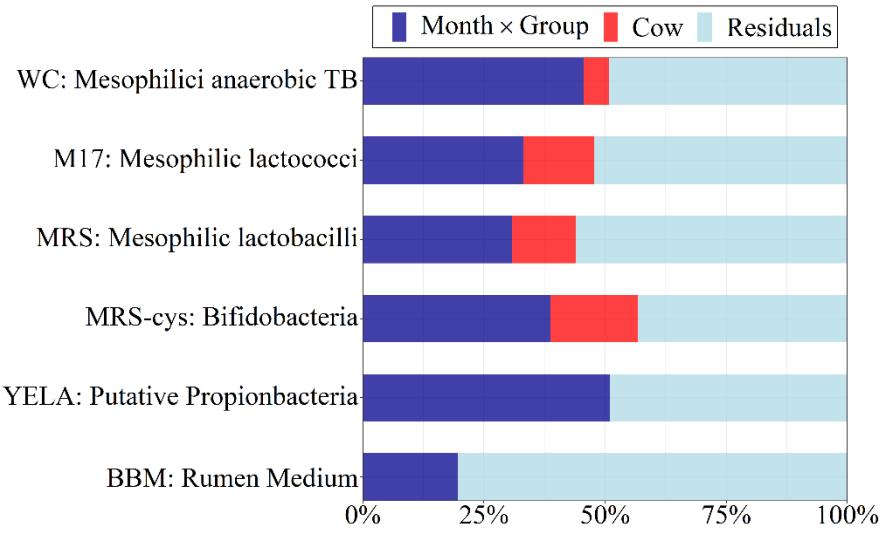
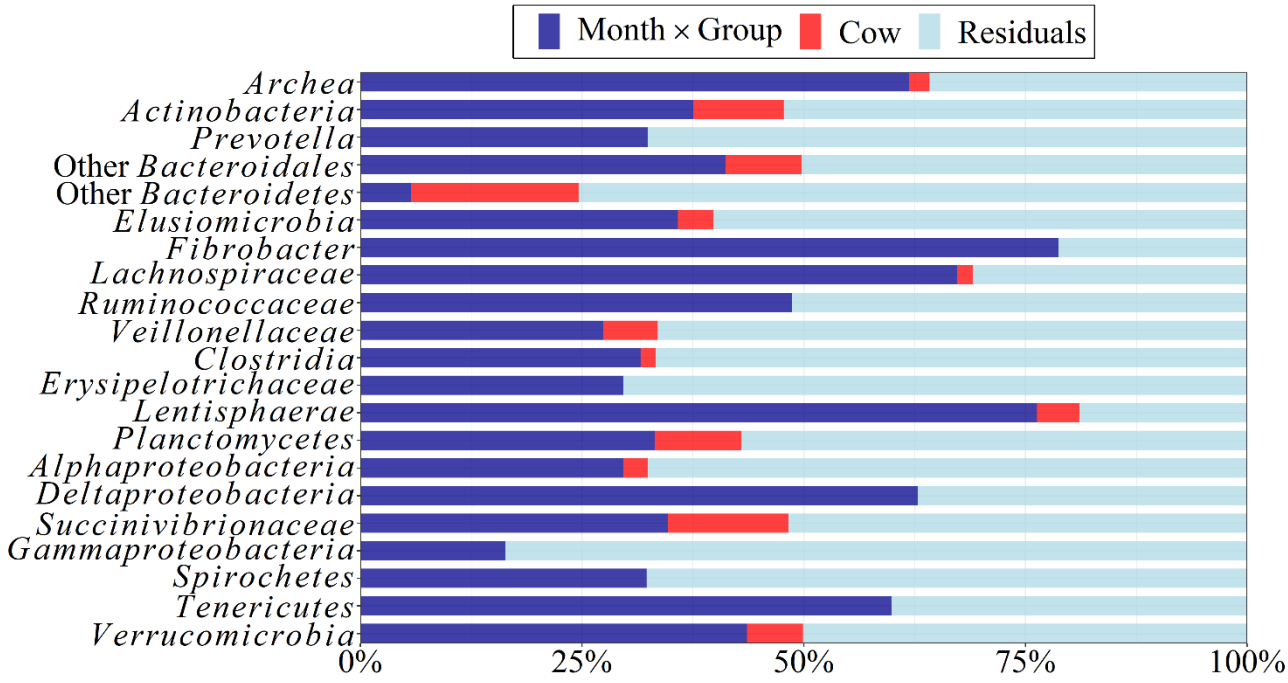
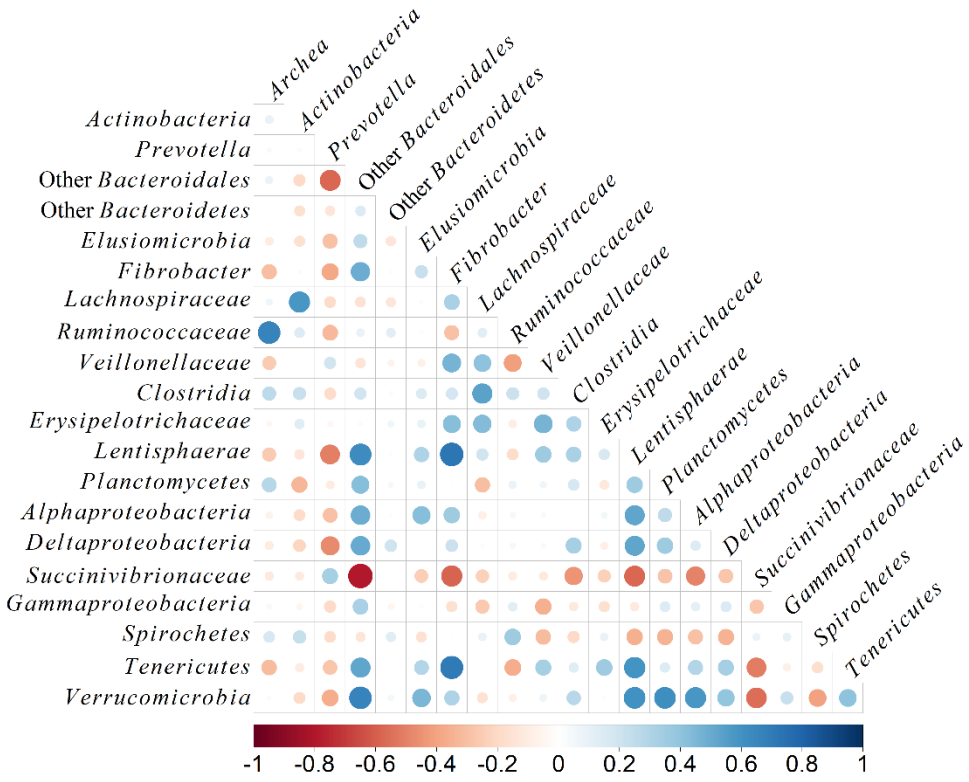


Figure 5. Rumen microbiota: major sources of variation (expressed as % of total variance) of individual rumen bacterial taxa relative abundances: Month × Group combined effects (dark blue), individual cow within group (red), and residual variability (light blue).



Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.*

Figure 6. Rumen microbiota: Heat-map plot of correlations among individual rumen bacterial taxa relative abundances.



Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.*

Figure 7. Rumen microbiota: Patterns of the scores of the latent explanatory factors during the experimental period. Blue circles represent LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent SE of estimates. Lines and curves represent significant linear, quadratic, or cubic patterns, with their R^2 values, for the cows kept solely indoors. Asterisks indicate the significance levels of the differences between the 2 groups in each month (*P < 0.05; **P < 0.01; ***P < 0.001).

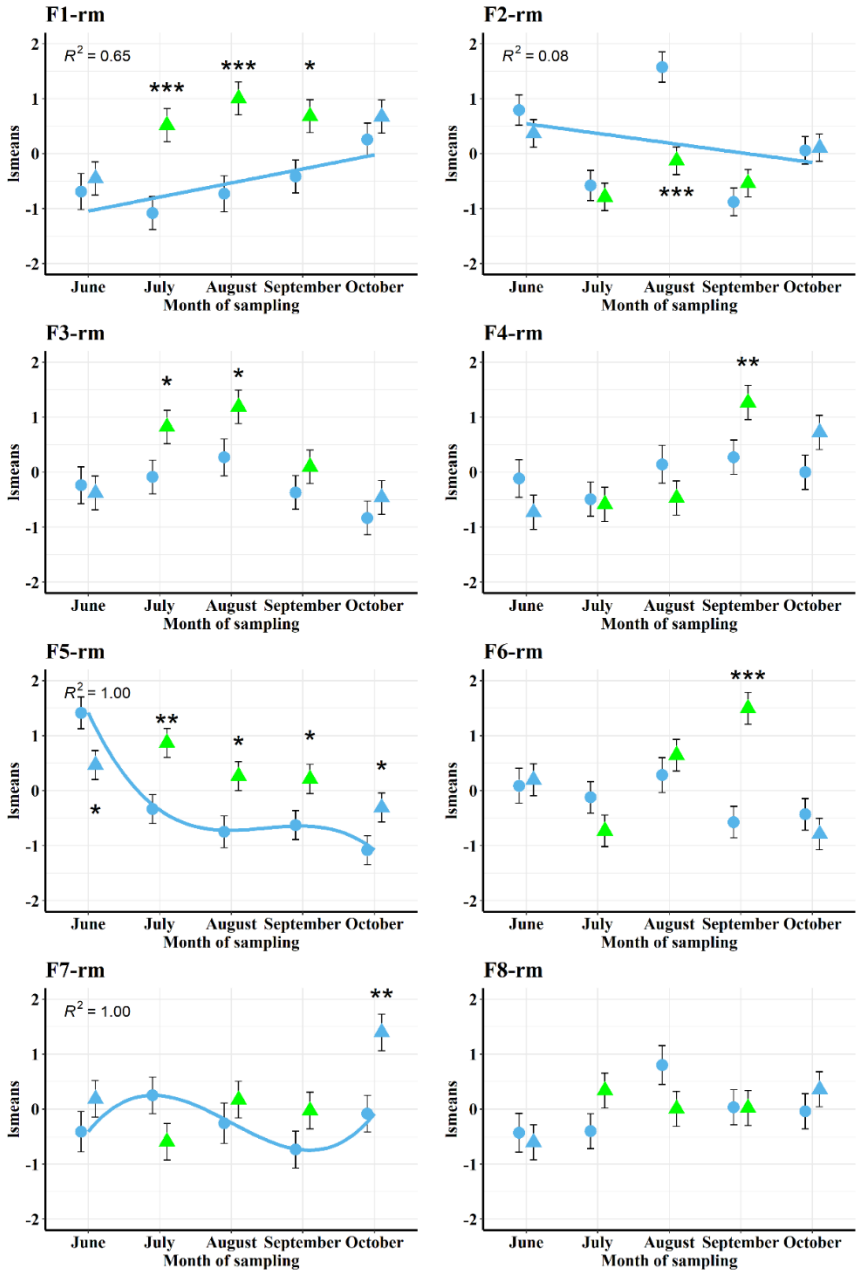
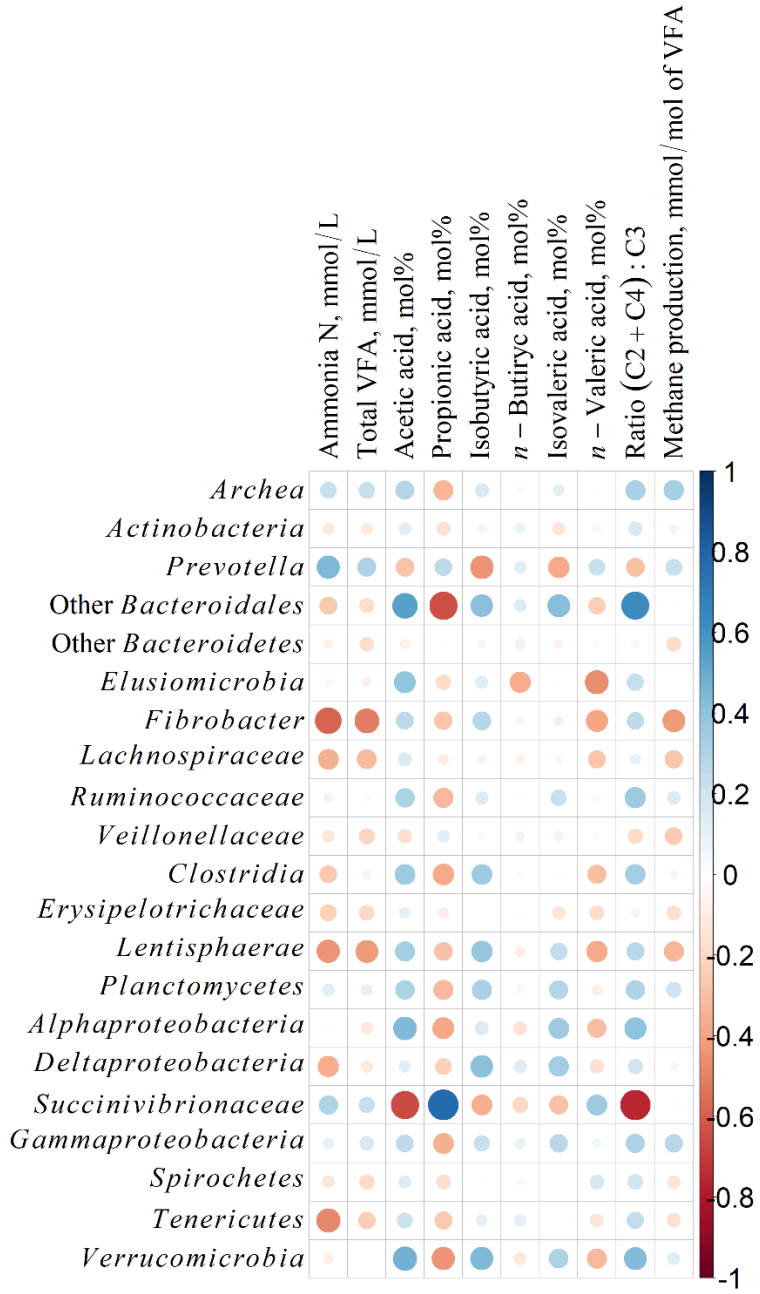
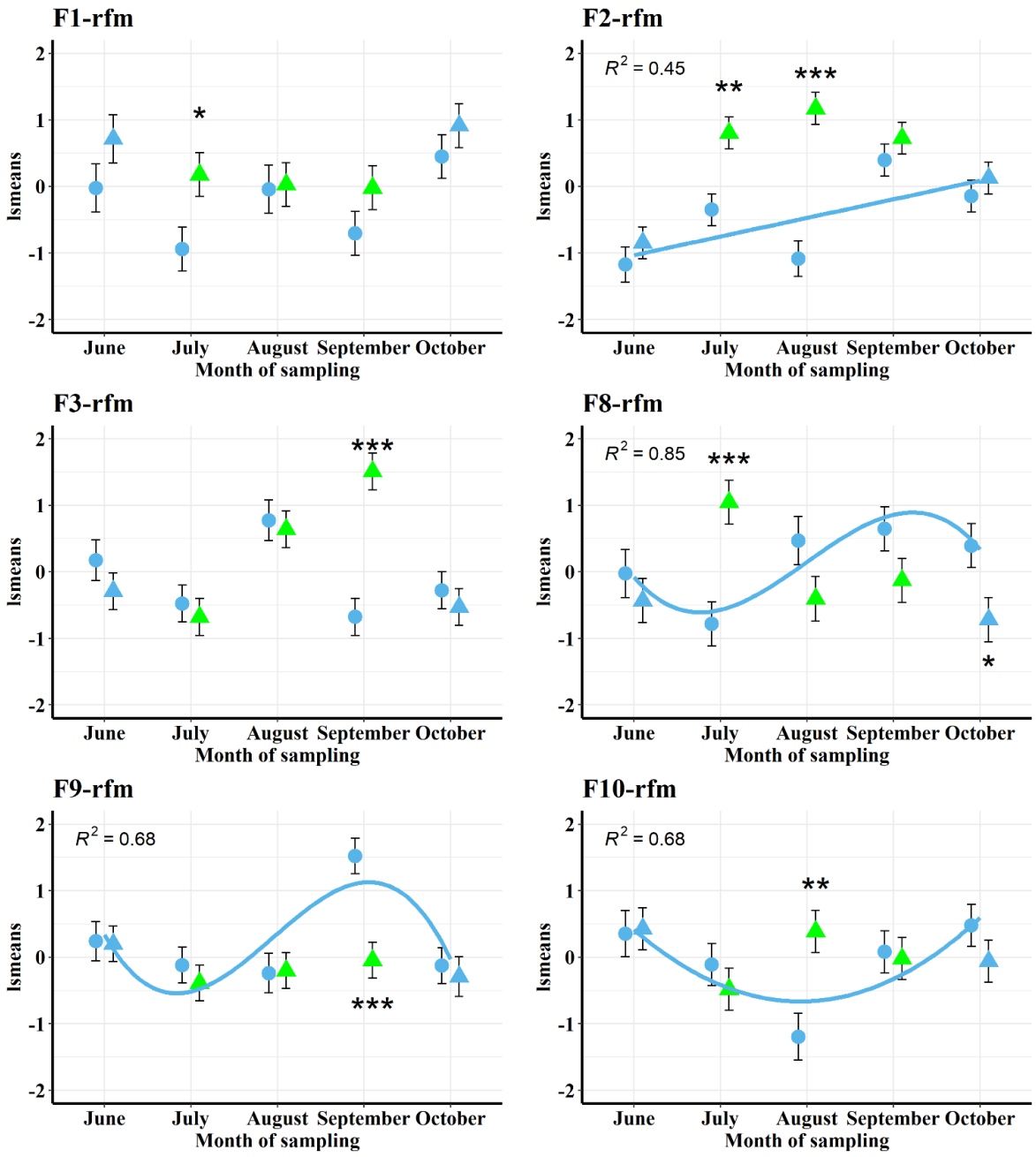


Figure 8. Rumen fermentation traits and microbiota: Heat-map plot of correlations between rumen bacterial taxa relative abundancies and rumen fermentation traits.



Prevotella: copri, melaninogenica; *Lachnospiraceae: Blautia et cetera; Ruminococcaceae: Oscillospira, Ruminococcus, Ethanoligenens; Veillonellaceae: Anaerovibrio, Selenomonas, Succiniclasticum; Succinivibrionaceae: Ruminobacter, Succinivibrio.* C2: acetic acid; C3: propionic acid; C4: Butyric acid.

Figure 9. Rumen fermentation traits and microbiota: patterns during the experimental period of the scores of the 6 out of 10 “mixed” latent explanatory factors (based each including rumen fermentation traits and rumen bacterial taxa. Blue circles represent LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent SE of estimates. Lines and curves represent significant linear, quadratic, or cubic patterns, with their R2 values, for the cows kept solely indoors. Asterisks indicate the significance levels of the differences between the 2 groups in each month (*P < 0.05; **P < 0.01; ***P < 0.001).



CHAPTER II

Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing

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Editor's Choice

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Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing

Secchi et al.

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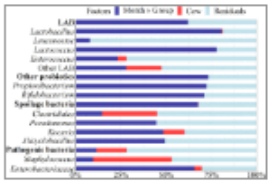

INTERPRETIVE SUMMARY

Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing

By Secchi et al., page 96-116. This study addresses the relationships between milk metagenomics, milk composition, and dairy efficiency of 2 groups (6 cows/group) of Brown Swiss cows sampled over 5 months under two types of rearing: indoor farming and summer highland grazing. A total of 44 bacterial taxa were obtained in milk, 13 of which are of interest to the dairy industry (lactic acid bacteria, LAB, and spoilage bacteria), and for human and animal health (other probiotics, and pathogenic bacteria). While on summer highland pastures the cows exhibited an increase in almost all the LAB, bifidobacteria and propionibacteria, and a reduction in spoilage taxa. All the bacterial changes disappeared when the cows were moved back indoors from the alpine pasture.

ABSTRACT

The study of the complex relationships between milk metagenomics and milk composition and cheese-making efficiency as affected by indoor farming and summer highland grazing was the aim of the present work. The experimental design considered monthly sampling (over 5 mo) of the milk produced by 12 Brown Swiss cows divided into two groups: the first remained on a lowland indoor farm from June to October, while the second was moved to highland pastures in July, and then returned to the lowland farm in September. The resulting 60 milk samples (2 kg each) were used to analyze milk composition, milk coagulation, curd firming, and syneresis processes, and to make individual model cheeses to measure cheese yields and nutrient recoveries in the cheese. After DNA extraction and Illumina Miseq sequencing, milk microbiota amplicons were also processed by means of an open-source pipeline called Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2; https://qiime2.org). Out of a total of 44 taxa analyzed, 13 bacterial taxa were considered important for the dairy industry (lactic acid bacteria, LAB, 5 taxa; and spoilage bacteria, 4) and for human (other probiotics, 2) and animal health (pathogenic bacteria, 2). The results revealed the transhumant group of cows transferred to summer highland pastures showed an increase in almost all the LAB taxa, Bifidobacteria, and propionibacteria, and a reduction in spoilage taxa. All the metagenomics changes disappeared when the transhumant cows were moved back to the permanent indoor farm. The relationships between 17 microbial traits and 30 compositional and technological milk traits were investigated through analysis of correlation and latent explanatory factor analysis. Eight latent factors were identified, explaining 75.3% of the total variance, 2 of which were mainly based on microbial traits: pro-dairy bacteria (14% of total variance, improving during summer pasturing) and pathogenic bacteria (6.0% of total variance). Some bacterial traits contributed to other compositional-technological latent factors (gelation, udder health, and caseins).

Keywords: milk microbiota, probiotic bacteria, dairy bacteria, milk spoilage, summer transhumance.

INTRODUCTION

The microbiota of milk has been studied for many decades because of the important relationships between milk microorganisms on one side, and milk characteristics, end product, economic impact, and nutritional and health values on the other (Quigley et al., 2013; Boor et al., 2017; Issa and Tahergorabi, 2019). The most interesting of the favorable relationships between microbes and the various milk characteristics are those that concern the role of microbial species, especially lactic acid bacteria (**LAB**), in relation to milk end products, particularly cheese (Skeie, 2007; Ardö et al., 2017; Nam et al., 2021), and digestion and intestinal functions and integrity in human consumers (prebiotics and probiotics; Aryana and Olson, 2017; Nyanzi et al., 2021). The most interesting of the unfavorable relationships are the potential effects of some microbial species on the spoilage of milk and dairy products (Quigley et al., 2013; Martin et al., 2021), and on the health of lactating animals (mastitis; Andrews et al., 2019) and of human consumers (pathogenic activities; Verraes et al., 2015).

The recent development of metagenomics is now expanding our knowledge of these aspects of milk microbiota (Addis et al., 2016; Parente et al., 2020). Traditional microbiological studies were based on identifying, isolating, characterizing, and counting individual microbial species or strains (Tilocca et al., 2020). With metagenomics, the entire milk microbiota composition can be identified and characterized. Alongside ecological studies of milk microbial communities, we can now gather new information on many microbial taxa involved in different compositional, technological, and nutritional properties of milk.

One of the most important, but also difficult to study, issues is the effect of dairy system, particularly pasture grazing, on the milk microbiota, and the effect of the microbiota on the properties associated with milk processing and end products, nutritional value, and consumer health (Doyle et al., 2017). The difficulties lie mainly in disentangling the confounding effects of environment, management, animal characteristics, season, feedstuffs, and hygiene (Du et al., 2020).

The opposite extremes of dairy farming are represented by the intensive indoor system, with all-year-round use of total mixed rations, and forage-based systems, where the cows are kept at pasture day and night, and have only limited access to compound feed during milking (O'Callaghan et al., 2017). Among the latter, farms that practice transhumance to temporary farms on highland summer pastures are very distinctive for both the extreme environmental conditions the animals face, and the renowned quality and nutritional value of their dairy products (Buchin et al., 1999). Little is known of the extent to which the specificity of mountain dairy products is due to the milk microbiota. We hypothesized that the summer transhumance to highland summer pastures would alter the microbial population of the milk, and that milk microbiota could affect the cheese-making process.

The general aim of this research, therefore, was to study the milk microbiota in indoor housing versus summer highland grazing and its relationships with milk quality and technological properties, with particular emphasis on the bacterial taxa related to various specific activities [cheese making, health maintenance (probiotics), milk spoilage, and pathogeny], and the effects of moving the cows from indoor conditions to the summer highland pasture, and then back to indoor conditions.

MATERIALS AND METHODS

Experimental Design and Milk Sampling

This study is part of a larger project studying the effects of the transhumance of cows to summer highland pastures on their productivity, and on the chemical, technological and microbiological characteristics of the milk produced. In this project, 2 groups of cows (one kept solely indoors, the other moved to summer pasture) were monitored before, during, and after summer transhumance. Details on the environmental conditions and methodology can be found in 2 previous studies: the first dealing with the cows' body condition and milk yield, milk composition, and cheese-making efficiency (Saha et al., 2019), and the second reporting some preliminary data on milk microbial counts (Carafa et al., 2020). All samples and measurements were obtained during the farms' normal milking procedures; therefore ethics commission approval was not required.

In line with the aims of this project, the present study was carried out at 2 farms in a mountain area (Trentino Province, northeastern Italian Alps): (1) a modern, permanent farm in a valley (Malè, Trento, Italy; 737 m above sea level), where lactating cows are loose housed indoors, fed TMR, and milked in a milking parlor; and (2) a temporary summer highland farm (Malga Juribello, within the "Paneveggio – Pale di San Martino" Nature Reserve, Passo Rolle, Trento, Italy; 1,860 m above sea level), where cows are kept at pasture day and night, and are milked and given a supplementary compound feed (3 to 6 kg/d, according to milk production) in a milking parlor in an old barn.

Briefly, the experimental design consisted of the following steps: the selection at the end of May of 12 healthy, multiparous, early-lactation Brown Swiss cows on the permanent lowland farm, and their random division into 2 groups of 6 cows each. The cows in both groups had similar ($P > 0.05$, based on t-test) parity numbers (2.5 and 2.8, respectively) and DIM (143 and 120, respectively); all 12 cows were kept together in the same indoor pen before the start of the experiment (beginning of June) and during the first (June) and last month of sampling (October). During summer (July, August, and September) 1 of the 2 groups was moved to the temporary highland farm (high group) at

the beginning of July, and returned to the permanent farm at the end of September; the other group remained indoors on the permanent lowland farm (low group). Monthly samples of milk from each cow at the evening milking, from mid-June to mid-October (5 samples per cow) were taken in both farms (60 samples in total).

The samples taken in June represent the initial condition of the 2 groups: having been reared together, non-significant differences for all traits were expected. The samples taken in July, August, and September represent the effects of the 2 farming conditions: indoors in the valley versus on highland pasture. The fifth sample, taken in October after the 2 groups had been together again for a month, represented potential carryover effects of summer pasturing on indoor rearing.

Each sample was divided into 2 aliquots: the first (50 mL) was immediately frozen in liquid nitrogen, taken to the Research and Innovation Centre, Food Quality and Nutrition Department of the Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy), and stored at -80 °C before microbiological analyses within 3 mo; the second aliquot (2 L) was immediately refrigerated at 4 °C and transported to the Milk Laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Padua, Italy) for evaluation of milk quality, cheese-making aptitude, and cheese yield.

Metagenomic Analyses

In a previous study (Carafa et al., 2020), we analyzed in detail the bacterial counts of milk regarding a preliminary comparison of the samples taken during summer pasturing (n = 18) with those taken indoors on the permanent farm (n = 42), without taking into account the effects of group and month. In the present study, the principal bacterial taxa identified by Qiime2 (version 2018.2; https://qiime2.org; Bolyen et al., 2019) were classified into four categories according to their potential activity in relation to the cheese-making (LAB), other probiotics, spoilage, and pathogenic properties of milk, and were statistically analyzed, disentangling the effects of individual cows,

groups of cows, month of sampling, and environmental and feeding conditions. The relationships between the metagenomic information and the qualitative and cheese-making properties of milk were also explored.

In brief, genomic DNA was extracted using the DNeasyPower Food Microbial Kit (Qiagen) and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific). The Miseq Library (Illumina) was prepared according to the authors' recommendations and followed by Illumina sequencing. All the sequencing data were processed using Qiime2, and the final data were deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA528228), where they can be accessed with accession number PRJNA528228.

Bacterial Categories

The 4 categories of the identified taxa are here briefly reported. The LAB category includes the taxa belonging to the *Lactobacillaceae* family, including *Lactobacillus*, *Leuconostoc; (*Zheng et al., 2020), *Lactococcus,* and *Enterococcus (*Gagnon et al., 2020); the "other probiotics" category includes all the taxa belonging to the *Propionibacterium* (Rabah et al., 2017) and *Bifidobacterium* genera (Prasanna et al., 2014). The "spoilage bacteria" category includes all the taxa belonging to the order *Clostridiales* (Burtscher et al., 2020) and the genera *Pseudomonas* (Meng et al., 2017), *Kocuria* (Ribeiro-Júnior et al., 2020), and *Alicyclobacillus* (Pornpukdeewattana et al., 2020). Finally, the "pathogenic" category includes all the taxa belonging to the genus *Staphylococcus* (Gebremedhin et al., 2022), and the family *Enterobacteriaceae* (Anand and Griffiths, 2011). The remaining 31 bacterial taxa were grouped as "other milk bacteria".

Milk Composition, Cheese-Making Aptitude, and Cheese Yield

Within 20 h of collection, the second aliquot was used to evaluate milk composition, traditional coagulation properties, whey composition, cheese yields, and milk nutrient recoveries in the curd, and for curd firming modeling and the manufacture of model cheeses.

Milk composition

In brief, milk composition traits (TS, fat, nonfat solids, protein, casein, lactose, and urea contents) were evaluated with a Milkoscan FT2 infrared analyzer (Foss A/S). Somatic cell counts were obtained with a Fossomatic Minor FC counter (Foss A/S) and then log-transformed to SCS. The fat/protein ratio and casein number (casein as a percentage of protein) were computed from the fat, protein, and casein contents. In the present study, we used the qualitative and technological characteristics of the milk samples to search for potential relationships with the metagenomic information from the same milk samples.

Milk coagulation properties and curd firming modeling parameters

Traditional milk coagulation properties were using a lactodynamograph (Formagraph; Foss A/S) according to Cecchinato et al. (2013), and consisted of the following: rennet coagulation time (**RCT**, min), curd-firming time ($\mathbf{k_{20}}$, min), and curd firmness (**a**) 30, 45, and 60 min after rennet addition (a_{30}, a_{45}, and a_{60}; mm). Estimates of the curd-firming and syneresis equation parameters of each individual milk sample were obtained by extracting 240 curd firmness values (one every 15 s for 60 min) from the lactodynamograph. The equation parameters were rennet coagulation time by equation ($\mathbf{RCT_{eq}}$, min), the curd-firming instant rate constant (k_{CF}, %/min), the syneresis instant rate constant ($\mathbf{k_{SR}}$, %/min), maximum curd firmness ($\mathbf{CF_{max}}$, mm), and time to reach CF_{max} ($\mathbf{t_{max}}$, min; Malchiodi et al., 2014).

Model Cheese Making, Cheese Yields, Milk Nutrient Recoveries in the Curd, and Whey Composition

A larger aliquot (1,500 mL) from each milk sample was used to manufacture a model cheese according to a procedure that replicates the process for making full-fat cheese (Stocco et al., 2018). Briefly, the procedure was as follows. (1) Each milk sample was poured into a stainless-steel laboratory vat. (2) The vat was placed in a water bath and heated to 35 °C for 30 min; (3) Rennet solution (8 mL; Hansen Standard 215 with 80 ± 5% chymosin and 20 ± 5% pepsin; Pacovis Amrein AG) freshly diluted to 4.29% (wt/vol) in distilled, water was added. (4) After coagulation, the curd was cut. (5) The curd was drained for 30 min. (6) The resulting whey was collected, weighed and sampled. (7) The chemical composition (TS, fat, protein, and lactose) of the whey samples was analyzed with a Milkoscan FT2 infrared analyzer (Foss A/S). (8) The curd was pressed for 30 min at 250 kPa in a cheese-pressing machine, turning every 10 min. (9) The pressed curd wheel was soaked in a brine solution (20% NaCl) for 30 min. (10) After brining, the cheese wheels were weighed and the pH measured with a Crison Basic 20 electrode (Crison Instruments SA). The percentage yields of fresh cheese (%CY_{CURD}) and cheese solids (%CY_{SOLIDS}) were determined, as well as the following nutrient recovery traits (**REC**; the quantity of a given nutrient in the cheese as a percentage of the same nutrient in the milk processed): milk fat (REC_{FAT}, %), milk protein ($REC_{PROTEIN}$, %), total milk solids (REC_{SOLIDS}, %), and milk energy (REC_{ENERGY}, %).

Statistical Analysis

All relative bacterial abundancies were $\log_{10}$ transformed. Two samples were excluded from the statistical analysis because of a lack of microbiological data in one and of qualitative-technological data in the other. All bacterial and qualitative-technological data were checked to identify and exclude outlier values (outside the interval ± 3 SD of the mean).

Mixed-Model ANOVA

The $\log_{10}$ transformed relative abundances obtained from the milk metagenomic analysis were analyzed with a linear mixed model in the R environment (R Core Team, 2016), which included the fixed effects of the month × group interaction (10 levels: 5 mo, June to October; and 2 groups, high and low), and the random effect of cow within group. Polynomial contrasts were estimated between the 5 least square means of month within low group to determine the response curve of each trait (linear, quadratic, and cubic components) during the 5 mo the cows were kept indoors on the permanent farm as a measure of the effect of season and advancing lactation in the control group. Contrasts between the high and low groups were estimated within each month to test for the following: homogeneity of groups in the same environment (indoors) at the beginning of the trial (June); the effect of transhumance to highland pasture during the summer months (July, August, and September) compared with the control indoor group; and the carryover effect of summer pasture on the high group after returning to indoor conditions on the permanent farm (October). A similar model with the month × group interaction treated as a random factor was run to quantify the relative importance of this environmental or diet factor, individual animal within group, and residual factors not accounted for by the model. The variances in these 3 sources of variation were expressed as percentages of their sum (total variance).

The model used here is the same model that we used in the previous study (Saha et al., 2019) to analyze the qualitative and technological properties of milk. Thus, those results are not reported and discussed here, except where they are useful for interpreting relationships with the metagenomics data.

Correlation analysis and latent explanatory factor analysis

The 2 datasets of metagenomic relative abundances (only for the bacterial categories of interest; i.e. LAB, other probiotics, spoilage, and pathogenic bacteria), and the qualitative and technological properties of milk were merged for the correlation and multivariate analyses to explore the relationships between bacterial and chemical/technological traits. Correlations were calculated

among the metagenomic relative abundances of the selected taxa and groups, and between the metagenomic relative abundances and the qualitative and technological milk traits.

Due to the high number and complexity of the relationships among all the traits, we used a multivariate factor analysis (FA) to summarize the interrelated measured traits in a small number of unmeasured latent independent explanatory variables (factors). Factor anlysis was performed on the selected traits as follows. First, we performed Kaiser–Meyer–Olkin and Bartlett's tests, which showed that the traits were suitable for FA. The FA was carried out with Varimax rotation in the R environment (R Core Team, 2016) using the psych package (available at CRAN: The Comprehensive R Archive Network, version 2.2.9; https://cran.r-project.org/web/packages/psych/index.html) in 3 steps: (1) extraction of factors such that the minimum number of uncorrelated latent factors explained the greatest proportion of common variance; (2) factor rotation until each factor was defined by a few variables with high loadings; and (3) biological interpretation of the factors based on the strength of the loadings of the variables. The eigenvalues of the factors and the communalities of the variables after rotation were also determined.

Eight latent explanatory factors were extracted from the 47 milk traits selected (17 bacterial and 30 qualitative or technological traits). To better understand their meaning, the sign of all the loadings of the second, sixth and eighth factors were inverted. The scores of each milk sample for each factor were analyzed using the same linear mixed model as that used for the metagenomic relative abundances.

RESULTS

Factors of Variation in Milk Metagenomic Relative Abundances

As a first step in examining the factors of variation in the relative abundances of the bacterial taxa of the 4 designated groups, we present in Figure 1 a summary of the percentages of total variance represented by the combined effects of group of cows (low vs. high) and of month of sampling (June to October; dark blue), the effects of individual cows within group (red), and the residual sources of variations (light blue). It is clear that the effects of the group × month interaction represent a major source of variation (>45% of total variance) in the relative abundances of about two-thirds of the bacterial traits, with the exception of *Leuconostoc*, *Enterococcus*, other LAB, *Clostridiales*, pathogenic bacteria, and *Staphylococcus*. The variability due to individual cows was negligible for 10 out of 17 traits, very important for *Staphylococcus* and *Clostridiales* taxa, and moderate for *Enterococcus*, other LAB, *Kocuria*, pathogenic bacteria and *Enterobacteriaceae*.

Combined Effects of Group of Cow and Month of Sampling on Milk Metagenomic Relative Abundances

Table 1 shows the levels of statistical significance of the combined effects of group and month of sampling for the relative abundances of the 44 bacterial taxa identified, and their sums in categories defined by their prevalent activity. The month × group interaction exerted a significant effect on the relative abundances of LAB, other probiotics, and spoilage bacteria, and of all the taxa within these categories except for the *Leuconostoc* taxa. The pathogenic bacteria group was not significantly affected, although the *Staphylococcus* and *Enterobacteriaceae* taxa within this group were; these two taxa went in opposite directions. The relative abundances of the 31 bacterial taxa belonging to neither the desired nor the undesired groups were significantly affected in fewer than half of cases.

Figure 2 shows the plots of the relative abundances of the sum of all the milk LAB taxa having a desired effect on cheese-making, and of the individual taxa (*Lactobacillus*, *Leuconostoc,*

Lactococcus, *Enterococcus*, and other LAB). In the first plot, we can see that the relative abundance of LAB exhibits a significant quadratic pattern in the low group during the 5 mo of the experiment, with the lowest value occurring in August. In June, the difference between the high and the low groups of cows was not significant, which is expected as all the cows were housed and fed together indoors on the permanent lowland farm. In contrast, during the three summer months (July, August, and September), when the high group was on the summer highland pastures, their values were always significantly higher than the low group. At the end of summer, when the high group returned to join the low group on the permanent farm in the valley, the 2 groups showed no significant differences, indicating the absence of carryover effects of summer transhumance.

Lactobacillus, Lactococcus, and *Enterococcus* showed a trend very similar to the LAB category, probably because of their high relative abundances during the summer highland grazing period. The situation is very different for *Leuconostoc* and the other LAB taxa: over the 5 mo of the experiment the low group exhibited a linear decreasing pattern in the case of *Leuconostoc*, and a linear increasing pattern for other LAB.

Other probiotics followed a cubic pattern (Figure 3) for the low group, with the highest relative abundances in August and September, mainly due to the pattern of *Bifidobacterium*. Throughout the study period the high group exhibited higher abundances than the low group, with the difference increasing month by month. Again, as for LAB, no carryover effect was observed after the HIGH cows returned to the indoor permanent farm (October).

Spoilage bacteria showed a complex pattern (Figure 4), and some seasonal variation in the low group according to bacterial taxa. With the exception of *Kocuria*, the relative abundances of all the spoilage bacteria taxa were lower in the high than in the low group during summer pasturing. This difference was significant in July and August for *Alicyclobacillus*, in August for *Clostridiales*, in September for *Pseudomonas,* and in August and September for the whole group. No carryover effect was observed in October.

Finally, the relative abundances of the pathogenic bacteria (Figure 5) followed a cubic pattern for the low group of cows (with the highest value in July), due to the pattern of *Staphylococcus*. The differences between the high and low groups were not significant for these bacteria because of the opposite patterns in *Staphylococcus* and *Enterobacteriaceae*: the high group had a lower relative abundance of *Staphylococcus* than the low group and a higher relative abundance of *Enterobacteriaceae* during summer pasturing. (No differences were observed before and after transhumance).

The results of the mixed-model ANOVA of the other 31 bacterial taxa detected in milk are summarized in Table 2. Regarding the seasonal pattern of the low cows, only 12 of the 31 taxa exhibited a significant trend: *Jeotgalicoccus*, *Aerococcaceae*, and *Acinetobacter* showed a linear increase over time; *Rhodococcus*, and *Delftia* showed a quadratic pattern with a maximum during summer; *Bacteroidales* showed a quadratic pattern with a minimum during summer; *Chitiniphagaceae*, *Solibacillus*, other *Bacillales*, *Ochrobactrum*, *Sphingomonas*, and other *Alphaproteobacteria* followed a cubic pattern with the maximum value in July and the minimum in September. The other 19 taxa showed no significant variation over time.

Comparing the high and low cows within each month, we found an unexpected significant difference for *Bacteroidales* in June. We detected some difference in 19 of the 31 taxa during the 3 summer months (i.e., when the high group was on highland pastures while the low group remained indoors): in 10 in July, 3 in August, and 10 in September. Higher values of 9 taxa were observed in the high group, and 14 taxa in the low group. Only other *Firmicutes* and *Xanthomonadaceae* showed significant carryover effects in October.

Correlations

Correlation analyses were carried out on the relative abundances of the bacterial taxa known to have some specific activity in the 4 designated categories, and their Pearson correlations are

summarized in a heat plot in Figure 6. The relative abundances of the individual taxa, and the LAB and other probiotics categories, were generally positively correlated with each other, with the exception of the *Leuconostoc* taxa, which is almost entirely independent of all the other taxa and categories.

The spoilage bacteria taxa exhibited low correlations with each other and negative correlations with the relative abundances of the taxa of the LAB and other probiotics categories (Figure 6). The 2 taxa included in the pathogenic bacteria category were negatively correlated with each other, and their correlations with other bacterial categories were in opposite directions: *Enterobacteriaceae* were positively correlated with the LAB and other probiotics categories and taxa, and *Staphylococcus* had low correlations with spoilage bacteria taxa.

The correlations among the constituents and technological properties of milk are not among the objectives of this study, as they are already well known; therefore, they are not illustrated in detail and discussed here.

The correlations between the bacterial taxa and the constituents and technological traits of milk are summarized in a heat plot in Figure 7. These correlations vary greatly according to taxa and milk trait. It is worth noting that the LAB and other probiotics categories and their individual taxa exhibited correlations with many of the 30 milk composition and technological traits that were often in opposite directions to those shown by the spoilage bacteria category and individual taxa.

Latent Explanatory Factors of the Bacterial, Compositional and Technological Traits of Milk

The multivariate FA carried out on the 47 selected bacterial, compositional, and technological milk traits identified 8 latent explanatory factors. The loadings of each factor, excluding those that were non-relevant (< 0.30), are reported in Table 3, with high loadings (> 0.50) indicated by asterisks. All 17 bacterial taxa were included in one (n=6) or more (n=11) factors. *Leuconostoc*, other LAB, *Clostridiales*, *Alicyclobacillus,* and *Enterobacteriaceae* had the lowest loading values (never higher

than 0.44) and therefore are not well represented by any latent explanatory factor. In contrast, all 4 bacterial categories presented high communality loading values (0.68 to 0.90).

The 30 compositional and technological traits of milk, with the exception of milk urea, SCS, and whey protein, often contributed to characterizing the factors and presented high communality values.

The 8 latent explanatory factors represented 75.3% of the total covariance of the whole matrix, with individual values ranging from 14.2% for the first factor to 5.3% for the eighth (Table 4). It is worth noting that the mixed-model ANOVA of the scores of each factor revealed that all the latent explanatory factors, except factor 7, were significantly affected by the combined effect of group of cows and month of sampling (Table 4).

The 10 least squares means values, the standard errors, the seasonal patterns of low cows, and the significance levels of the differences between the 2 groups of cows within each month are illustrated in Figure 8 (one plot for each of the 8 latent explanatory factors). Factor 1 was characterized by a quadratic trend with the maximum value in October for the cows kept solely indoors (low group), and presented no significant differences between the 2 experimental groups of cows in any month of sampling. Factor 2 presented a quadratic seasonal trend with the maximum value in August, and the high group having significantly greater values during the 3 summer months. Factor 3 presented a quadratic seasonal pattern for low cows, but with the minimum in August and the high group having significantly higher values in July and August. Factor 4 presented a pattern that was almost the opposite of that of Factor 3: low cows followed a cubic pattern with the maximum in August, and high cows had significantly lower values in September. Factor 5 presented a linearly increasing pattern for low cows and significantly lower values in the high group only in August. Factor 6 presented a cubic seasonal pattern with the maximum in July and no differences between the 2 groups of cows. Factor 7 presented a linearly increasing pattern for the low group and no differences between

the 2 groups. Factor 8, like factor 7, presented a linearly increasing pattern for the low group, and no differences between the 2 groups.

DISCUSSION

Effects of Group of Cows and Individual Cows

In this study, we compared 2 groups of cows kept in the same physiological, environmental, nutritional, and management conditions only in June, when they were all kept indoors on the lowland permanent farm. At $P < 0.05$ there is a 5% probability that the differences between the high and low are due to chance, and at $P < 0.01$ the probability is 1%. Of the 56 contrasts tested (the relative abundances of 44 taxa and four categories of bacteria shown in Figure 2, 3, 4, and 5 and Table 2, and the scores of 8 explanatory latent factors shown in Figure 8), 3 were significant at $P < 0.05$ (*Bacteroidales* taxa, factor 3, factor 5) and one at $P < 0.01$ (*Pseudomonas* taxa), so we can assume the 2 groups were homogeneous. No information is available in the literature on the variability in bacterial traits in different, randomly composed groups of cows. The increasing variability among groups of cows may be due to permanent differences among different cows observed in subsequent samplings (animal effect). As seen in Figure 1, the permanent animal effect is generally small or not observable for the majority of bacterial taxa. This means that the (high) variability observed among different milk samples is due to the effects of other common factors (environment, diet, hygiene practices, milking routine, etc.) or individual, nonpermanent factors (temporary diseases, cleanness of teat surface, feed selection, etc.; Du et al., 2020; Parente et al., 2020). It is not surprising that the bacterial taxa with the highest animal effect was *Staphylococcus* that is a potential pathogen generally considered to be a cause of clinical mastitis (Verraes et al., 2015; Bobbo et al., 2017; Keane, 2019). It is worth noting that the cows selected for this study were all healthy, and that none of them developed clinical mastitis during the study. On the other side, single episodes of clinical mastitis do not influence the (permanent) animal effect, only the residual variance, and repeated cases of clinical mastitis normally result in the cows being culled. The high animal effect observed in Figure 1 for *Staphylococcus* could be interpreted as the predisposition of some healthy cows to carry greater or lesser quantities of these bacteria. Whether this indicates a predisposition for subclinical mastitis is

not known, and is an issue worth investigating with a much larger number of animals. Cows have a (modest) heritability for both the incidence of clinical mastitis (Koeck et al., 2014), and the level of the mastitis indicator represented by the somatic cell content in the milk (Urioste et al., 2010; Pegolo et al., 2021). This indicates an interaction between the cow's genome and the infectiousness of the pathogens causing mastitis, so the variability in the relative abundances of *Staphylococcus* taxa among different animals observed here could also depend on their genome.

Other bacterial taxa shown in Figure 1 exhibiting a nontrivial animal effect are other LAB, *Clostridiales,* and *Kocuria*. We found no information in the scientific literature on animal repeatability of the relative abundances of these taxa, so this, too, could be an interesting line of research with respect to cheese-making efficiency or cheese defects (de Paiva Anciens Ramos et al., 2021).

Associations Between Milk Microbiota, Milk Composition, and Cheese-Making Properties

Association studies between milk microbiota and composition and cheese-making properties are infrequent and deal mainly with the potential effects of pathogenic bacteria on udder health, and mastitis in particular (Leitner et al., 2006; Bobbo et al., 2017).

Multivariate statistical analyses are often used to study associations among different bacterial taxa (Rodrigues et al., 2017) and between these and other milk or cheese characteristics (Nyman et al., 2014). The most commonly used method is principal component analysis, as it is very efficient, although the results are not always easy to interpret. Factor analysis has the advantage of better clustering the observed traits so that the latent explanatory factors can be related to a small number of important traits. Unfortunately, this method is seldom used with metagenomics data sets, and we are not aware of any FA combining the microbiological, compositional, and technological properties of milk, which means that it is not possible to compare our results with those of other authors.

It is worth noting that in this analysis we did not obtain any latent factors based on the simultaneous strong influence of bacterial and other milk characteristics. This means that milk

characteristics do not seem to be highly dependent on microbiological populations, nor *vice versa*. Two of the 8 factors were based mainly on bacterial taxa, and the other 6 on milk composition and cheese-making properties.

As seen in Table 4, the first latent explanatory factor (14.2% of total variation) is based on traits obtained mainly from lactodynamographic tests and modeling, and particularly, with negative loadings, on the time from rennet addition to coagulation (RCT and RCT_{eq}), and the time to reach a given (k_{20}) or maximum (t_{max}) curd firmness. Early gelation is obviously positively correlated with an increase in the traits measuring curd firmness (a_{30}, a_{45}, a_{60}, and CF_{max}) and allows more time for estimating curd syneresis (k_{SR}; Table 3). This is why we named this factor the "milk gelation factor". It also includes the protein and casein contents of milk and whey with positive loadings; protein, especially casein, are known to have a favorable effect on the rapidity and intensity of coagulation (Amalfitano et al., 2019). Among the bacterial traits, only 2 LAB taxa are involved in the gelation factor, but with opposite signs: negative in the case of *Leuconostoc,* positive in the case of other LAB. Finally, milk urea is also included with a negative loading.

The second most important latent factor, obtained from the analysis (14.0% of total variation), shown in the Table 4, is substantially based on milk metagenomics, as it includes, with positive loadings, the 2 categories (and their major taxa) that have putative positive effects on the commercial and nutritional value of milk: LAB and other probiotics. However, it also includes the spoilage bacteria taxa with a negative loading, and spoilage microorganisms are well known to have a negative impact on the value and quality of dairy products (Martin et al., 2021). The pathogenic bacteria category is not included in this factor because of the opposite sign of the loadings of the two taxa it includes. It is evident that this important latent factor can be considered an index of the favorableness of the microbiological profile of milk, and for this reason we named it "pro-dairy bacteria". This factor also includes, with a negative sign, some traits related to milk and whey composition, but none of these characterizes the latent factor (Table 3).

The third latent explanatory factor in Table 4 (11.7% of total variation) is based on milk fat content and cheese yield. It is worth noting that fat is a major component of curd solids but is, in particular, the component with the highest variability, much greater than that of protein. This explains why milk fat is so closely associated with milk solids (and the fat/protein ratio) and with cheese yield expressed as cheese solids as a percentage of the TS of the processed milk. This last trait is obviously associated with the recovery of milk solids and energy in the curd, and also with water retained in the curd and fresh cheese yield. This is why in Table 3 we named this latent explanatory factor "Cheese yield factor". It is also associated with, but not characterized by, whey fat, because with the increasing fat content of milk we expect an increase in both fat retained in the curd and fat lost in the whey, although proportionally in favor of the former due to the positive relationships between the fat content of milk and fat recovery in the curd. The other traits (negatively) associated with the cheese yield factor were casein number and milk lactose content. Because lactose is the major component of milk solids and ends up being mainly lost with the whey, it is evident that, as it increases in milk, it causes a reduction in the yield of cheese solids. The meaning of the negative loading of casein number in this factor is less evident, but we should bear in mind that this trait is at the same time also included in 2 other factors, discussed later. A cheese yield factor was also identified in a previous large data set that did not include metagenomic information (Dadousis et al., 2018b). It was the most important factor in that study, representing 14% of all variation and, as in this study, was characterized by yield of cheese solids, fat content, and recovery of milk energy in the curd, as well as by protein content. A cheese yield latent factor was also identified from analysis of a large data set obtained from observations on dairy ewes (Manca et al., 2016).

The fourth factor presented in Table 3 is characterized by the lactose content of milk and whey, milk nonfat solids, and whey solids (lactose being the major constituent of both the latter). As expected, because lactose is retained in very small proportions in the curd, this factor included recovery of milk solids and energy in the curd, with negative loadings. An increase in lactose content

is frequently associated with a lower somatic cell content (see the negative loading of SCS) and whey proteins (see the positive loading of casein number), and, taken together, there are interpreted as indicators of good udder health (Macciotta et al., 2012). This is why we named this factor "udder health factor", as others have done for cattle (Macciotta et al., 2012; Cecchinato et al., 2012; Dadousis et al., 2018a,b; Cecchinato et al., 2019), and for goats and sheep (Manca et al., 2016; Vacca et al., 2016). Note that the udder health factor is associated with the variations in 5 bacterial taxa: the decrease in *Enterococcus*, *Propionibacterium,* and *Enterobacteriaceae*, and the increase in other LAB and *Alicyclobacillus*. The meaning of these associations is clear for *Enterobacteriaceae*, but not for the other taxa.

In a previous study we found relationships between the presence of a few bacterial groups causing mastitis and the qualitative and technological properties of milk (Bobbo et al., 2017). *Staphylococcus aureus* was the contagious bacteria most frequently isolated in milk samples from individual cows. The presence of this pathogen was associated with decreases in daily milk yield, casein number, and milk lactose (i.e. the udder health factor), but not in other milk constituents. The contagious milk bacteria were also associated with a worsening of milk coagulation and curd firmness properties, and a decrease in cheese-making efficiency and, in particular, recovery of milk fat and protein in the curd (Bobbo et al., 2017). More information on the relationships between milk SCC or SCS and milk properties can be found in Bobbo et al. (2016).

The fifth factor listed in Table 3 is characterized by milk casein, milk protein (whey protein), nonfat solids, casein number, and protein recovery in the curd. It is evident that casein content has a central role in this factor, so we named it "casein factor". It also includes some traits related to the rate and degree of curd firming (k_{20}, a_{60}, and CF_{max}), confirmation that caseins play an important role in curd firming, much more so than in milk coagulation time (Jõudu et al., 2008). Protein is also more correlated than fat with the retention of water in the curd, which explains the positive loading of curd cheese yield (Cipolat-Gotet et al., 2020). In any case, we should bear in mind that different protein

fractions have different effects on milk coagulation and curd firming traits (Amalfitano et al., 2019), as well as cheese yield and milk nutrient recovery in cheese (Cipolat-Gotet et al., 2018). It is worth noting that "other LAB taxa" is the only microbiological trait positively included in this factor.

As seen in Table 3, the sixth factor is, like the pro-dairy bacteria factor, based only on bacterial traits. It is characterized mainly by the pathogenic bacteria category, specifically by the *Staphylococcus* taxa, but not by *Enterobacteriaceae* (included negatively in the udder Health factor). This is why we named it "pathogenic bacteria". It is worth noting that mastitis has been described as a dysbiosis, an imbalance in the healthy mammary gland microbiome (Andrews et al., 2019). This latent factor is also associated with a negative loading to the LAB (*Leuconostoc* and *Enterococcus* taxa) and spoilage bacteria categories (particularly the *Kocuria* and *Pseudomonas* taxa), and with a positive loading to *Alicyclobacillus*.

The seventh factor (Table 3) is also mainly characterized by traits obtained during the lactodynamographic test, but here the time intervals from rennet addition to coagulation are not included (earliness of coagulation, as in the case of the gelation factor), whereas the rapidity of the curd firming (k_{CF}, k_{20} and t_{max}) and syneresis processes (k_{SR}) is central, and explains the positive loading of a_{30}, the small loading of a_{45}, and the negative loading of a_{60}. This is why we named this the "curdling factor". Small correlations between gelation time, rapidity of curd firming and syneresis were previously reported by Macciotta et al. (2012). It is worth noting that 2 independent latent factors representing coagulation traits and curd firming traits were also obtained on goats milk (Todaro et al., 2005).

The eighth factor in Table 3 is characterized by positive loading of the recoveries of milk fat (REC_{FAT}) and total milk solids (REC_{ENERGY}) in the curd, and a negative loading of the fat and TS contents in the whey, so we named this the "fat recovery factor".

Effects of Season or Lactation Stage, Farming System, and Pasture Carryover

We were unable in this study to disentangle the effects of season and lactation stage, because, as is often the case in pasture-based farming systems, the cows at the beginning of pasturing are generally in the first half of lactation, and it progresses together with the advancing season. Having initially created 2 homogeneous groups of cows for lactation stage, and having kept their composition constant during the experiment, we found a similar overlap in advancing season and lactation stage in the high (moved to summer pastures) and the low group (kept solely indoors). In the case of the high group, the overlap also went hand in hand with the gradual maturation of the forage on the summer pasture. This should be borne in mind when interpreting the results, as the often significant seasonal pattern of the low cows (Figures 2, 3, 4, and 5, and Table 2) represents the simultaneous change in season and lactation stage, but not feeding regime (constant TMR).

We were also unable to model the same pattern in the case of the high group, because we cannot assume continuous evolution along the 5 experimental months. This group, in fact, underwent 2 abrupt changes: the move from the permanent farm in the valley to the highland pastures, and then the return to indoor conditions. In this case, we considered the low group as the control, and compared the high group against them month by month. As seen before, the substantially low incidence of significant contrasts between the 2 groups in June supports the assumption of initial homogeneity of the 2 groups.

The large number of significant differences observed in July, August, and September for the bacterial traits and the latent factors confirms the hypothesis of very large effects of farming system (permanent indoor housing vs. summer highland pasture), and is consistent with results previously obtained by the same project using bacterial culture-dependent and -independent approaches (Carafa et al., 2020). Other authors have observed large difference in the microbiota of milk produced by cows kept indoors and cows at pasture (Bonizzi et al., 2009; Doyle et al., 2017). The autochthonous LAB of milk produced on highland pastures are known to be important for the cheese-making process

and the final quality of traditional mountain cheeses (Carafa et al., 2019). As the metagenomics results are expressed as relative abundances (i.e. proportions among different taxa and not their population sizes), it would be useful to draw comparisons with bacterial plate counts on different selective media to obtain a clearer picture of the actual amounts of viable bacterial populations in the milk samples analyzed. In our previous study (Carafa et al., 2020), we found that summer highland grazing increased the counts of all bacterial categories. The increases in aerobic (+41%), anaerobic (+54%), and mesophilic lactococci (+45%) and Bifidobacteria counts (+47%) from the low to the high group were similar, whereas the increases in mesophilic lactobacilli (+411%), *Propionibacteria* (+125%), and coliform counts (+631%) were several times greater, largely congruent with the increase in their relative abundances found here using metagenomics. The substantial increases in the relative abundances of the LAB category and its main taxa (Figure 2), and of the pro-dairy factor (Figure 8) during summer highland pasturing, confirm that the improvement in milk chemical composition observed in this project and in several other studies (Bergamaschi et al., 2016; Bergamaschi and Bittante, 2018; Bittante et al., 2021) is due to pasturing. It is well known that different farming systems, as well as individual farms (Bokulich and Mills, 2013; Skeie et al., 2019; Priyashantha and Lundh, 2021) and dairy plants, affect cheese-making efficiency and product quality (Falardeau et al., 2019; Nam et al., 2021). This also supports the claimed specificity of cheeses produced on temporary highland summer farms in Alpine regions (Bittante et al., 2011a; 2011b) and signals the possibility of authenticating the origin of dairy products according to farming system (Bergamaschi et al., 2020).

It is worth noting that, in a previous large survey, a significant effect of dairy system was found for latent factors named “cheese yield”, “udder health” and “yield” (Dadousis et al., 2018b; production traits were not included here). The effect of farming system favored modern indoor farming for cheese yield and yield, but favored traditional farming for udder health.

The potential carryover effects of summer pasture after the cows return to indoor rearing have not been extensively studied at the microbiological level. It is worth noting that, in this study, the

carryover effect on milk bacteria was negligible, whereas some effects on milk quality, composition, and cheese-making aptitude have been observed (Saha et al., 2019).

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CONCLUSIONS

We can conclude that the milk microbiota is very complex, and the majority of bacterial taxa are strongly influenced by farming system as well as by the advancement of season and lactation stage. Transhumance of dairy cows from indoor conditions to summer highland pastures may increase the relative abundances of LAB, and other probiotic bacteria (bifidobacteria and propionibacteria) and decrease the abundances of spoilage bacteria, thereby improving the milk in terms of cheese-making aptitude and benefits to human health. This effect disappears after the cows return indoors in the autumn. Systematic differences in milk microbiota among different cows concern some bacterial taxa, particularly the pathogenic bacteria and *Clostridiales*, signaling the need for new studies on the relationships between the cow genome and milk microbiota. Metagenomic analysis of milk microbiota appears to be a powerful tool for studying the complex relationships between farming system, individual cow characteristics, and the value of milk for cheese-making and for human and animal health.

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TABLES AND FIGURES

Table 1. Descriptive statistics, significance levels of the Month × Group interaction, and the root mean square error (RMSE) of the log_{10} relative abundances of milk bacterial taxa with known dairy (LAB), other probiotic, spoilage and pathogenic activities, and of other bacteria found in the milk

traits	Samples, N	Descriptive statistics		Month x Group F-value	RMSE
		Mean	±SD		
Lactic acid bacteria (LAB)	57	0.780	0.528	10.0***	0.335
Lactobacillus	57	0.277	0.452	25.0***	0.200
Leuconostoc	57	0.082	0.165	1.5	0.158
Lactococcus	57	0.294	0.487	20.6***	0.237
Enterococcus	58	0.384	0.389	2.8*	0.335
Other LAB	58	0.191	0.288	3.9**	0.214
Other probiotics	57	0.287	0.426	15.8***	0.230
Propionibacterium	55	0.059	0.140	10.6***	0.086
Bifidobacterium	57	0.245	0.399	14.2***	0.224
Spoilage bacteria	58	1.182	0.585	12.9 ***	0.342
Clostridiales	58	0.308	0.346	2.4*	0.258
Pseudomonas	58	0.745	0.631	5.4***	0.482
Kocuria	58	0.659	0.654	7.7***	0.425
Alicyclobacillus	58	0.111	0.178	6.6***	0.129
Pathogenic bacteria	58	0.656	0.522	1.8	0.447
Staphylococcus	57	0.404	0.494	2.4*	0.341
Enterobacteriaceae	57	0.229	0.395	11.9***	0.220
Other bacteria					
Actinomyces	56	0.019	0.096	3.0**	0.083
Corynebacterium	55	0.016	0.071	0.9	0.072
Rhodococcus	57	0.215	0.314	2.9*	0.250
Other *Actinomycetales*	56	0.153	0.243	4.1***	0.197
Bacteroidales	57	0.283	0.279	5.0***	0.207
Flavobacterium	57	0.094	0.189	2.2*	0.173
Chryseobacterium	58	0.536	0.515	3.8**	0.378
Other *Flavobacteriales*	57	0.094	0.144	1.4	0.139
Sphingobacterium	57	0.152	0.195	3.6**	0.154
Other *Sphingobacteriales*	57	0.028	0.077	1.7	0.073
Chitinophagaceae	57	0.015	0.061	1.0	0.056
Solibacillus	57	0.055	0.163	1.8	0.153
Jeotgalicoccus	56	0.041	0.122	1.9	0.103
Exiguobacterium	57	0.013	0.071	0.9	0.071
Other *Bacillales*	57	0.112	0.283	3.2**	0.220
Aerococcaceae	58	0.148	0.286	3.1**	0.229
Carnobacteriaceae	56	0.045	0.151	1.8	0.128
Other *Firmicutes*	56	0.004	0.020	1.0	0.020
Ochrobactrum	57	0.068	0.159	3.2**	0.118

Paracoccus	57	0.080	0.194	7.9***	0.132
Sphingomonas	57	0.050	0.093	2.6*	0.080
Other *Alphaproteobacteria*	58	0.276	0.313	1.6	0.278
Delftia	58	0.148	0.230	1.7	0.213
Other *Betaproteobacteria*	57	0.177	0.225	1.0	0.225
Deltaproteobacteria	55	0.001	0.007	0.9	0.007
Epsilonproteobacteria	56	0.015	0.042	0.9	0.042
Ruminobacter	55	0.022	0.066	0.8	0.066
Acinetobacter	58	0.751	0.507	6.7***	0.326
Enhydrobacter	56	0.073	0.169	0.7	0.174
Xanthomonadaceae	58	0.599	0.465	4.8***	0.363
Other *GammaProteobacteria*	57	0.187	0.280	1.9	0.260

*P<0.05; **P<0.01; ***P<0.001;

Table 2. Significance levels and order and shape of the patterns observed for microbial abundances of bacterial taxa, with no specific dairy, probiotic, spoilage or pathogenic activity in milk samples collected from cows kept permanently indoors, and differences between these and the cows moved to summer highland pastures in the months before (June), during (July, August, and September) and after (October) transhumance, in terms of $\log_{10}$ relative abundance

	Pattern on indoor cows[1]		Difference between transhumant vs. indoor cows:				
Item	P-value	Order:shape	June	July	August	September	October
Actinomyces	NS	-	0.00	0.01	0.00	0.20***	0.00
Corynebacterium	NS	-	-0.01	0.01	0.00	0.09	0.04
Rhodococcus	0.005	Q:up-down	-0.01	-0.34*	-0.36*	0.12	-0.10
Other *Actinomycetales*	NS	-	0.11	0.20	-0.07	0.51***	-0.08
Bacteroidales	0.017	Q:down-up	-0.36*	-0.19	-0.26	0.19	-0.04
Flavobacterium	NS	-	0.00	0.07	0.03	-0.08	0.03
Chryseobacterium	NS	-	0.02	0.71**	0.52	0.98***	-0.23
Other *Flavobacteriales*	NS	-	-0.13	0.08	-0.13	0.01	-0.17
Sphingobacterium	NS	-	-0.07	-0.15	-0.05	-0.33**	0.11
Other *Sphingobacteriales*	NS	-	-0.02	0.10*	0.00	0.07	-0.02
Chitinophagaceae	0.046	C:up-down-up	-0.01	-0.02	0.00	0.00	-0.03
Solibacillus	0.015	C:up-down-up	-0.11	-0.18*	0.00	0.00	-0.09
Jeotgalicoccus	0.002	L:up	0.05	-0.01	0.00	-0.15*	-0.11
Exiguobacterium	NS	-	0.00	0.00	0.00	0.00	-0.02
Other *Bacillales*	<0.001	C:up-down-up	0.02	-0.47**	-0.02	-0.06	-0.27
Aerococcaceae	0.001	L:up	0.10	-0.13	-0.09	-0.32*	-0.04
Carnobacteriaceae	NS	-	-0.06	0.00	0.01	-0.10	0.13
Other *Firmicutes*	NS	-	0.00	0.00	-0.01	0.00	-0.03*
Ochrobactrum	0.04	C: up-down-up	-0.03	-0.29**	-0.10	0.01	0.02
Paracoccus	NS	-	0.00	0.52***	0.14	0.17*	0.01
Sphingomonas	<0.001	C:up-down-up	-0.01	-0.15**	-0.05	-0.03	-0.06
Other *Alphaproteobacteria*	0.031	C:up-down-up	0.08	-0.02	-0.43*	0.23	-0.20
Delftia	0.033	Q:up-down	-0.09	-0.22	-0.23	-0.27*	0.14
Other *Betaproteobacteria*	NS	-	0.16	0.11	-0.18	0.07	0.03
Deltaproteobacteria	NS	-	0.00	-0.01*	0.00	0.00	0.00
Epsilonproteobacteria	NS	-	0.00	0.00	0.01	0.02	-0.02
Ruminobacter	NS	-	-0.04	-0.05	-0.03	0.00	0.00
Acinetobacter	0.003	L:up	-0.10	0.21	0.81**	-0.28	0.72
Enhydrobacter	NS	-	0.07	-0.09	0.00	-0.07	-0.11
Xanthomonadaceae	NS	-	0.08	-0.57*	-0.28	-0.74***	0.45*
Other *GammaProteobacteria*	NS	-	0.04	0.30	-0.05	0.43**	0.03

[1] L: up = linear pattern increasing from June to October; Q: up-down = zenithal quadratic pattern rising to a maximum during summer and then decreasing; Q: down-up = nadir quadratic pattern decreasing to a minimum during summer and then increasing; C: up-down-up = cubic pattern rising to a maximum in July, decreasing to a minimum in September, and then increasing again. Dashes indicate absence of a significant pattern for indoor cows.
*P<0.05; **P<0.01; ***P<0.001.

Table 3. Loadings on the latent explanatory factors of the relative abundances of milk bacterial taxa, milk and whey constituents, milk coagulation, curd firming, and cheese yield.

Item[1]	Factor 1: Gelation	Factor 2: Pro-dairy	Factor 3: Cheese yield	Factor 4: Udder health	Factor 5: Caseins	Factor 6: Pathogens	Factor 7: Curdling	Factor 8: Fat recovery	Communality
Milk bacterial groups									
LAB		0.63*				-0.39			0.68
Lactobacillus		0.83*							0.82
Leuconostoc	-0.40					-0.33			0.37
Lactcoccus		0.65*							0.61
Enterococcus				-0.53		-0.37			0.48
Other LAB	0.33			0.31	0.33				0.44
Other probiotics		0.91*							>0.90
Propionibacterium		0.52*		-0.46					0.55
Bifidobacterium		0.92*							>0.90
Spoilage bacteria		-0.79*				-0.42			0.85
Clostridiales		-0.31							0.24
Pseudomonas		-0.57*				-0.31			0.43
Kocuria		-0.48				-0.52*			0.57
Alicyclobacillus				0.38		0.41			0.36
Pathogenic bacteria						0.80*			0.74
Staphylococcus		-0.35				0.78*			0.77
Enterobacteriaceae		0.48		-0.36					0.43
Milk technological traits									
Milk composition:									
TS			0.89*						>0.90
Milk fat			0.95*						>0.90
Nonfat solids		-0.36		0.52	0.70*				>0.90
Milk protein	0.35	-0.32			0.81*				>0.90
Fat/Protein ratio			0.84*		-0.44				>0.90
Milk casein	0.30				0.86*				>0.90
Casein number			-0.38	0.33	0.53*				0.61
Milk urea	-0.43								0.43
Udder health traits:									
SCS				-0.40					0.25
Milk lactose			-0.31	0.75*					>0.90
Coagulation properties:									
RCT	-0.96*								>0.90
k_{20}	-0.47				-0.31		-0.56		0.69
a_{30}	0.87*						0.31		>0.90
a_{45}	0.79*								>0.90
a_{60}	0.44	-0.36			0.41		-0.59		>0.90
Curd firming modeling:									
RCT_{eq}	-0.96*								>0.90
k_{CF}							0.90*		>0.90
k_{SR}	0.67*						0.48		0.84
CF_{max}	0.81*				0.38				>0.90
t_{max}	-0.75*						-0.54*		>0.90
Cheese yields:									
$\%CY_{CURD}$			0.58*		0.50*				0.64
$\%CY_{SOLIDS}$			0.85*		0.31				>0.90
REC_{FAT}								0.87*	>0.90
$REC_{PROTEIN}$					0.62*				0.45
REC_{SOLIDS}			0.65*	-0.56*					>0.90
REC_{ENERGY}			0.68*	-0.38				0.47	>0.90
Whey composition:									
Whey total solids		-0.35		0.75*				-0.36	>0.90

Whey fat				0.47			-0.83*	>0.90
Whey protein	0.39	-0.41			0.34			0.57
Whey lactose		-0.36		0.88*				>0.90

[1]LAB = lactic acid bacteria; RCT = rennet coagulation time; K_{20} = curd-firming time; a_{30}, a_{45}, a_{60} = curd firmness 30, 45, 60 min after rennet addition, respectively; RCT_{eq} = rennet coagulation time by equation; k_{CF} = curd-firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness; t_{max} = time to reach CF_{max}; %CF = percentage cheese yields; REC = nutrient recovery traits.

*High loading, >0.50.

Table 4. Descriptive statistics, significance levels of the Group × Month interaction, and root mean square error (RMSE) of the latent explanatory factors of milk bacterial taxa, milk and whey constituents, milk coagulation, and curd firming properties, and cheese yield.

Latent explanatory factor	Explained variance (%)		Group × Month F-value	RMSE
	By each factor	Cumulative		
Factor 1: “Gelation factor”	14.2 %	14.2 %	7.3***	0.49
Factor 2: “Pro-dairy bacteria”	14.0 %	28.2 %	23.1***	0.46
Factor 3: “Cheese yield factor”	11.7 %	39.9 %	2.4*	0.84
Factor 4: “Udder health factor”	9.4 %	49.3 %	13.0***	0.44
Factor 5: “Casein factor”	9.2 %	58.5 %	6.5***	0.55
Factor 6: “Pathogenic bacteria”	6.0 %	64.6 %	2.8*	0.69
Factor 7: “Curdling factor”	5.5 %	70.1 %	1.8	0.58
Factor 8: “Fat-rec factor”	5.3 %	75.3 %	3.5**	0.76

*P<0.05; **P<0.01; ***P<0.001.

Figure 1. Sources of the variation (expressed as percentage of total variance) in individual milk bacterial taxa relative abundances and their categories (in bold): effects of the Month × Group interaction (dark blue), individual cow within group (red), and residual variability (light blue). LAB = lactic acid bacteria.

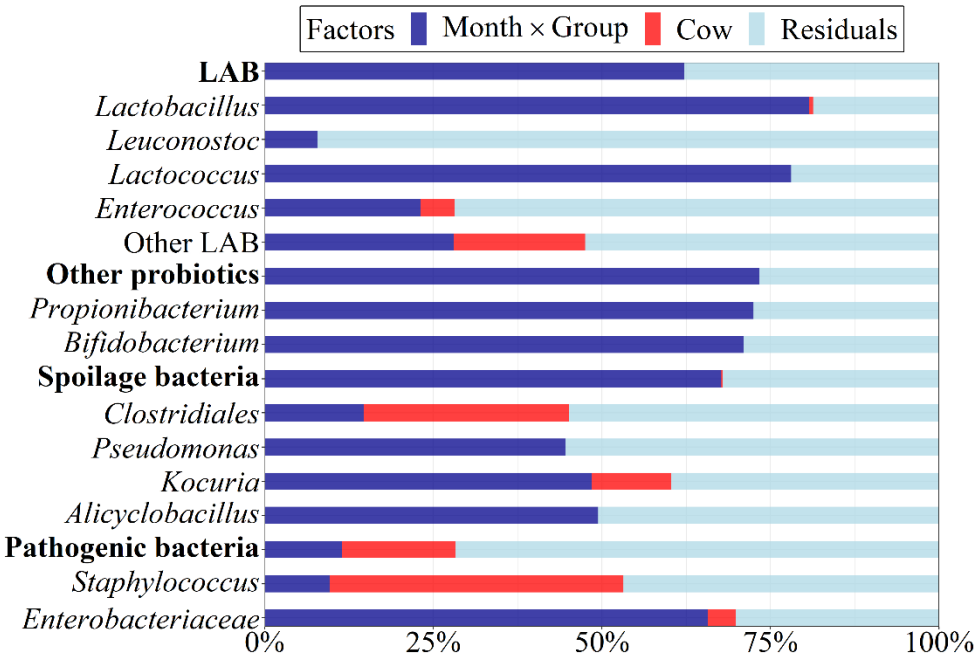


Figure 2. Patterns of the relative abundances of the milk lactic acid bacteria (LAB) taxa having desired dairy characteristics (the "LAB" category and individual bacterial taxa) during the experimental period. Blue circles represent the LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent the SE of estimates. Lines and curves represent significant linear, quadratic or cubic patterns, with their R^2 values, for cows kept solely indoors. Asterisks indicate the significance levels of the differences between the 2 groups in each month (*P<0.05; **P<0.01; ***P<0.001).

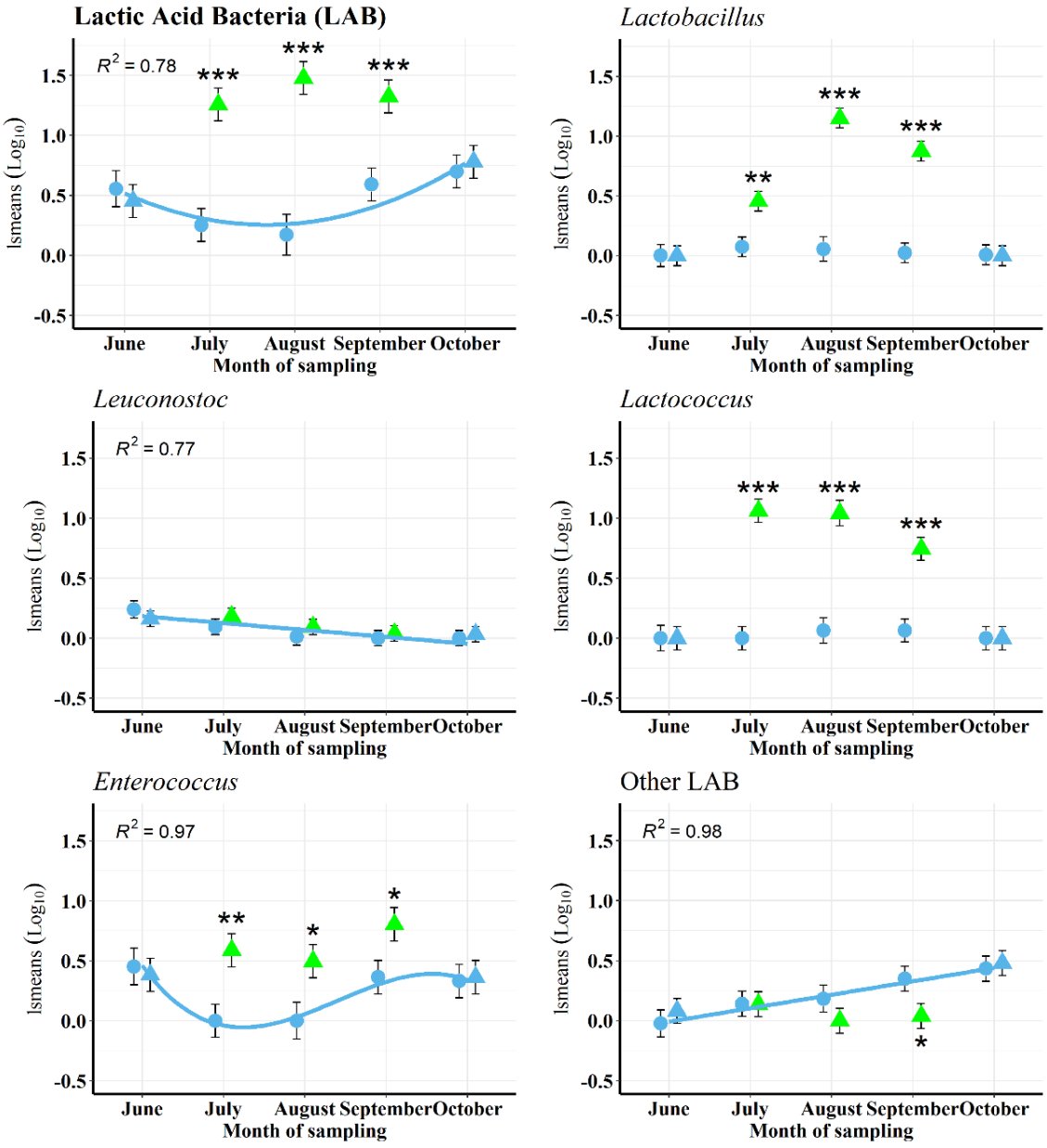


Figure 3. Patterns of the relative abundances of the other milk bacterial taxa having desired probiotic characteristics “other probiotics” category and individual bacteria taxa) during the experimental period. Blue circles represent the LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after summer transhumance. Bars represent the SE error of the estimates. Lines and curves represent significant linear, quadratic or cubic patterns, with their R^2 values, for the cows kept solely indoors. Asterisks indicate the significance levels of the differences between the two groups in each month (*P<0.05; **P<0.01; ***P<0.001).

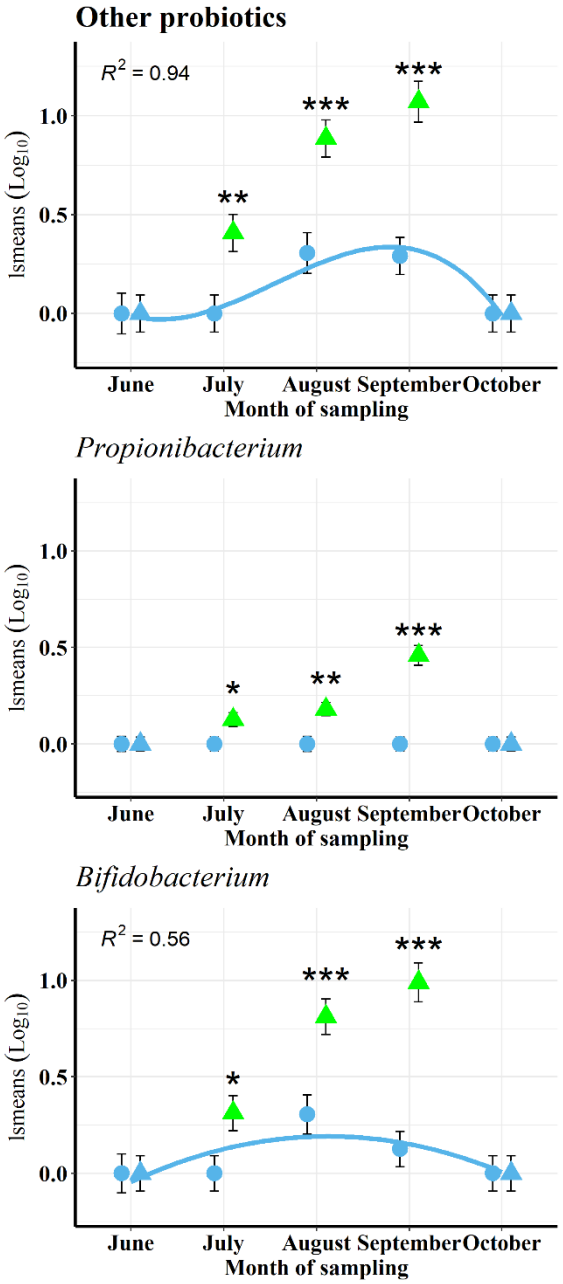


Figure 4. Patterns of the relative abundances of the milk bacterial taxa having undesired spoilage characteristics ("spoilage bacteria" category and individual bacteria taxa) during the experimental period. Blue circles represent the LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent the standard error of estimates. Lines and curves represent significant linear, quadratic or cubic patterns, with their R^2 values, for cows kept solely indoors. Asterisks indicate the significance levels of the differences between the two groups in each month (*P<0.05; **P<0.01; ***P<0.001).

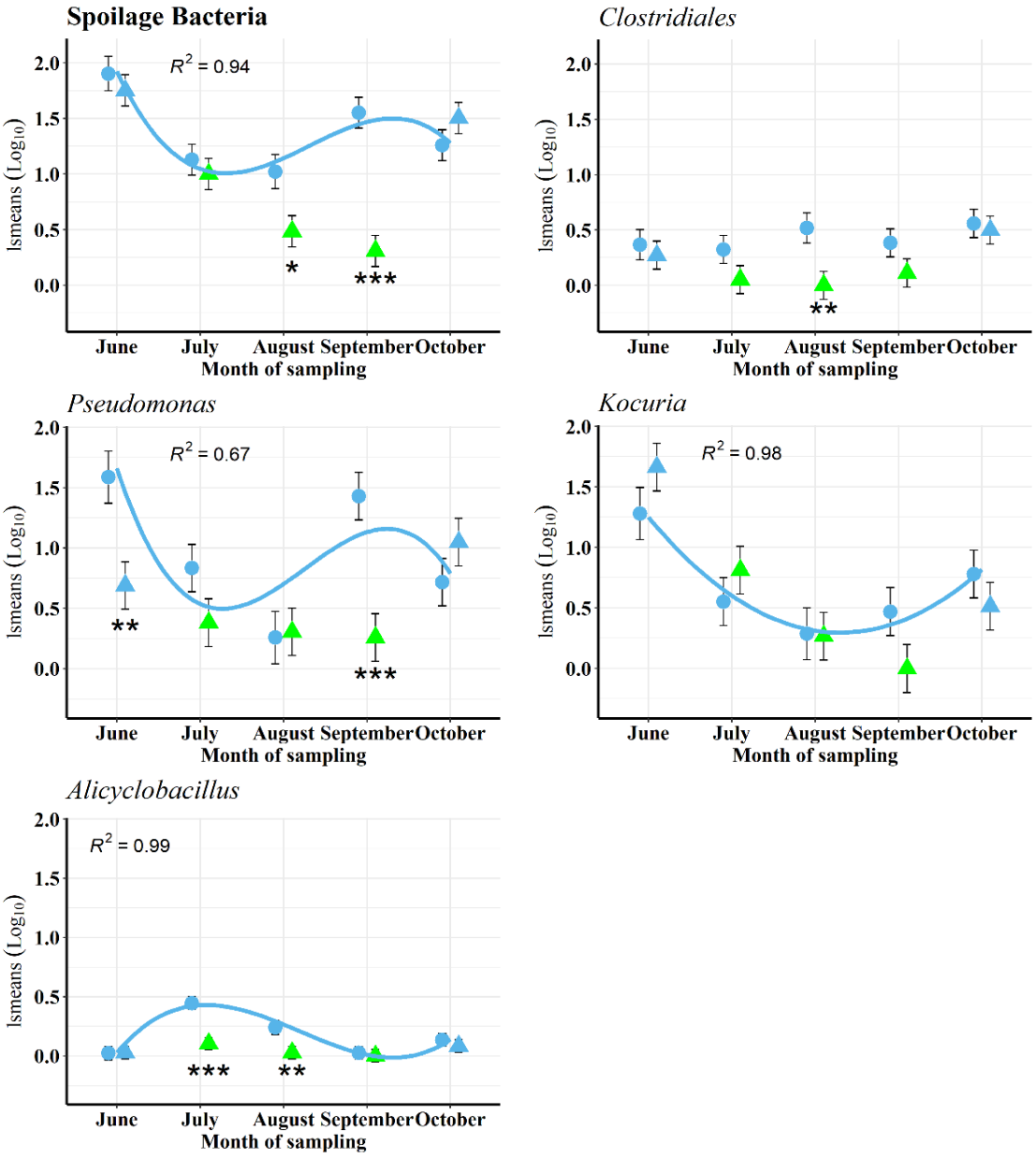


Figure 5. Patterns of the relative abundances of the milk bacterial taxa having undesired pathogenic characteristics ("pathogenic bacteria" category and individual bacteria taxa) during the experimental period. Blue circles represent the LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows kept indoors before and after the summer transhumance. Bars represent the SE of estimates. Lines and curves represent significant linear, quadratic or cubic patterns, with their R^2 values, for cows kept solely indoors. Asterisks indicate significance levels of the differences between the two groups in each month (*P<0.05; **P<0.01; ***P<0.001).

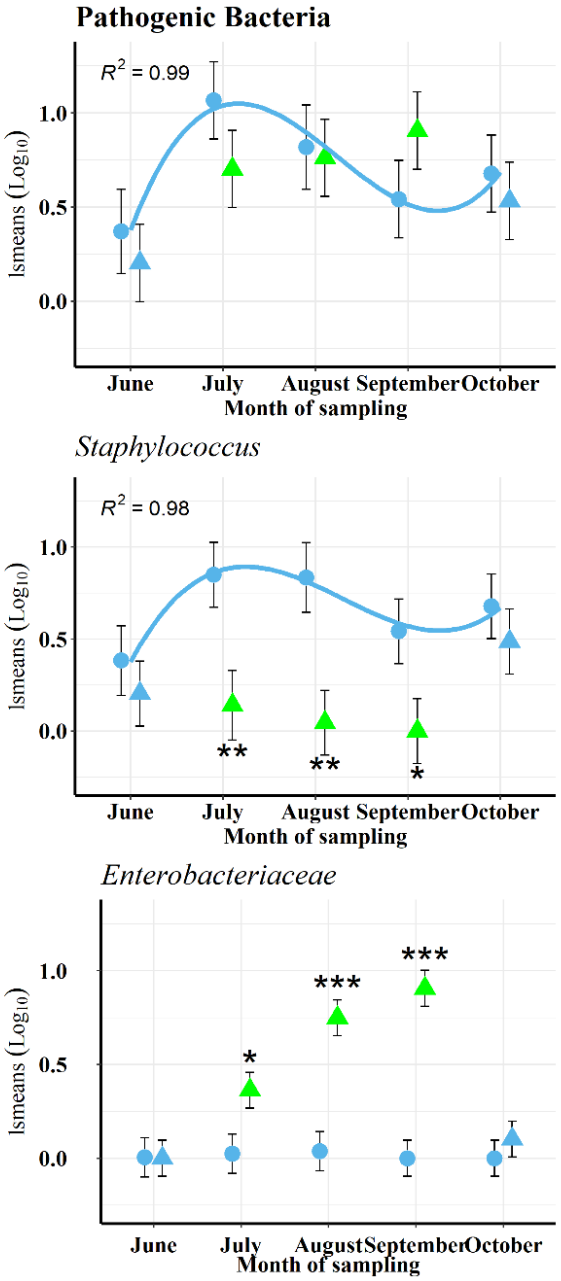


Figure 6. Heat plot of the correlations among the bacterial traits included in the factor analysis. LAB =lactic acid bacteria.

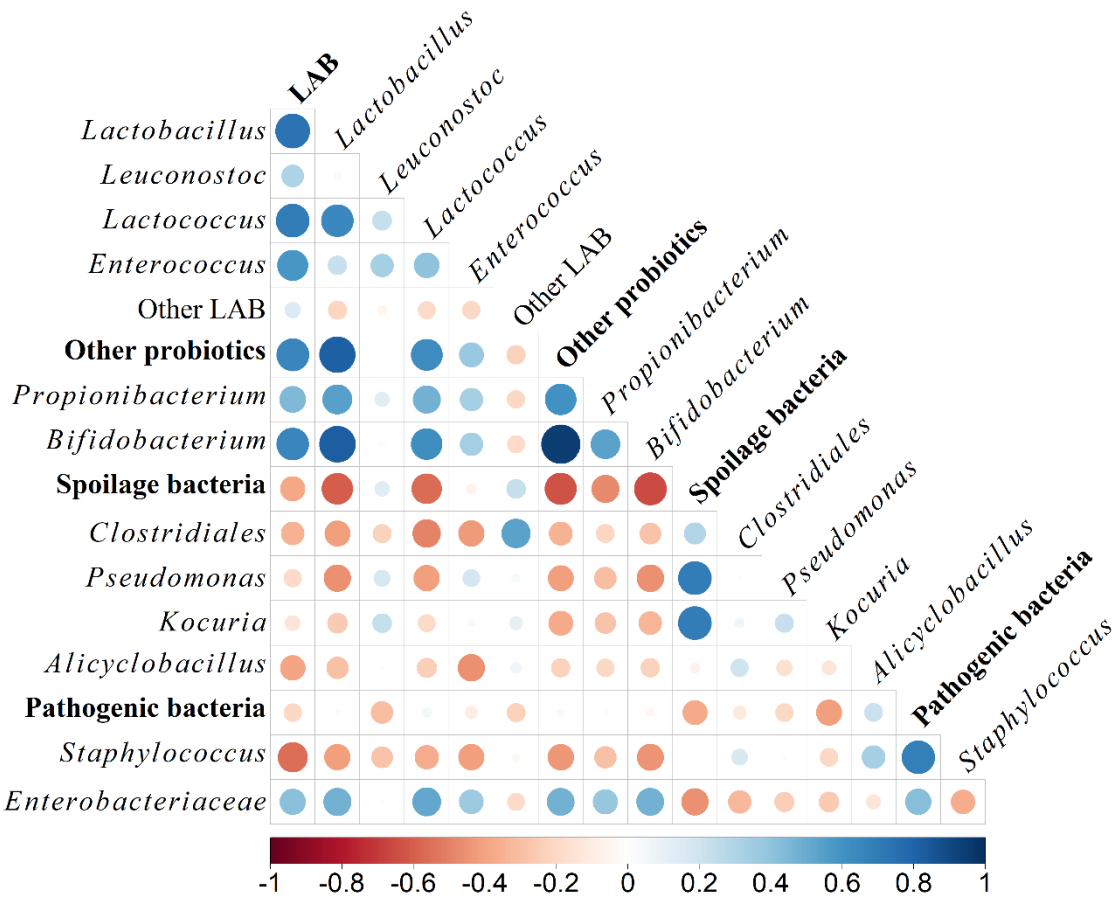


Figure 7. Heat plot of the correlations between the bacterial and chemical-technological traits included in the factor analysis. LAB = lactic acid bacteria; RCT = rennet coagulation time; K_{20} = curd-firming time; a_{30}, a_{45}, a_{60} = curd firmness 30, 45, 60 min after rennet addition, respectively; RCT_{eq} = rennet coagulation time by equation; k_{CF} = curd-firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness; t_{max} = time to reach CF_{max}; %CF = percentage cheese yields; REC = nutrient recovery traits.

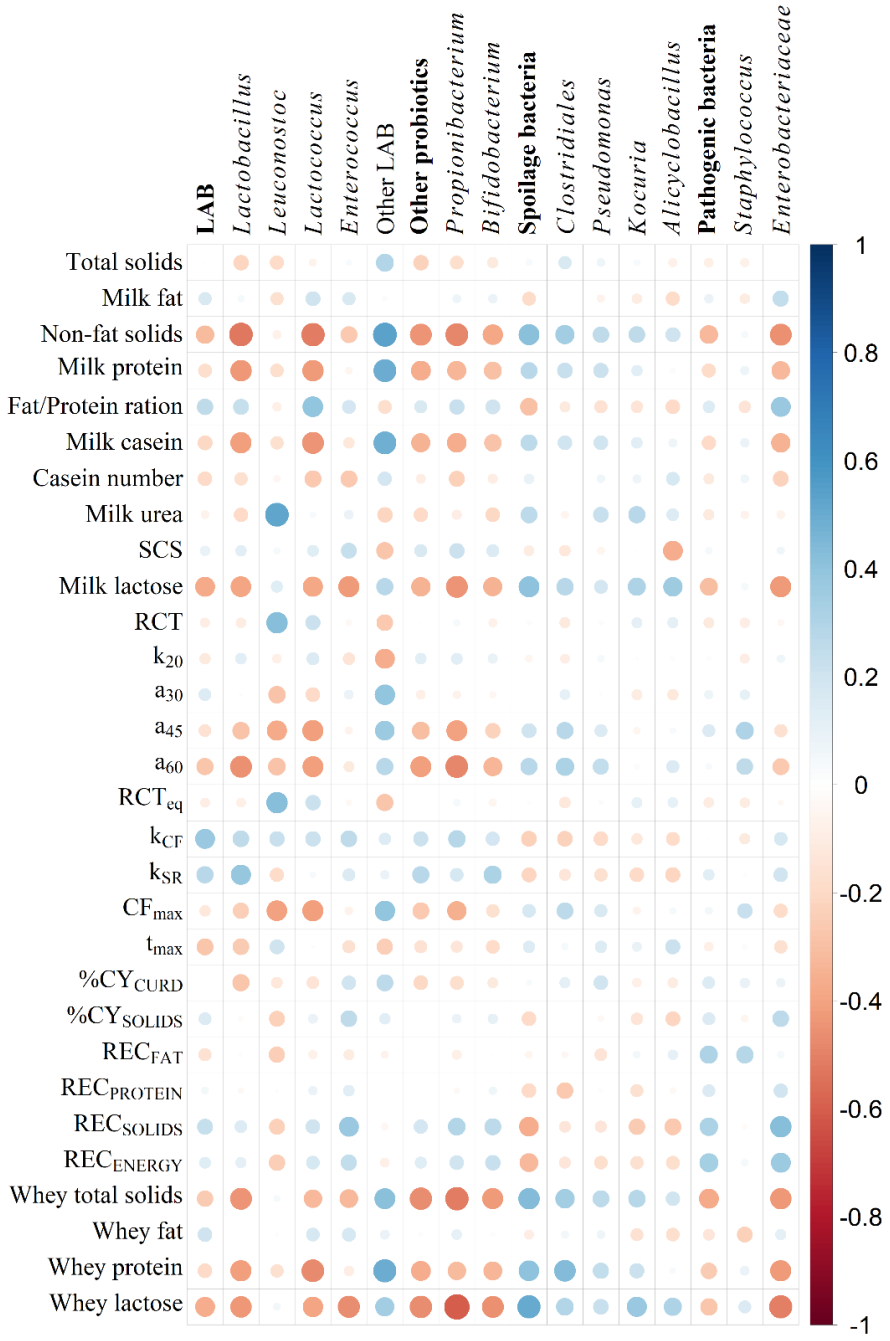
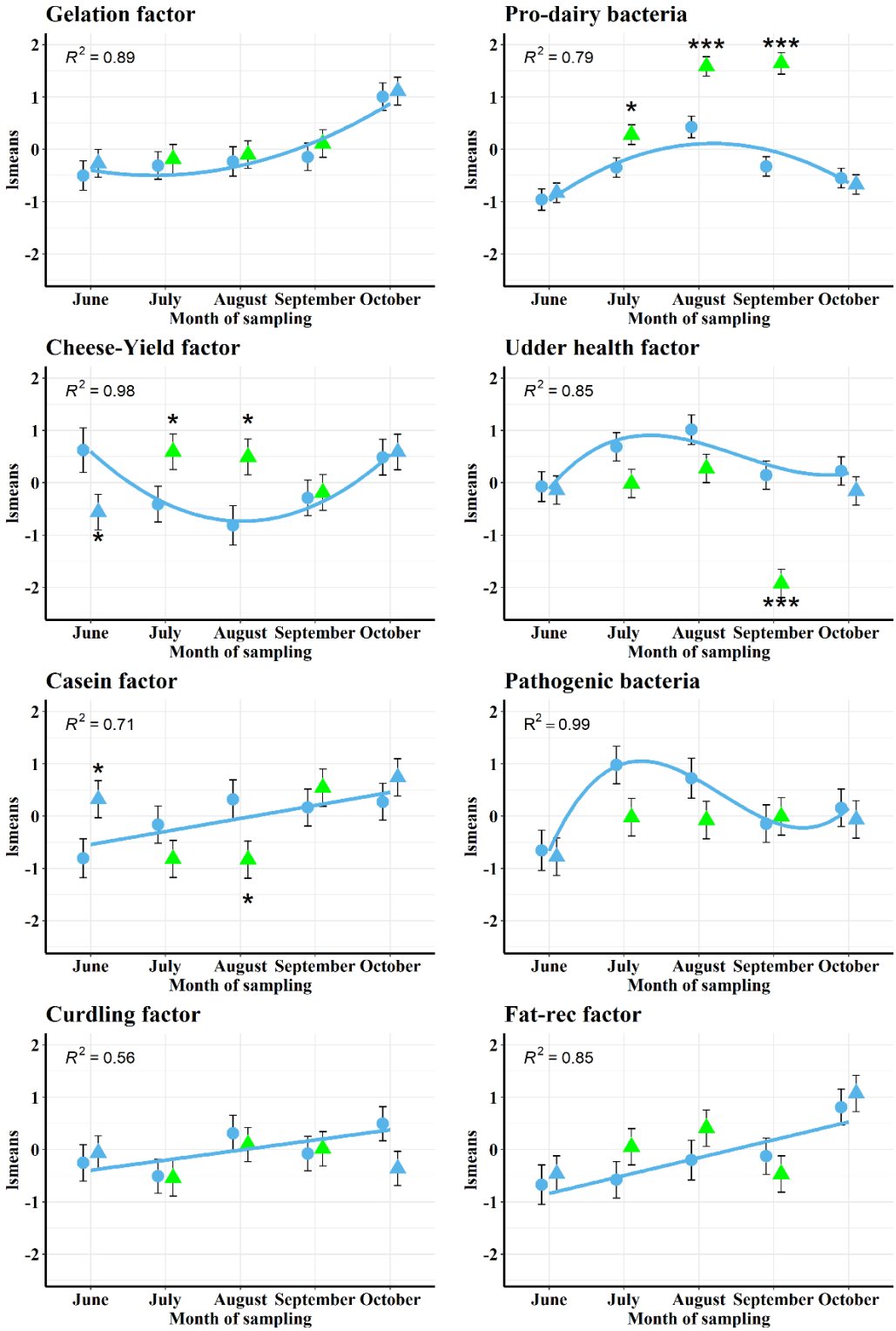


Figure 8. Patterns of the scores of the latent explanatory factors during the experimental period. Blue circles represent the LSM of the cows kept solely indoors, green triangles represent the cows moved to summer highland pastures, and blue triangles represent the latter cows when indoors before and after the summer transhumance. Bars represent the SE of estimates. Lines and curves represent significant linear, quadratic or cubic patterns, with their R^2 values, for the cows kept solely indoors. Asterisks indicate the significance levels of the differences between the two groups in each month (*P<0.05; **P<0.01; ***P<0.001).



CHAPTER III

Variation of microbiota, chemical composition and B-vitamins in milk from alpine pasture and indoor dairy cows

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INTERPRETIVE SUMMARY

Variation of microbiota, chemical composition and B-vitamins in milk from alpine pasture and indoor dairy cows

By Secchi et al., page 000. This study addresses the complex relationships between metagenomics, composition, B-vitamin and Lactoferrin in milk of 26 Italian Simmental cows from four farms during the summer transhumance to alpine pasture, and successively the same cows when moved back to lowland permanent indoor farms. In the milk, 8 traits were obtained for the composition, 4 regarding udder health, 4 B-vitamins, 8 for the bacterial counts, and 41 bacterial taxa, 11 of which with special interest for the dairy industry (LAB and spoilage bacteria), and human and animal health (other probiotics and pathogenic bacteria). The metagenomics analysis confirmed the high variability of the milk microbiota during and after summer transhumance, but also a large influence of different herd management.

ABSTRACT

The investigation of the complex connections between metagenomics with milk quality, B-vitamins and Lactoferrin, influenced by indoor farming and summer grazing by comparing different farms, was the aim of this work.

In detail, this study compared the milk obtained from 26 Italian Simmental cows of four herds during summer transhumance to four Alpine pastures (ALP) and milk of the same cows obtained during the following permanence in the indoor permanent farms (PF). The milk composition (8 traits), the udder health traits (4 traits, including lactoferrin), the B-vitamins content (4 traits), the bacterial counts (8 traits) and the relative abundances of milk bacterial taxa obtained (41 individual taxa and 5 groups) were analyzed on 52 milk samples. Results obtained using a linear model including herd, ALP vs PF and their interaction showed that there are many significant differences in milk composition and microbiological traits and that interaction represents an important source of variability. The large number of traits considered (70) and the complex matrix of variance-covarince between them suggested to perform a latent factor analysis on the dataset. Eight latent explanatory factors explained 75% of all variance. Seven latent factors (the most important of which being "Pro-dairy", "Probiotics", and "Caseins") combined contemporarily milk composition and microbial traits and characterized the differences observed between the milk obtained during summer transhumance to Alpine pasture and during the following indoor rearing, among the different herds and their interaction. The exception was the "Udder health" latent factor.

Keywords*:* raw milk, milk metagenomics, MiSeq Illumina, B-vitamins, lactoferrin, summer transhumance, lactic acid bacteria.

INTRODUCTION

In the European Alps and other mountainous regions, semi-natural pastures are the main food resources for ruminants during the summer season. Transhumance of dairy cows to temporary summer farms on high mountain pastures is an important ancient practice with a long-standing traditional heritage, also relevant to the maintenance and preservation of the environment and biodiversity (Eriksson, 2011; Sturaro et al., 2013).

When dairy cows are moved to summer pastures, they undergo several changes, which could cause nutritional imbalances and affect the production, quality, and composition of milk and dairy products (Bergamaschi et al., 2016; Zendri et al., 2016; O'Callaghan et al., 2017). These changes are influenced by a combination of different feed quality, availability, and botanical composition of pastures, increased physical activity during grazing, pasture adaptation, and different climatic conditions (Gorlier et al., 2012).

The summer transhumance to highland pasture is responsible of deep modification of composition, technological properties, microbiological profile and human health effects of milk and other dairy products.

We have carried out a previous research (Juribello project) on the changes before, during, and after summer transhumance on the nutritional and technological aspects, but also from the microbiological profile of milk of two groups of cows, a control group that remained indoor on a permanent lowland farm during all the experiment and a transhumant group which moved from lowland to Alpine highland pasture during summer and returned in the permanent farm in autumn (Saha et al., 2019; Carafa et al., 2020).

Milk is a rich medium for the development of a wide variety of microorganisms and in accordance with the definition proposed by Marchesi and Ravel, (2015), the milk microbiota refers to the set of microorganisms present in milk. The milk microbiota has been extensively investigated principally in cows (Addis et al., 2016; Doyle et al., 2017; Taponen et al., 2019), but also in other

dairy species such as sheep (Blanco et al., 2020), goats (Mcinnis et al., 2015), donkeys (Papademas et al., 2021), buffalo (Catozzi et al., 2017), and women (Butts et al., 2020; Lyons et al., 2022).

During the Juribello project we have analyzed the milk microbiota and we have seen that during summer transhumance there was increasing in lactic acid bacteria and other probiotics, and a decreasing in spoilage bacteria (Secchi et al., 2023). These changes reflect an improvement of technological properties of milk and a benefit for human health, but all the changes disappeared after summer transhumance.

But the health effect of milk is not limited to microbiological profile or to the fatty acid profile (Bergamaschi and Bittante, 2017), but also in the content of vitamins. The B vitamins are a group of water-soluble vitamins abundant in milk, and they have key functions as enzyme cofactors or intermediate components in major metabolic reactions (Graulet and Girard, 2017); they are also synthesized by the microorganisms in the rumen, so B vitamin deficiency is limited to situations where an antagonist is present or the rumen lacks the precursors to produce the vitamin. The knowledge about the effect of grazing on the profile of water-soluble vitamins in cows' milk is scarce. Magan et al. (2020), for example, investigated how the use of pasture or concentrate-based cow feeding systems significantly affected the relative concentrations of a limited number of water-soluble vitamins in the skim milk and whey protein powders ingredients analyzed.

Currently, there is a lack of studies regarding the milk microbiota during and after summer transhumance and the B-vitamin profile of milk, even from individual animals of different farms in the same area, because often experimental trials are limited to single farms. The purpose of this study is to investigate, in 4 different farms, the response to summer transhumance of dairy cows on variation in the dairy microbiota and its relationship to milk quality, B vitamins, and Lactoferrin.

MATERIALS AND METHODS

Experimental design

The experimental design was a 4 × 2 factorial design: 4 herds and two locations/seasons. Each herd was housed in a different permanent farm (**PF**) from autumn to spring, and was moved in a different temporary farm in the highland for Alpine pasture (**ALP**) during summer transhumance.

The four PF are located in Veneto (Villaverla, Vicenza, Italy) and Trentino Alto Adige (Levico Terme, Trento, Italy) provinces, and are at an altitude between 70 to 500 m above sea level. The corresponding four ALP are located on the Vezzena highland (Trento, Italy) between 1380-1700 m above sea level.

Twenty-six healthy Italian Simmental cows in mid-lactation, representative of their herds, were selected (5 to 7 animals per herd). Two PF herds (C, and P) were loose-housed, while the other two PF (M, and F) were tie-stall housed. Animals were fed meadow and alfalfa hay and compound feeds. At the end of June 2020, the herds were moved from the PF to their respective ALP, where the cows were free to graze day and night on Alpine pastures in different areas according to grass availability, without a rigid rotation plan. Each farm was also giving a compound feed supplement in the milking parlor according to milk yield.

Milk sampling

Individual milk samples were collected once at the end of July, four weeks after the beginning of Alpine pasture, and once at the end of October four weeks after cows were moved back to the PF for a total of 52 milk samples.

Two of the four PF and all ALP are equipped with a milking parlour and perform pre-milking cleaning of the teat skin and foremilk stripping. The two PF with tied cows used mobile single bucket milking machine. All the milk samples were obtained using the equipment adopted for official milk

recording by the Provincial Federation of Trento Breeders and approved by ICAR (International Committee for Animal Recording https://www.icar.org/). The sampling was based on a complete milking of all the four udder quarters during the evening milking.

None of the cows showed clinical symptoms of mastitis during the whole experiment. Two aliquots from each milk sample was taken: 50 mL for quality traits and microbial count analysis, were stored at 4°C until analyses, carried out within 24 h; 50mL for the metagenomic analysis were immediately frozen in liquid nitrogen and then stored at –80 °C until analyses were carried out within three months.

Milk composition traits

The milk composition was performed at the milk laboratory of the Department of Agronomy, food, Natural Resourced, Animals and Environment (DAFNAE) of the University of Padova.

Each individual milk samples were analyzed for protein, fat, lactose, and urea content with a MilkoScan FT2 infrared analyzer (Foss Electric A/S, Hillerød, Denmark) calibrated according to the following reference methods: ISO 8968-2/IDF 20-2 for protein, ISO 1211/IDF for fat, and ISO 26462/IDF 214 for lactose. Somatic cell counts (SCC) were obtained with a Fossomatic Minor FC counter (Foss Electric A/S) and log-transformed to somatic cell score (SCS) using the formula SCS = $\log_2$(SCC/100,000)+3 as proposed by Ali and Shook, (1980).

Determination of lactoferrin by ELISA

The content of lactoferrin in the milk was determined by commercial enzyme-linked immunosorbent assay (ELISA) test kits from Cloud-Clone, (Cat. SEA780Bo 96 test for Lactoferrin (LTF), organism species: Bos Taurus, CLOUD-CLONE CORP. Houston, USA), according to the manufacturer's recommendations. Briefly, milk samples were centrifuged for 15 minutes at 10,000 × g at 4°C, collect the aqueous fraction and centrifuged twice more for a total of 3 cycle. After. The

samples were diluted for 500–1000 times with MilliQ water (Heidebrecht and Kulozik, 2019). 100 μL of the samples or reaction standards was added into a 96-well plate pre-coated; the solutions were removed after an incubation at 37 °C for 1 h; the wells were then washed three times with Wash solution. Then, 100 μL of Detection Reagent B was added to each well, and incubated at 37 °C for 30 min; the wells were washed as before for 5 times.

Next, 90 μL of substrate solution was added to each well and incubated at 37 °C for 10-20 min to allow for the color to develop. Finally, 50 μL of Stop solution was added to each well to stop the reaction; the plates were immediately placed in a Multiskan FC plate reader (Thermo Fisher Scientific, Karlsruhe, Germany) the absorbance of three replicates was recorded at 450 nm immediately. The standard curves were generated as a 7-parameter curve fit using Soft-Max Pro. The final concentrations of lactoferrin were expressed as the average of three replicates.

Vitamins B analysis by LC-MS/MS

The B vitamins analysis were performed at the Metabolomic Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). All the reagents and chromatographic solvents (methanol, acetonitrile, formic acid and B-vitamins standards) were HPLC or LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), MilliQ water was used for the chromatography. The vitamins separation was performed with an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) using an Acquity UPLC BEH C18 (1.7 μm, 2.1 mm × 50 mm) column (Waters corporation, Milford, MA, USA) at 40 °C. The mobile phase consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B). Elution was performed at 0.25 mL/min with the following gradient: 0 – 3.00 min increase B 0% to 48%, 3.01 – 4.50 min hold 100% B, and 4.51 – 7 min hold 0% B. An integrated valve was scheduled to release the analytes into the mass spectrometer only from 0.5 to 3.5 min in order to keep source and analyzer free from dirt. An AB Sciex LLC QTRAP 6500+ was operated in positive ion multiple reaction monitoring (MRM) mode using a Turbo V ion

source with the following settings: Curtain Gas (CR) 35 °C, IonSpray Voltage (IV) 4500 V, Temperature 400 °C, Collision Gas (CAD) Medium, Ion Source Gas 1 (GS1) 45 psi, and Ion Source Gas 2 (GS2) 55 psi. Each period was scheduled with 600 cycles of 0.2 s cycle time each. The signal was acquired only in the analyte elution window (from 0.5 to 3.5 min). All the detailed settings for the MS/MS method are summarized in Table S1. Stock solutions of each individual standard were prepared in water/methanol (1:0.25), while vitamins were prepared as 10X - 100X or 1000X stock solutions. All the solutions were stored and refrigerated at -20°C until used. Briefly the samples preparation was prepared as following, 2 mL of each frozen milk samples were centrifuged at 16,000 g for 30 min, 100 µL of clear surnatant was placed in a HPLC vial, 2 µL was the injection volume. MultiQuant and Analyst from AB Sciex LLC were used for data acquisition and elaboration, respectively.

Metagenomic analyses

Milk samples microbiological counts and isolation

Microbiological analysis were performed at the microbiological laboratory of the Research and Innovation Center, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Milk samples were decimally diluted in sterile peptone water and plated onto the following agar media: Plate Count Agar (PCA) with skim milk (1 g/L) for the total bacterial count (TBC), in aerobic conditions for 24 h at 30 °C; Wilkins Chalgren (WC), for the anaerobic total bacteria count, incubated in anaerobic conditions for 48 h at 37 °C; de Man, Rogosa and Sharpe (MRS) agar acidified to pH 5.5 with 5 M lactic acid, for cultivating mesophilic lactobacilli, incubated in anaerobic conditions (in a jar with an "Anaerogen" anaerobic system) for 48 h at 30 °C; MRS agar with 0.05% (w/v) L-cysteine (MRS-*cys*), for cultivating bifidobacteria, incubated in anaerobic conditions for 48 h at 37 °C; M17 agar, for cultivating mesophilic lactococci, incubated in aerobic conditions for 48 h at 30 °C; Yeast Extract Lactate Agar (YELA), for counting propionibacteria (brown colonies), incubated in anaerobic

conditions for one week at 37 °C; Violet Red Bile Agar (VRBA) for counting coliforms, following the overlay method as suggested by manufacturer's instruction, for 24 h at 37 °C; Columbia Blood Agar (CBA) containing 5% defibrinated sheep blood for the count of hemolytic Streptococci incubated aerobically at 37 °C, and examined after 24 and 48 h; Columbia Blood Agar (CBA) supplemented with 5% defibrinated sheep blood with the addition of Chloramphenicol for growing algae, and suppress the growth of microorganisms, incubated aerobic conditions at 37°C, and examined after 48 and 72h. For CBA+sheep blood only colonies showing greenish discoloration (partial hemolysis) or clear zone (total hemolysis) around the colonies were counted; while for CBA+sheep blood+chloramphenicol only the dark grey opaque colonies were counted after a microscopic inspection for Prototheca spp. All culture media and anaerobic system were purchased from Oxoid (Thermo Fischer, Waltham, MS, USA).

One to three colonies were randomly isolated from WC and MRS-cys plates (plates with a number of colonies in the range of 10 - 300), while only brown colonies were isolated from YELA, which are reported to belong to *Propionibacterium* genus (Thierry and Madec, 1995). Each isolate was purified by subsequent culturing in the proper broth culture (the broth version of the same agar medium used for plate counting and isolation). Pure cultures were stored at –80 °C in glycerol (40% v/v) stocks. Cell morphology was determined by microscopic observation.

DNA extraction and genotypic identification of the milk-resident bacteria

All bacterial strains were grown overnight in the proper broth culture at 37 °C before DNA extraction. The bacterial DNA was isolated using Quick-gDNA™ MicroPrep (Zymo Research, Italy) following the manufacturer's instructions. A fragment of the 16S rRNA gene was amplified using the primers 27F (50-GAGAGTTTGATCCTGGCTCAG) and 1495R (50-CTACGGCTACCTTGTTACGA), designed by Grifoni et al. (1995).The PCR products were purified using the Exo-SAP-IT™kit (USB Co., Cleveland, OH), and sequenced in an ABI PRISM 3100

sequencer (Applied Biosystems, Italy), using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI, USA). All amplifications were run in a T100™ ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA).

The pools were always successfully amplified in the bacterial V3-V4 16S rRNA gene region. 2,023,319 paired-end sequences (an average of 38,910 reads per sample) were obtained.

Total DNA extraction from milk samples

For total genomic DNA extraction, 4 mL of milk were centrifuged at 4,000 g for 10 min at 4 °C, and the supernatant was discarded. Genomic DNA was extracted from the pellet using the DNeasyPower Food Microbial Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific, USA).

Preparation of the MiSeq library

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3-V4 region (Baker et al., 2003; Claesson et al., 2010), of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a

Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2020.11). Sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013)); Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA903798 (https://www.ncbi.nlm.nih.gov/sra/PRJNA903798).

Bacterial categories

On the basis of our previous study (Secchi et al., 2023), also in this work we decided to classify the relative abundances of the principal bacterial taxa identified by Qiime2 into four categories: the Lactic Acid Bacteria (LAB) category includes the taxa belonging to the Lactobacillales order (*Lactobacillus*, *Lactococcus* and *Enterococcus*), and Streptococcus (Khalid and Department, 2011; Gagnon et al., 2020); the "Other probiotics" category includes all the taxa belonging to the

Propionibacterium (Rabah et al., 2017) and Bifidobacterium genera (Prasanna et al., 2014); the "Spoilage bacteria" category includes all the taxa belonging to the Clostridiaceae (Burtscher et al., 2020) and the Pseudomonas (Meng et al., 2017); and at the end the "Pathogenic bacteria" category includes all the taxa belonging to the Staphylococcus genus (Gebremedhin et al., 2022), and the Enterobacteriaceae family (Anand and Griffiths, 2011).

The remaining 30 bacterial taxa were grouped as "other milk bacteria".

Statistical analysis

The data regarding microbiological counts were analyzed as means expressed in log CFU/mL, while relative bacterial abundancies were $\log_{10}$ transformed. One sample was excluded from the statistical analysis due of a lack of milk composition data. All bacterial and qualitative data were checked to identify and exclude outliers value (outside the interval ± 3 SD of the mean).

Mixed-model analysis of variance

The milk composition, the milk bacterial count, and the $\log_{10}$ transformed relative abundancies were analyzed according to a linear mixed model (RStudio version 1.4.1106) including the fixed effects of Herds (four levels: C, M, P, F), Location (two levels: ALP and PF), their interaction, and the random effect of cow nested in the Herd. It worth noting that the ALP vs PF contrast reflects not only the effects of location (geographical area, altitude, management, feeding, etc) but also the effect of season (July vs October) and of the lactation stage (mid-lactation vs late-lactation). We used a function to estimate R^2_{GLMM} statistic, *r.squaredGLMM*, included in the *MuMln* package for the R statistical software, we consider the conditional R-squared because concern variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013).

Contrast were estimated to examine the difference between ALP and PF to test for the effect of summer transhumance on the main effects, but also within each herd to explain the interaction between herd and location. A similar model with all effect as random factor was run for quantifying

the relative importance of the different herds and locations (and their interaction), of individual animals within herd and of residual factors non accounted by the model. The variance of these five sources of variation were expressed as percentage of their sum (total variance).

Regarding Miseq Illumina data: Alpha-diversity was performed with Goods coverage, observed OTUSs number and Shannon diversity index and statistical significance of between-group alpha diversity metrics were evaluated by Kruskal–Wallis H test in QIIME2; Beta-diversities were calculated using Unweighted and Weighted dissimilarity distance matrix in QIIME2. Beta-diversity distance matrix indicates differences in taxa composition between samples based on either presence-absence or quantitative species abundance data. Output matrix was ordinated using principal coordinate analysis (PCoA) and visualized using EMPeror (Vazquez-Baeza et al., 2013). Statistical significance of beta-diversity distances between groups was assessed using PERMANOVA with 999 permutations in QIIME2. For differential abundance test, taxonomy information was provided for each OTU sequence using ANCOM method (Mandal et al., 2015) implemented in QIIME2.

Correlation analysis and latent explanatory factor analysis

The dataset regarding relative abundances (only the bacterial categories of our interest), the milk composition, and the microbiological traits were merged for the correlation and multivariate analysis to explore the relationship between bacterial and chemical traits.

Correlation were carried out among the metagenomic relative abundancies of the selected taxa and group, and between the metagenomic relative abundancies and bacterial counts, and milk composition, including B vitamins and Lactoferrin.

For the high number and complexity of the relationship between all the traits, we used a multivariate factor analysis (FA) to summarize the interrelated measured traits in a small number of unmeasured latent independent explanatory variables (factors). FA was performed on the selected traits as follow: first, we performed KMO (Kaiser-Meyer-Olkin) and Barlett's tests, which showed

that the traits were suitable for FA. The factor analysis was carried out with Varimax rotation in the R environment (R Core Team, 2016) using the psych package (available at CRAN: The Comprehensive R Archive Network) in three steps: (i) extraction of factors such that the minimum number of uncorrelated latent factors explained the greatest proportion of common variance; (ii) factor rotation until each factor was defined by a few variables with high loadings; and (iii) biological interpretation of the factors based on the strength of the loadings of the variables. The eigenvalues of the factors and the communalities of the variables after rotation were also determined.

Eight latent explanatory factors were extracted from the 39 milk traits selected (8 milk composition, 4 udder health traits, 4 vitamins, 8 milk microbial counts, and 15 metagenomic traits). The scores of each milk sample for each factor were analysed using the same linear mixed model as that used for the metagenomic relative abundancies.

RESULTS AND DISCUSSION

Milk composition, udder health traits and lactoferrin

Descriptive statistics and results of ANOVA of the mixed model for the milk composition, udder health and Lactoferrin are presented in Table 1.

As our knowledge, transhumance to summer alpine pasture has notable effects on the cows' physiological, social, feeding, and nutritional status, which are reflected in modification to milk quality traits of the milk produced (Leiber et al., 2006; Saha et al., 2019) (MY), specifically the deficiency in nutrients and energy, and changes in the environment and management may explain the decreasing MY that was 19 kg/d during summer transhumance, and increase to 21 kg/d after ALP at the PF.

The average milk composition was in the range of that found in the Alpine region for Simmental dual purpose area (Zendri et al., 2016; Bittante et al., 2021) and also in different production system and in different European countries (Perišić et al., 2009). Regarding the different effects that we considered, the herd affected all composition traits with the exception of total solids, casein index, SCS and lactoferrin (Table 1). The effect of ALP vs PF and of its interaction with herds affect the same traits excluding milk fat and fat/protein ratio. The contrasts between ALP and PF reported in the Table 2 highlighted that the milk composition traits were quite always lower during the ALP, respect to the PF, but also reflected the considerable differences among the herds. It worth noting that the decrease of nutrients content between ALP and PF is probably due mainly to the advancing of lactation (Amalfitano et al., 2021). In our previous study on summer transhumance, we found that cows transferred on highland pasture were producing milk with more fat and protein than the cows of the same herd maintained indoor during summer, and both groups increased the content of fat and protein in the following October, when both groups were in late-lactation and were kept indoor in the PF (Saha et al., 2019).

An exception to this interpretation is the pattern of milk urea, which decreased on average between the ALP and the PF samplings (Table 2), but with very large differences among different herds. This trait reflect much more the protein content of the diet (Bittante, 2022) than physiological aspects of the cow, like lactation stage (Amalfitano et al., 2021). .

Regarding udder health traits, it is possible to see that milk pH and lactose content are much different in different herds and also the comparison between ALP and PF is different in different herds (interaction between herd and location) whereas the average effect of location (ALP vs PF) was not significant (Table 1). The importance of the interaction between herd and location could also explain the differences observed with other studies, based on single herds (Romanzin et al., 2013). SCS was lower in ALP samples than in PF samples (Table 2), and also this effect could be probably attributed more to the advancement of lactation than to the feeding and environment. Lastly the lactoferrin, which is a milk protein with antimicrobial and immunomodulatory activities, was not affected by the factors included in the statistical model, but this seems due to the very large variability of this trait (Table 1).

In Figure 1b we can found the various sources of variability for milk composition traits. It is possible to see that the effect of herd is dominant for the fat/protein ratio, and moderate for the milk fat. While null effect for protein, casein and casein number, where instead the comparison between ALP and PF was important. The interaction between the individual herds and the period/location was relevable in some cases, and moderate in urea and pH. The random effect of individual cows, nested within the herds, has some visible effect in the case of somatic cell score and casein number. Lastly, the residual variation was prevalent in fat/protein ration and milk fat.

B-Vitamins composition

Descriptive statistics and results of ANOVA of the mixed model for B vitamins content are presented in Table 1.

B vitamins were water-soluble compounds, and they were produced by plants (except B_{12}), by microorganisms (all B vitamins complex), and by animal tissues in minimum quantities (Nicotinic Acid). All B vitamins are present in milk and dairy products; depending to consumption habits, milk and dairy products can be significant source of B vitamins and occupy a major place in a healthy and well-balanced diet. B vitamins are essential for cell life and metabolism, but also additional biological activities. Cow milk contains all B vitamins except biotin, because they are supplied to cows by feedstuffs ingested and, moreover, because they are synthesized by rumen and milk microorganisms (Leblanc et al., 2011). Different factors can affect the B vitamins concentration in cow milk, i.e. stage of lactation, breed, season, dietary factors and genetic factors. On average the milk content found here for riboflavin was higher, while for thiamine and nicotinic acid lower than the data reported by Graulet and Girard, (2017). It is evident that the four vitamins analyzed are all characterized by a large variability among different milk samples, and this explain the low proportion of effects reaching the statistical significance. None of the four vitamins were significantly affected by herd, nicotinic acid (vit B_3) was affected by location being higher in ALP samples than in PF samples (Table 2), and riboflavin (vit B_2) showed different, and also opposite contrasts between ALP and PF (Table 1: significant interaction; Table 2: different contrasts in different herds).

Bovine dietary riboflavin is primarily sourced from green, leafy forage, in facts riboflavin is present in considerably lower concentrations in cereal grains, compared to fresh leafy forage (i.e., grass) (Edelman and Colt, 2016). Riboflavin provides pigmentation in leaves, conferring a yellow color similar to β-carotene, the relative abundance of which is primarily responsible for the intensity of yellow color in fat-containing dairy systems. These results could be in agree with Poulsen et al. (2015) study that compared the riboflavin content of bulk milk from three dairies in Denmark, recording higher riboflavin concentrations in milk from an organic dairy derived from high dietary proportions of grass and legume-based forage, than in milk from two conventional dairies.

We detected a very low quantity of folic acid in our milk samples which plays an essential role in DNA and methionine metabolism, and the highest milk folate concentrations are found in the colostrum. Presence of folate-binding protein in bovine milk has been detected many years ago. This protein seems to play a role in secretion of folates into milk (Ford et al., 1972). Milk folate–binding protein does not improve efficiency of intestinal absorption of folates in humans, but the protein present in cow milk protects the labile forms of folates naturally present in milk from degradation during the gastrointestinal transit (Nygren-Babol and Jägerstad, 2012) and reduces folate uptake by intestinal bacteria (Ford, 1974), which overall can contribute to increase the amount of folates available for absorption. The random effect of individual cows, nested within the herds, in the case of the folic acid was dominant (see Figure 1b).

Microbial counts

Descriptive statistics and outcome of ANOVA on microbial counts and metagenomics relative abundancies of raw milk samples are shown in Table 3, while contrasts between ALP and PF in general and in specific for each herd are presented in Table 4.

All plate counts presented large differences due to herds, location (ALP vs PF, with the only exception of those onto YELA and WC) and to their interaction (Table 3). The loads of all microbial counts measured in milk, were always higher in PF samples than in ALP samples (Table 4). In fact, the TBC (total bacteria count) on PCA, as well as the LAB counts on MRS and M17, hemolytic streptococci on CBA, and coliforms in VRBA were significantly higher in PF than in ALP samples. This superiority in October PF milk samples respect to July ALP samples seem to be related to advancement of lactation and season more than to environment and feeding condition. In fact, in the previous study (Carafa et al., 2020) on two groups of cows (one kept always indoor and the other moved to highland pastures and returned indoor), we found that in July the milk samples of the second group were characterized by higher counts than the first group for aerobic TB (PCA), anaerobic TB

(WC), mesophilic lactobacilli (MRS), mesophilic lactococci (M17), putative bifidobacteria (MRS-cys), putative propionibacteria (YELA), and coliforms bacterial counts (VRBA).

The Figure 1a shows that the different sources of variability for the milk bacterial counts. The comparison between ALP samples and PF samples was prevalent for aerobic TBC and mesophilic lactobacilli, and moderate for anaerobic TBC, mesophilic Lactococci and proteolytic. The interaction between the individual herds and the period/location was almost always dominant. The random effect of individual cows, nested within the herds, was of scares-null importance with some visible effect only in the case of hemolytic streptococci and coliforms. Lastly, the residual variation not accounted by the former factors is notable in the case of anaerobic TBC, proteolitics and hemolytic streptococci. While, the residual variation is very small for the putative propionibacteria.

High-throughput 16S rDNA sequencing analysis of milk samples

In total, we obtained the relative abundancy of 41 microbial taxa in milk samples analyzed. The 11 taxa more interesting for milk use and value were grouped in 4 groups of taxa, illustrated in Table 3: those more related to cheese-making properties of milk (LAB, lactic acid bacteria, 5 taxa), those potentially favorable to human health (Other probiotics, 2 taxa), those involved in milk deterioration (Spoilage bacteria, 2 taxa), and those potentially affecting negatively humans and animals health (Pathogenic bacteria, 2 taxa). These 11 taxa represented all together the 3.30-98.66 % of all DNA extracted. The remaining 30 taxa were grouped in “Other bacteria”. Of the 46 taxa and groups 38 presented significant differences among the 4 herds, 31 were different in between the ALP (July) and PF (October) samples, and 38 were affected by a significant interaction. All taxa were significantly affected by at least one of these three fixed factors included in the model.

For the 11 taxa useful for evaluating the value of milk and for the four groups in which they have been grouped it is possible to analyze deeply the relative importance of the different sources of variation. The Figure 1a shows clearly that the sources of variability are very different for individual

taxa and also for their groups. It is possible to see that the effect of herd is dominant for *Enterococcus* and *Enterobacteriaceae*, and moderate for *Streptococcus*, Other LAB, and *Pseudomonas*. The comparison between ALP samples taken in July and PF samples taken in October, across all herds, was relatively important in the case of *Lactococcus*, *Streptococcus*, *Propionibacteriaceae*, *Staphylococcus* and *Enterobacteriaceae*. The interaction between the individual herds and the period/location was always very important and in some cases dominant (*Lactobacillus*, *Lactococcus*, *Propionibacteriaceae*, and *Clostridiaceae*). The random effect of individual cows, nested within the herds, was of minor importance with some visible effect in the case of *Lactococcus*, *Enterococcus*, and *Staphylococcus*. Lastly, the residual variation not accounted by the former factors is dominant in the case of Other LAB, *Bifidobacteriaceae*, *Pseudomonas*, and *Staphylococcus* taxa and in Spoilage and Pathogenic bacteria groups. On the contrary, the residual variation is very small for the majority of LAB taxa, the Propionibacteriaceae taxa and Other probiotic group.

It worth noting that the relative importance of different sources of variation of groups of taxa are not simply equivalent to the weighted means of their individual taxa (Figure 1a). This is due to the fact that relative abundancies are log transformed to search for Gaussian distribution and to the fact that they are not independent among different taxa. The positive correlations between the individual taxa of a group tend to increase the importance of the source in the group of taxa. An example is the higher importance of individual cows for the LAB group respect to the importance of cows in the 5 individual taxa of this group. On the contrary, negative correlations between taxa can compensate their effects (see herd importance in LAB group and in Pathogenic bacteria group).

It is well known that raw milk is a complex microbial ecosystem (Quigley et al., 2013). Changes in rearing conditions and in seasonality have an impact the quality and safety of milk. In our previous work on the Juribello project we have found this peculiarity. In particular, Secchi et al., (2023) has shown during the summer months of transhumance a positive increasing on the growth of

LAB and other probiotics, as well as a decreasing in spoilage bacteria. In the present study, we are unable to distinguish the effect of advancing season and the lactation stage.

Mallet et al., (2012) found considerable quantitative and qualitative microbial diversity in raw milk in the region of Basse-Normandie, France. This diversity was influenced by a combination of milk collection practises and farm management practises considered, milk samples were collected in a winter period with cows kept indoors, and in a spring period where the cows were outdoor at pasture.

Each animal's milk is different, but each farm's milk is also different. Currently, experimental trials ono milk microbiology is often limited to individual farms and individual highland pastures, so it is difficult to compare this present work that include 4 different farms and pasture, with the studies in the literature.

Correlations among the bacterial and compositional traits of milk

All the milk traits considered in this study are not independent with each other but present variable correlations. The correlations among the composition, udder health and B-vitamins traits are shown graphically in the heat map of Figure 2. As expected, total solids, fat, non fat solids, protein, fat/protein ratio and casein are generally positively correlated. Casein number is, on the contrary, negatively correlated with the former traits. Correlations of milk urea, pH, SCS and lactoferrin content with other composition traits are moderate to low. Lastly, milk lactose is correlated negatively with milk fat (and fat/protein ratio) and positively with non-fat solids and casein number, but, especially, is negatively correlated with SCS.

Moving to the four B-vitamins considered, it could be seen from Figure 2 that they tend to be positively correlated with each other, with the exception of folic acid (vitamin B_9). The correlations between the B-vitamins and the other compositional traits are generally very modest, with the exception of that between folic acid and SCS (positive) and lactose (negative).

While the correlations among milk compositional traits found here are generally in agreement with the results of previous studies (Macciotta et al., 2012), those among B-vitamins, and between them and the milk compositional traits are not reported in the literature, the authors are aware of.

The correlations among the milk bacterial count and the relative abundancies of the milk microbial taxa and their groups of interest obtained from metagenomic analysis are depicted in the heat-map of Figure 3. First of all, we can see that the microbial counts tend to be positively correlated among them. As expected, it is possible to see that the four groups considered are generally positively correlated with their individual taxa, and particularly with those characterized by the largest abundancy: LAB group with *Lactococcus*, Other probiotics with *Propionibacteriaceae*, Spoilage bacteria with *Clostridiaceae* and *Pseudomonas*, and Pathogenic bacteria with *Staphylococcus*. Considering the correlations between the individual bacterial taxa, it could be seen that positive moderate correlations are found between *Lactobacillus* and *Propionibacteriaceae*, and between other LAB and *Clostridiaceae*, whereas negative correlations are found especially between *Lactococcus* and the large majority of the other microbial taxa. Lastly, it could be observed the negative correlations between the two taxa grouped in the Pathogenic bacteria (*Staphylococcus* and *Enterobacteriaceae*), which, moreover, were characterized by opposite correlations with all the other groups and taxa (Figure 3). This confirms fully the results obtained in a previous study on a different database (Secchi et al., 2023).

The relationships between microbial counts and metagenomic taxa and groups on one side and milk composition and B-Vitamins contents on the other traits are more complex and variable and are summarized in the heat-map of Figure 4.

First of all, we can see that some traits are relatively independent from the others and tend to present modest correlation coefficients: YELA and CBA counts and *Bifidobacteriaceae* and *Clostridiaceae* bacterial taxa among the microbial traits, and the fat/protein ratio, SCS, milk lactose, lactoferrin and folic acid among the compositional traits.

The other microbial counts tend to be correlated positively with the milk nutrient contents and negatively with the casein number, milk pH and B-vitamins. The situation of the 240 correlation coefficients between metagenomic and milk composition traits is much more variable (Figure 4) and require a different, multivariate, analysis for being studied. A similar situation was found in our previous study on Juribello project (Secchi et al., 2023) which, differently from this study, included also cheese-making traits, but not the microbial counts.

Latent explanatory factors of the bacterial and compositional traits of milk

The multivariate factor analysis carried out on the 39 selected compositional and bacterial milk traits identified eight latent explanatory factors that all together explained 70% of total variance-covariance matrix among all traits. The loadings of each factor, excluding those non-relevant (< 0.30), are reported in Table 7, while high loading (>0.50) are shown with an asterisk. The explained variance (as % of total variance, and the analyses of variance of the scores obtained for each factor and milk sample are summarized in Table 8 and, lastly, the contrasts between ALP and PF samplings are reported in Table 9. The latent explanatory factors obtained are described and discussed individually. We will compare often this study with our previous research (Juribello project) as it is the only work reporting latent explanatory factors including milk compositional and microbiological traits (Secchi et al., 2023).

Latent factor 1: Pro-dairy

The first latent explanatory factor explained 14.4% of all variance and is based especially on microbial traits (10 traits) and only on two compositional traits. The dominant traits of this latent factor are the relative abundances of LAB group and its major taxa *Lactococcus* (Table 7) showing very high and positive loadings: 0.86 and 0.89, respectively. Also the two pathogenic taxa are included in this latent factor, again with opposite sign, negative for *Staphylococcus* and positive for *Enterobacteriaceae*. Due to these opposed sign of the loadings of its two taxa, it could be expected

that the group, Pathogenic bacteria, presents a modest loading. This factor included also 5 of the 8 bacterial counts (PCA, WC, MRS, VRBA and Proteolytics) all with positive loadings. It worth noting that microbial counts, even though expressing different microbial groups, are quantitative traits, so they are related to the total bacterial count of the milk sample. On the contrary, the relative abundances obtained from metagenomics are qualitative data expressing the proportions of different taxa. Being the total a constant (100%), it is expected in this case that an increase in some taxa be compensated by the decrease of other taxa. The fact that this latent factor relies mainly on the number of several microbial groups and on the proportion of the major taxa considered could explain the inclusion, with a negative sign, of the milk pH. Less clear is the meaning of the inclusion in this factor of the milk urea, which is often considered an indicator of the dietary availability of protein for the lactating cow (Broderick and Clayton, 1997).

In the previous study, the first latent explanatory factor was based on milk coagulation and curd firming traits, non included in this study, but the second factor (representing 14% of variance and called Pro-Dairy) was based (positively) on LAB group and taxa, but also on Other probiotics (positively), on Spoilage bacteria (negatively) and on the two Pathogenic taxa (*Staphylococcus,* negatively, and *Enterobacteriaceae*, positively). Due to these similarities, also the latent factor 1 in this study was named Pro-Dairy.

The analysis of variance showed that the Pro-Dairy latent factor was affected significantly by all the three fixed factors included in the statistical model (Table 8), with a very high R^2c (0.80). In fact, the contrasts reported in Table 9 highlighted that this factor was significantly higher in PF than in ALP samples, except for the farm P.

Latent factor 2: Probiotics

Differently from the previous study, where Other probiotics group and taxa were associated with LAB group and taxa, in this study they represented the major loadings (+0.93, see Table 7) of the second latent factor, named Probiotics, which represented 10.3% of total variance. In particular,

this latent factor is associated with the relative abundancy of *Propionibacteriaceae* taxon but also with the YELA (putative propionibacteria) bacterial count (+0.39). It worth noting that factor 2 is associated positively (+0.78) with the relative abundancy of *Lactobacillus* taxon, but negatively with PCA (aerobic TB), WC (anaerobic TB), M17 (mesophilic lactococci) and PCAp (Proteolitics). Beyond the 8 microbial traits only one milk composition trait is included in Factor 2: the content of nicotinic acid (+0.39, see Table 7). Even though several microbial taxa are known to be involved in the synthesis of vitamins, especially of B group, no specific information is available on relationships between the microbiota and the nicotinic acid content of milk (Leblanc et al., 2011).

The latent factor 2 Probiotics is not directly affected by the main factors of Herd and Location (ALP vs PF) but by their interaction (Table 8). This interaction is so important that the determination coefficient of this latent factor is the highest among all latent factors identified (R^2c: 0.85). In fact, among the single herds in general were lower during ALP respect PF, and significant opposite in herd M (Table 9).

Latent factor 3: Caseins

The third latent explanatory factor is based on the milk content of casein, total protein, and non-fat solids (loadings +0.80 to +0.90, see Table 7), and to a minor degree (+0.40) to total solids. On the contrary, the loading of casein number is negative (-0.39), even though both numerator and denominator (casein and total protein) of this ratio are included positively and with similar loadings in this latent factor. This seems especially to be due to the lower variability of the numerator than of the denominator. This imply that the increase of milk protein seems related more than proportionally to an increase of whey proteins than of caseins, and this interpretation is confirmed by the Pearson correlations (Figure 2) of milk protein with casein (strong and positive) and casein number (moderate and negative). Consequently, this latent factor was named “Caseins” and it explained 9.1% of total variance (Table 8).

Also in the previous study a “Caseins” latent factor with similar major loadings was found (Secchi et al., 2023), explaining 9.2% of total variance. In that case, the factor included also some trait relative to milk coagulation and curd firming, cheese yield and the recovery of milk protein in the curd.

This Casein latent factor of this study involves also two microbial traits, both moderate and positive: the CBA count and the relative abundancy one of the LAB taxa: the *Streptococcus* (Table 7). In the previous research, the only microbial trait included was the relative abundancy of Other LAB taxa.

Differently from the previous latent factor (Probiotics), in the case of Caseins both Herd and ALP vs PF factors showed a highly significant effect, whereas their interaction was more moderate as the coefficient of determination (R^2c: 0.66, Table 8). The Caseins factor tend to be always lower during ALP than the PF (see in Table9).

Latent factor 4: Udder health

An Udder health latent factor was identified in this and previous study. In both the major loading was that of milk lactose content followed by non-fat solids and casein number (all positively) and that of SCS and of milk pH (negatively). The lactose content is often found to be negatively correlated with somatic cell count or score (Alessio et al., 2021). The association of mastitis pathogens infection of cows udder with milk SCS (positive), lactose content (negative), casein number (negative), milk pH (positive), was clearly found by (Pegolo et al., 2022). The meaning of the positive loading of casein number is due to the fact that the increase of this trait is linked to a relative decrease of whey proteins (mainly lacto-globulin and lacto-globulin) which are known to increase in cows with mastitis. Generally, the contrast reported in Table 9 showed a positive differences, location either the single herds were higher during ALP than the PF.

Latent factor 5: Cheese yield

The fifth latent factor is based on milk fat, total solids, and fat/protein ratio (Table 7), all strictly correlated with each other's (Figure 2) and represents the 8.4% of total variance (Table 8). This factor included also two moderate negative loadings with milk lactose and relative abundancy of *Streptococcus* taxa (Table 7). We named this factor "Cheese yield" following the results obtained in the previous study where, beyond milk fat, total solids and fat/protein ratio, this factor was also strongly and positively related (+0.85) to cheese yield expressed as weight of cheese solids per 100 kg of milk processed (Secchi et al., 2023). Obviously, this result is due to the fact that milk fat is the milk nutrient more represented in cheese solids in terms of average value, and particularly it is the nutrient with the highest contribution to cheese solids variability (Stocco et al., 2022). In the previous research, the Cheese yield factor was also strongly related to the efficiency of recovery of milk solids and energy in the curd. It worth noting that the Cheese yield factor of the previous study included also a positive loading of cheese yield expressed as weight of fresh cheese per 100 kg of milk processed, but this loading was slightly lower than that found for cheese yield expressed in cheese solids (0.58 vs 0.85, respectively). These two measures of cheese yield were also related positively to the "Caseins" factor, but with lower loadings and an inverse ranking (0.50 vs 0.31, respectively). This is due to the fact that the water retained in fresh curd is much more related to the cheese content of casein that of fat (Stocco et al., 2022).

The fixed effects included in the statistical model explained a relatively small proportion of total variability of this factor ($R^2c = 0.27$, see Table 8), and the only significant source of variation was the herd.

Latent factor 6: Spoil-1 and latent factor 7: Spoil-2

In this study we found that the relative abundancy of the Spoilage bacteria group of taxa was positively related contemporarily to two latent factors, named respectively Spoil-1 and Spoil-2. The difference between the two factors was that, beyond the group of taxa, the Spoil-1 was related especially to relative abundancy of *Clostridiaceae* taxon and Spoil-2 to that of *Pseudomonas* taxon

(Table 7). In addition, Spoil-1 presented positive loadings of relative abundancy of Other LAB and of *Bifidobacteriaceae* taxa and negative loadings of PCA, PCAp, and VRBA microbial counts, whereas Spoil-2 factor included positive loadings of PCAp counts and relative abundancy of *Enterococcus* taxa and negative loadings of Pathogenic bacteria group, and also of milk urea content (Table 7). They represented 7.4% and 5.8% of total variance, respectively, and were both affected significantly by all the three fixed effects included in the model (Table 8). The LSM differences between ALP and PF reported in Table 9 showed a significant opposite average effect. Spoil-1 tends to be greater during PF than ALP, while Spoil-2 was moderate greater during ALP.

In the previous research, the Spoilage group and individual taxa were not the base of specific latent factors, but were included, with negative loadings, in the Pro-Dairy factor (Secchi et al., 2023).

Latent factor 8: B-vit

The last latent explanatory factor is based (Table 7) on two B-vitamins, B_1 Thiamine and B_2 Riboflavin, and on some microbial traits, namely: the microbial counts WC (negative loading), M17 (negative) and YELA (positive, and the relative abundancy of LAB group (positive), *Enterococcus* taxon (positive), and *Enterobacteriaceae* taxon (negative).

This latent factor represents 5.8% of total variance and is affected by the herd and, strongly, by the interaction of herd with location (Table 8). Lastly, the contrast (Table 9) were quite always lower in PF than in ALP. No previous study on the factor analysis of milk traits including B-vitamins are available, the authors are aware of, so no comparison with other studies can be done.

Lactoferrin

All the traits related to milk composition and udder trait, except lactoferrin, were included in one or more latent factors and presented a communality coefficient >0.50 (Table 7) and than could be considered represented by latent factors, even though milk urea, pH and SCS were still retaining an individual variability worthing to be considered (communality 0.51 to 0.56). In our previous study, their communality coefficients were even lower than in this study (Secchi et al., 2023).

Lactoferrin didn't contribute to any of the 8 factors and presented a communality of only 0.17 (Table 7), so it should be regarded substantially as an independent trait. Lactoferrin is a milk glycoprotein that exerts multiple antimicrobial, antiviral, antifungal properties (Ciccaglione et al., 2019) and anti-inflammatory and immunostimulatory effects by increasing the synthesis of IgA and IgG antibodies and stimulating the production of T and B cells (Bielecka et al., 2022). A anticarcinogenic potential of bovine lactoferrin has also been observed. Lactoferrin is considered a mastitis marker in dairy ruminants (Giagu et al., 2022), but, even though we have considered it in the group udder health traits, it was not included in the latent factor based on the other traits of the same group (milk pH, SCS and lactose content, see Table 7). On the other side it worth noting that Bisutti et al. (2022) found no correlation between milk lactoferrin and the differential somatic cell count (PNM and lymphocytes, and macrophages). On the other side lactoferrin was not affected by any of the three fixed effects included in the model (Table 1), probably because of the high residual variability. The real meaning of lactoferrin in relation to mastitis of cows should be better indagated.

Variability of microbial traits not explained by latent factors

All the microbial traits (8 bacteria counts and 15 relative abundancies of individual bacterial taxa and their groups) were included in one or more explanatory latent factors. Only two bacterial counts (YELA and CBA) and three relative abundancies (*Streptococcus*, *Bifidobacteriaceae*, and Pathogenic bacteria as a group) showed a communality coefficient <0.50 and worth then some individual attention.

Lastly, it should have considered that 30 bacterial taxa "Other bacteria" were excluded *a priori* from latent factor analysis because of the limit in the number of traits imposed by this methodology which cannot be greater than the number of samples analysed. This means that further research on larger number of samples are needed for testing for including "other bacteria" taxa in the 8 latent factors obtained here and/or for identifying new latent factors.

CONCLUSIONS

We confirmed that milk produced in summer during Alpine grazing of cows and that obtained indoor giving them preserved foodstuffs are very different in terms of composition, udder health traits, B-vitamin content and microbiological traits (bacterial counts and metagenomics relative abundancies). We have also highlighted that the comparison between Alpine pasture and indoor milk is different in different herds, so the interaction between herd and location is very important for the large majority of traits analysed. The large number of traits considered and the complexity of their relationships suggested to analyse data for identifying some latent explanatory factors. Seven of the eight latent factors obtained (the most important of which being “Pro-dairy”, “Probiotics”, and “Caseins”) combined contemporarily milk composition and microbial traits and characterized the differences observed between the milk obtained during summer transhumance to Alpine pasture and that obtained during the following indoor rearing, among the different herds and their interaction. The exception was the “Udder health” latent factor.

Conflicts of interest

None.

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TABLES AND FIGURES

Table 1. Descriptive statistics (mean ± standard deviation) and statistical analysis of the fixed effects (F-value and significance levels) of Herds, Location (ALP *vs* PF) and interaction between Herds and Location of milk composition, udder health traits, and B-vitamins of milk of cows from 4 permanent farms moved in June to four Alpine temporary farms (ALP, summer pasture) and moved back to lowland permanent farms (PF, indoor system) at the end of September.

Traits	Samples N	Descriptive statistics: Mean	±SD	Herd	ALP *vs* PF	Interaction	R^2c	RMSE
Milk composition:								
Total solids, %	50	12.66	1.37	2.2	2.1	0.8	0.46	1.07
Milk fat, %	50	3.63	1.37	4.3 **	0.3	0.9	0.39	1.13
Non-fat solids, %	50	9.14	0.53	7.0 ***	19.3 ***	5.4 **	0.86	0.21
Milk Protein, %	50	3.57	0.42	7.5 ***	20.1 ***	8.7 ***	0.83	0.18
Fat/Protein ratio, %	50	1.01	0.36	5.4 **	0.0	1.0	0.32	0.31
Milk casein, %	50	2.85	0.29	8.9 ***	12.1 **	6.5 **	0.68	0.17
Casein number, %	50	79.95	3.48	0.1	1.5	0.6	0.60	2.37
Milk Urea, mg/dL	50	20.92	7.93	16.9 ***	4.6 *	9.6 ***	0.73	4.21
Udder health traits:								
pH	51	6.45	0.14	10.7 ***	3.2	3.2 **	0.49	0.10
SCS, unit	51	3.45	2.01	2.7	5.3 *	0.9	0.83	0.95
Milk lactose, %	50	4.83	0.34	6.9 ***	1.4	3.1 *	0.32	0.30
Lactoferrin (mg/L)	50	181	152	0.9	0.1	0.8	0.50	114
Vitamins, (µg/L):								
Thiamine (B_1)	51	187	119	0.8	3.6	2.3	0.39	97.50
Riboflavin (B_2)	51	2,225	1,302	0.6	1.2	3.5 *	0.32	1,125
Nicotinic Acid (B_3)	50	2.72	2.43	0.1	4.6 *	1.8	0.38	2.00
Folic Acid (B_9)	49	0.37	0.43	0.6	0.3	2.4	0.80	0.20

*P<0.05; **P<0.01; ***P<0.001;
SCS= somatic cell score. RMSE= root mean square error. R^2c = conditional R^2.

Table 2. Contrast estimates between the values obtained during summer transhumance on Alpine pastures (mid-july) respect to lowland indoor permanent farms (mid-october) on the overall trial and within each herd (interaction) of milk composition, udder health traits and B-vitamins content

Traits	Difference of ALP respect PF:				
	Overall effect	Single herds			
		C	M	P	F
Milk composition:					
Total solids, %	-1.30	-0.92	-1.98 **	-0.82	-1.48 *
Milk fat, %	-0.91	-0.35	-1.74 *	-0.96	-0.56
Non-fat solids, %	-0.58 ***	-0.62 ***	-0.41 **	-0.26	-1.02 ***
Milk Protein, %	-0.55 ***	-0.51 ***	-0.67 ***	-0.07	-0.94 ***
Fat/Protein ratio, %	-0.08	0.00	-0.30	-0.13	0.11
Milk casein, %	-0.33 **	-0.34 **	-0.37 **	0.02	-0.64 ***
Casein number, %	3.20	1.81	4.39 **	3.56 *	3.00
Milk Urea, mg/dL	-5.90 *	-5.31 *	-14.09 ***	-9.32 **	5.08
Udder health traits:					
pH	0.09	0.10	0.26 ***	-0.02	0.00
SCS, unit	-1.14 *	-1.46 *	-0.87	-1.81*	-0.42
Milk lactose, %	0.06	-0.19	0.40 *	0.22	-0.18
Lactoferrin (mg/L)	109	22.2	113.3	115.8	181.8
Vitamins, (µg/L):					
Thiamine (B_1)	72	106.0	101.1	146.2 *	-62.4
Riboflavin (B_2)	966	669	1852 **	2018 **	-676
Nicotinic Acid (B_3)	2.26 *	2.44 *	4.22 **	0.66	1.68
Folic Acid (B_9)	-0.03	-0.07	-0.20	-0.08	0.25

*P<0.05; **P<0.01; ***P<0.001;
SCS= somatic cell score.

Table 3. Descriptive statistics (mean ± standard deviation) and statistical analysis of the fixed effects (F-value and significance levels) of the Herds, Location (ALP *vs* PF) and interaction between Herd and Location of milk bacterial counts (expressed as log CFU/mL), the log10 relative abundances of milk bacterial taxa with known dairy (LAB), other probiotics, spoilage and pathogenic activities, and of other bacteria found in the milk of cows from 4 permanent farms moved in June to four Alpine temporary farms (ALP, summer pasture) and moved back to lowland permanent farms (PF, indoor system) at the end of September.

Traits	Samples N	Descriptive statistics: Mean	±SD	Herd	ALP *vs* PF	Interaction	R^2c	RMSE
Milk bacterial counts:								
PCA: aerobic TB	51	4.45	0.94	23.7 ***	66.8 ***	36.5 ***	0.82	0.40
WC: anaerobic TB	50	4.46	0.92	8.7 ***	0.8	6.2 **	0.57	0.62
MRS: mesophilic lactobacilli	50	3.71	1.26	27.3 ***	48.82 ***	24.1 ***	0.85	0.50
M17: mesophilic lactococci	50	4.17	1.32	11.9 ***	9.7 **	21.3 ***	0.74	0.69
YELA: putative Propionibacteria	49	1.06	1.18	502.1 ***	0	367.3 ***	0.98	0.16
VRBA: coliforms	49	1.83	1.25	231.2 ***	175.4 ***	158.7 ***	0.97	0.23
PCAp: proteolytics	51	3.04	1.37	4.0 *	46.4 ***	17.2 ***	0.68	0.79
CBA: hemolytic streptococci	50	3.18	1.12	7.7 ***	75.1 ***	17.1 ***	0.73	0.59
Metagenomic relative abundances:								
Lactic acid bacteria (LAB):	50	1.240	0.478	15.9 ***	17.5 ***	43.5 ***	0.89	0.172
Lactobacillus	49	0.222	0.374	8.4 ***	0.2	44.1 ***	0.80	0.171
Lactococcus	50	0.880	0.672	54.7 ***	48.9 ***	113.6 ***	0.93	0.185
Enterococcus	51	0.227	0.305	11.3 ***	1.16	11.1 ***	0.85	0.119
Streptococcus	51	0.330	0.353	15.4 ***	28.3 ***	4.9 **	0.57	0.238
Other LAB	51	0.203	0.243	7.8 ***	16.9 ***	6.7 **	0.49	0.181
Other probiotics:	49	0.248	0.394	2.6	0.3	70.7 ***	0.89	0.134
Propionibacterium	50	0.169	0.398	0.2	0.0	127.1 ***	0.94	0.097
Bifidobacterium	50	0.095	0.119	4.7 **	0.3	4.9 **	0.37	0.099
Spoilage bacteria:	51	0.399	0.256	8.1 ***	5.3 *	5.2 **	0.39	0.209
Clostridiaceae	49	0.181	0.215	12.8 ***	41.3 ***	27.4 ***	0.73	0.115
Pseudomonas	50	0.232	0.195	4.0*	1.1	3.9 *	0.42	0.155
Pathogenic bacteria:	51	0.748	0.517	3.3 *	12.9 **	4.9 **	0.34	0.442
Staphylococcus	51	0.528	0.577	4.6 **	14.2 ***	7.5 **	0.49	0.427
Enterobacteriaceae	51	0.291	0.363	41.9 ***	0.6	10.0 ***	0.76	0.180
Other bacteria:	50	1.660	0.260	5.5 **	1.0	2.4	0.69	0.152
Corynebacterium	51	0.327	0.424	11.5 ***	27.3 ***	22.9 ***	0.77	0.211
Dermabacteraceae	50	0.172	0.275	18.9 ***	28.8 ***	28.9 ***	0.72	0.148
Intrasporangiaceae	50	0.122	0.229	13.7 ***	1.7	13.6 ***	0.53	0.161
Microbacteriaceae	50	0.340	0.277	8.9 ***	23.6 ***	12.2 ***	0.63	0.174
Micrococcaceae	51	0.503	0.418	18.2 ***	8.0 **	9.8 ***	0.69	0.244
Propionicimonas	50	0.085	0.165	4.1 *	0.8	2.0	0.45	0.128
Other *Actinobacteria*	50	0.265	0.230	2.9 *	18.1 ***	2.7	0.58	0.156

Porphyromonas	49	0.003	0.009	0.0	11.6 **	2.5	0.32	0.008
Flavobacteriaceae	51	0.238	0.282	7.1 ***	1.7	5.2 **	0.88	0.099
Chryseobacterium	50	0.480	0.391	58.0 ***	177.6 ***	87.8 ***	0.92	0.112
Wautersiella	49	0.100	0.221	104.6 ***	253.1 ***	57.4***	0.89	0.072
Chitinophagaceae	49	0.140	0.274	269.3 ***	2.3	8.1 **	0.97	0.045
Other *bacteroidetes*	51	0.212	0.196	6.6 ***	5.8 *	2.8	0.46	0.150
Cyanobacteria	50	0.044	0.105	0.0	14.3 ***	2.7	0.37	0.088
Solibacillus	50	0.189	0.310	40.7 ***	30.3 ***	39.6 ***	0.78	0.146
Aerococcus	50	0.063	0.131	2.2	4.6 *	3.8 *	0.28	0.117
Trichococcus	50	0.074	0.121	10.3 ***	16.9 ***	14.0 ***	0.55	0.084
Turicibacter	50	0.220	0.270	15.3 ***	54.2 ***	31.7 ***	0.74	0.139
Peptostreptococcaceae	49	0.401	0.389	31.4 ***	80.8 ***	45.1 ***	0.81	0.171
Ruminococceae	50	0.091	0.139	7.6 ***	32.9 ***	16.4 ***	0.58	0.093
Tissierellaceae	49	0.020	0.054	0.6	9.4 **	3.3 *	0.23	0.050
Other *Firmicutes*	50	0.213	0.259	2.4	33.6 ***	11.3 ***	0.66	0.154
Agrobacterium	50	0.118	0.224	15.1 ***	19.2 ***	82.5 ***	0.93	0.058
Paracoccus	50	0.189	0.352	127.7 ***	1.5	18.8 ***	0.92	0.099
Sphingomonadaceae	50	0.079	0.103	5.8 **	2.1	0.3	0.55	0.072
Comamonadaceae	49	0.190	0.175	26.1 ***	160.1 ***	49.7 ***	0.94	0.045
Acinetobacter	51	0.939	0.469	11.4 ***	31.0 ***	43.3 ***	0.79	0.216
Enhydrobacter	51	0.242	0.327	25.3 ***	62.9 ***	13.3 ***	0.73	0.173
Xanthomonadaceae	50	0.210	0.239	1.1	3.5	20.1 ***	0.79	0.111
Other *Proteobacteria*	51	0.363	0.279	10.1 ***	1.4	0.8	0.62	0.177

*P<0.05; **P<0.01; ***P<0.001

RMSE= root mean square error. R^2c = conditional R^2.

Table 4. Contrast estimates between the values obtained during summer transhumance on Alpine pastures (mid-July) respect to lowland indoor permanent farms (mid-October) on the overall trial and within each herd (interaction) of milk bacterial counts (expressed as log CFU/mL), and the log10 relative abundances of milk bacterial taxa (categories and other bacteria)

Traits	Differences ALP respect PF				
	Overall effect	Single herds			
		C	M	P	F
Milk bacterial counts:					
PCA: aerobic TB	-1.16 ***	-1.80 ***	-2.50 ***	0.62 **	-0.96 **
WC: anaerobic TB	-0.83	-0.32	-2.13 ***	-0.37	-0.51
MRS: mesophilic lactobacilli	-1.72 ***	-1.99 ***	-2.37 ***	0.30	-2.82 ***
M17: mesophilic Lactococci	-1.25 **	-1.22 **	-3.82 ***	0.11	-0.07
YELA: putative propionibacteria	-0.10	0.00	2.35 ***	-0.90 ***	-1.86 ***
VRBA: coliforms	-0.85 ***	-1.88 ***	-1.86 ***	1.75 ***	-1.39 ***
PCAp: proteolytics	-1.18 ***	-2.94 ***	-2.63 ***	0.46	0.40
CBA: hemolytic streptococci	-0.86 ***	-2.93 ***	-0.10	0.05	-0.57
Metagenomics:					
Lactic acid bacteria (LAB):	-0.38 ***	-0.45 ***	-0.58 ***	0.63 ***	-1.13 ***
Lactobacillus	0.20	0.04	1.13 ***	-0.42 ***	0.06
Lactococcus	-0.70 ***	-0.74 ***	-1.66 ***	0.97 ***	-1.34 ***
Enterococcus	0.14	-0.07	0.1	0.46 ***	0.08
Streptococcus	-0.29 ***	-0.69 ***	0.00	-0.22	-0.24
Other LAB	0.04 ***	0.40 ***	0.04	-0.15	-0.15
Other probiotics:	0.32	0.04	1.29 ***	-0.16 *	0.09
Propionibacterium	0.36	0.01	1.31 ***	0.02	0.09
Bifidobacterium	-0.02	0.03	0.08	-0.20 **	0.01
Spoilage bacteria:	0.01 *	0.26 *	0.05	-0.36 **	0.07
Clostridiaceae	-0.02 ***	0.42 ***	0.09	-0.33 ***	-0.27 **
Pseudomonas	0.05	-0.09	0.00	-0.04	0.32 **
Pathogenic bacteria:	0.13 **	0.86 **	0.16	-0.38	-0.13
Staphylococcus	0.41 ***	0.89 ***	0.97 ***	-0.43	0.22
Enterobacteriaceae	-0.23	-0.07	-0.65 ***	0.08	-0.26 *
Other milk bacteria:					
Corynebacterium	-0.07 ***	0.67 ***	0.11	-0.82 ***	-0.25
Dermabacteraceae	0.10 ***	-0.43 ***	0.68 ***	0.06	0.09
Intrasporangiaceae	-0.04	0.12	0.32 **	-0.07	-0.54 ***
Microbacteriaceae	-0.02 ***	-0.46 ***	0.17	0.30 **	-0.08
Micrococcaceae	-0.16 **	-0.41 **	0.38 *	-0.63 ***	0.02
Propionicimonas	0.06	-0.06	0.15	0	0.14
Other *Actinobacteria*	0.20 ***	0.38 ***	0.14	0.27 **	0.02
Porphyromonas	0.01 **	0.02 **	0.017 *	0	0
Flavobacteriaceae	0.08	-0.09	0.01	0.15 *	0.24 **
Chryseobacterium	-0.40 ***	-0.88 ***	-0.88 ***	0.50 ***	-0.35 ***

Wautersiella	-0.21 ***	-0.69 ***	0.02	-0.15 ***	-0.02
Chitinophagaceae	-0.02	0.04	0.03	0.004	-0.16 ***
Other *bacteroidetes*	0.09 *	0.20 *	0.05	-0.09	0.22 *
Cyanobacteria	0.09 ***	0.19 ***	0.06	0	0.13 *
Solibacillus	-0.13 ***	0.44 ***	0	-0.80 ***	-0.17
Aerococcus	-0.02 *	0.14 *	0	-0.17 *	-0.02
Trichococcus	-0.01 ***	0.19 ***	0	-0.23 ***	-0.018
Turicibacter	0.02 ***	0.58 ***	0.11	-0.46 ***	-0.14
Peptostreptococcaceae	0.06 ***	0.87 ***	0.20	-0.65 ***	-0.19
Ruminococceae	0.06 ***	0.30 ***	0.06	-0.21 ***	0.09
Tissierellaceae	0.02 **	0.09 **	0.03	-0.01	0
Other *Firmicutes*	0.19 ***	0.50 ***	0.18	-0.22 *	0.30 **
Agrobacterium	0.11 ***	-0.14 ***	-0.01	-0.03	0.61 ***
Paracoccus	-0.15	-0.07	-0.012	0.06	-0.56 ***
Sphingomonadaceae	0.06	0.06	0.05	0.03	0.09
Comamonadaceae	-0.10 ***	-0.43 ***	-0.09 **	-0.02	0.12 **
Acinetobacter	-0.19 ***	-0.70 ***	-1.1 ***	0.53 ***	0.53 **
Enhydrobacter	-0.26 ***	-0.78 ***	0.0448	-0.18	-0.11
Xanthomonadaceae	0.19	-0.12	-0.0125	0.40 ***	0.50 ***
Other *Proteobacteria*	0.21	0.12	0.33 **	0.20	0.21

*P<0.05; **P<0.01; ***P<0.001
LAB= lactic acid bacteria.

Table 7. Loadings on the latent explanatory factors of the milk composition, udder health traits, B-Vitamins, milk bacterial counts and groups.

Items	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Communality
	Pro-dairy	Probiotics	Caseins	Udder health	Cheese yield	Spoil-1	Spoli-2	B-vit.	
Milk composition:									
Total solids, %			0.40		0.86*				>0.90
Milk fat, %					0.93*				>0.90
Non-fat solids, %			0.80*	0.49					>0.90
Milk Protein, %			0.88*						>0.90
Fat/Protein ratio, %					0.93*				>0.90
Milk casein, %			0.90*						>0.90
Casein number, %			-0.41	0.66*					0.77
Milk Urea, mg/dL	0.39						-0.49		0.56
Udder health traits:									
pH	-0.53*			-0.36					0.54
SCS, unit				-0.66*					0.51
Milk lactose, %				0.87*	-0.35				>0.90
Lactoferrin (µg/mL)									0.17
Vitamins, (µg/L):									
Thiamine (B_1)								0.46	0.34
Riboflavin (B_2)								0.57	0.45
Nicotinic Acid (B_3)		0.39		0.32					0.34
Folic Acid (B_9)				-0.73*					0.59
Milk bacterial counts:									
PCA: aerobic TB	0.73*	-0.37				-0.32			0.87
WC: anaerobic TB	0.34	-0.39						-0.45	0.54
MRS: mesophilic lactobacilli	0.75*								0.67
M17: mesophilic lactococci		-0.64*						-0.45	0.81
YELA: putative propionibacteria		0.39						0.37	0.46
VRBA: coliforms BC	0.71*					-0.39			0.80
PCAp: Proteolytics	0.52*	-0.31				-0.40	0.40		0.80
CBA: hemolytic streptococci			0.33						0.41
Milk bacterial groups:									
LAB	0.86*							0.31	0.87
Lactobacillus		0.78*							0.70
Lactcoccus	0.89*								>0.90
Enterococcus							0.30	0.75*	0.79
Streptococcus			0.40		-0.34				0.31
Other LAB						0.78*			0.76
Other probiotics		0.93*							>0.90
Propionibacteriaceae		0.91*							>0.90
Bifidobacteriaceae						0.46			0.39
Spoilage bacteria						0.71*	0.62*		>0.90
Clostridiaceae						0.90*			>0.90
Pseudomonas							0.93*		>0.90
Pathogenic bacteria	-0.34						-0.33		0.35
Staphylococcus	-0.70*								0.66
Enterobacteriaceae	0.66*							-0.31	0.66

*High loadings, >50.

Table 8. Descriptive statistics, and statistical analysis of the fixed effects (F-value and significance levels) of the Herds, Location (ALP *vs* PF) and interaction between Herd and Location of the latent explanatory factors of the milk bacterial taxa and milk composition.

Latent explanatory factor	Explained variance:		Herd	ALP *vs* PF	Interaction	R^2c	RMSE
	Individual	Cumulative					
Factor 1: "Pro-dairy"	14.4 %	14.4 %	28.4 ***	27.3 ***	26.7 ***	0.80	0.43
Factor 2: "Probiotics"	10.3 %	24.7 %	0.8	1.7	55.9 ***	0.85	0.39
Factor 3: "Caseins"	9.1 %	33.8 %	7.8 ***	16.6 ***	4.1 *	0.66	0.55
Factor 4: "Udder health"	8.7 %	42.4 %	2.6	0.2	0.3	0.24	0.69
Factor 5: "Cheese yield"	8.4 %	50.9 %	4.3 *	0.2	1.1	0.27	0.73
Factor 6: "Spoil-1"	7.4 %	58.3 %	6.1 **	16.0 ***	11.8 ***	0.52	0.69
Factor 7: "Spoil-2"	5.8 %	64.1 %	3.4 *	6.0 *	6.2 **	0.47	0.62
Factor 8: "B-vit"	5.8 %	70.0 %	3.4 *	0.1	16.4 ***	0.79	0.44

*P<0.05; **P<0.01; ***P<0.001
RMSE= root mean square error. R^2c = conditional R^2.

Table 9. Contrast estimates between the values obtained during summer transhumance on Alpine pastures (mid-July) respect to lowland indoor permanent farms (mid-October) in the overall trial and within each herd (interaction) of the latent explanatory factor.

Latent explanatory factor	Differences ALP respect PF				
	Overall effect	Single herds			
		C	M	P	F
Factor 1: “Pro-dairy”	-0.93 ***	-1.23 ***	-1.59 ***	0.92 ***	-1.84 ***
Factor 2: “Probiotics”	0.46	-0.27	2.99 ***	-0.64 **	-0.24
Factor 3: “Caseins”	-0.92 ***	-1.32 ***	-0.29	-0.41	-1.65 ***
Factor 4: “Udder health”	0.28	0.18	0.58	0.28	0.08
Factor 5: “Cheese yield”	-0.266	0.16	-0.71	-0.57	0.05
Factor 6: “Spoil-1”	-0.332 ***	1.50 ***	-0.21	-1.26 **	-1.36 **
Factor 7: “Spoil-2”	0.04 *	-0.84 *	0.01	-0.35	1.35 **
Factor 8: “B-Vit”	0.93	0.08	1.03 ***	1.58 ***	-0.92 **

*P<0.05; **P<0.01; ***P<0.001

Figure 1a. Sources of the variation (expressed as percentage of total variance) in individual milk bacterial counts, and milk taxa relative abundances (in italic) and their categories (in bold): effects of the Herds (dark blue), Location (ALP *vs* PF) (red), interaction between Herd and Location (orange), individual cow nested in the Herd (green), and the residual variability (light blue).

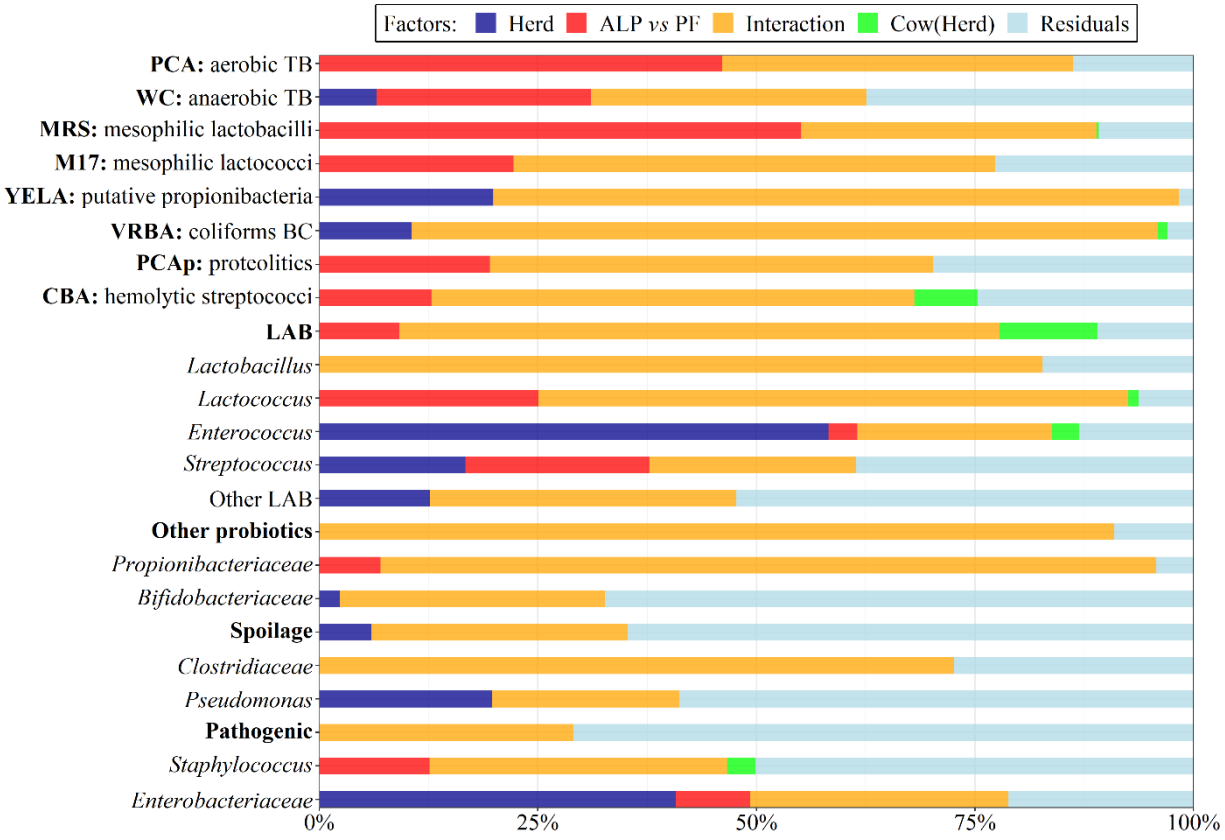


Figure 1b. Sources of the variation (expressed as percentage of total variance) in milk composition, udder health traits and B-vitamins: effects of the Herds (dark blue), Location (ALP *vs* PF) (red), interaction between Herd and Location (orange), individual cow nested in the Herd (green), and the residual variability (light blue).

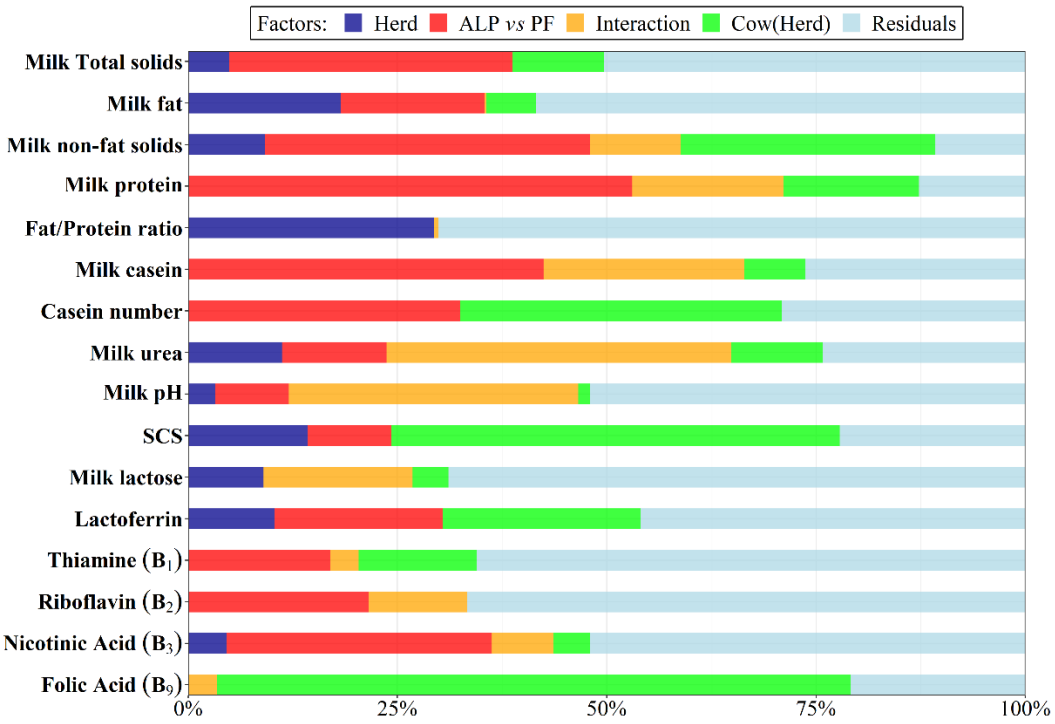


Figure 2. Heat plot of the correlations among the milk composition traits, udder health traits and B-vitamins included in the factor analysis.

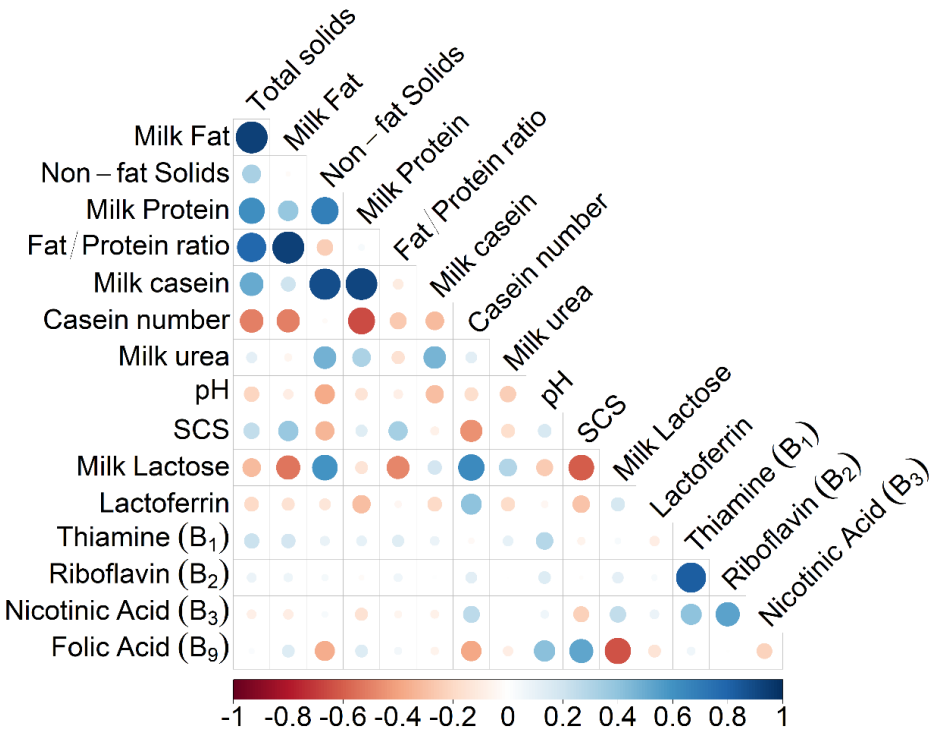
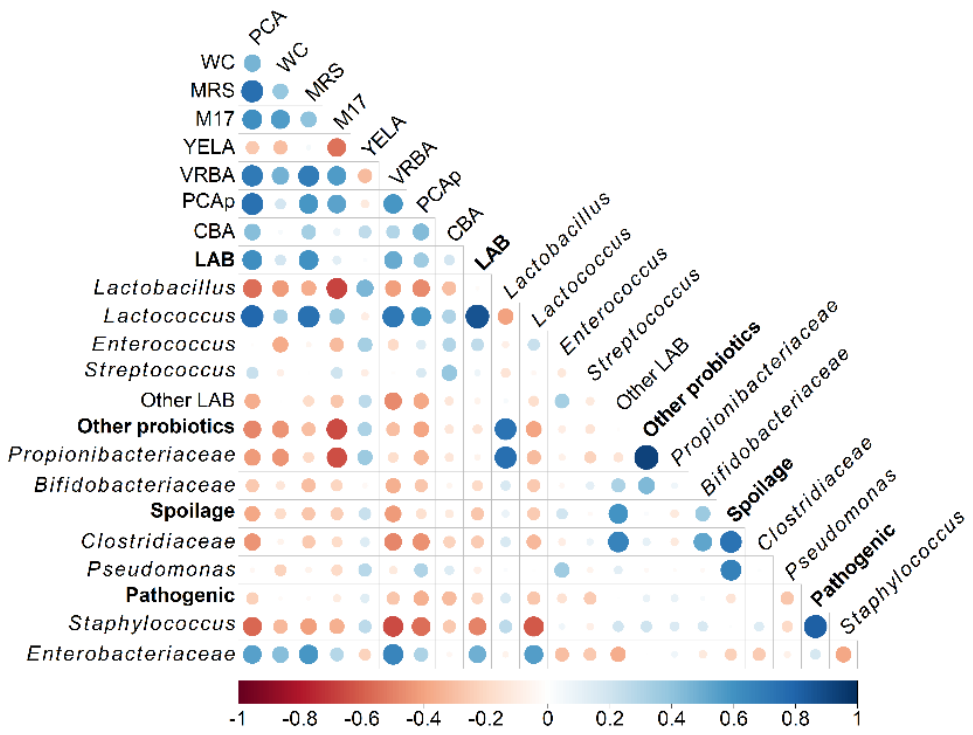
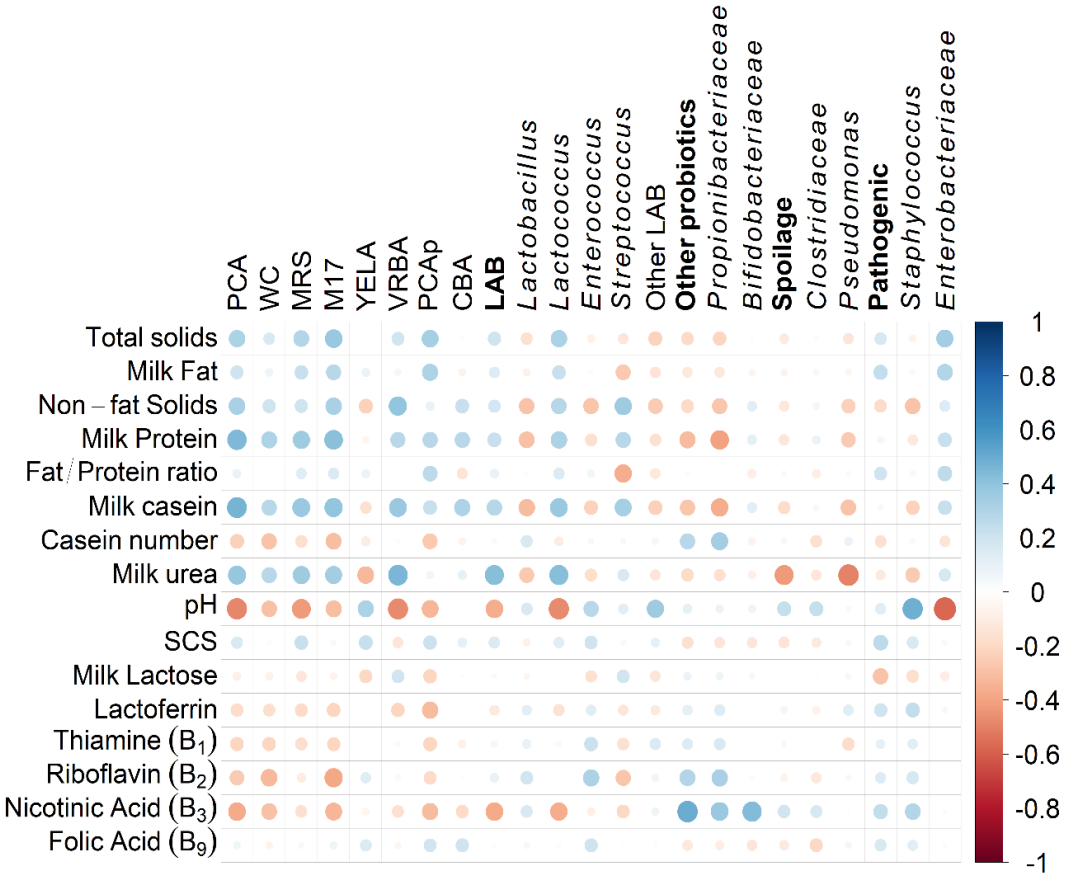


Figure 3. Heat plot of the correlations among the milk bacterial counts and milk bacterial groups included in the factor analysis.



PCA: mesophilic aerobic TB; WC: mesophilic anaerobic TB; MRS: mesophilic bacilli; M17: mesophilic lactococci; YELA: Propionibacteria; VRBA: Coliforms; PCAp: proteolytics and CBA: hemolytic Streptococci.

Figure 4. Heat plot of the correlation between the bacterial and chemical traits included in the factor analysis.



PCA: mesophilic aerobic TB; WC: mesophilic anaerobic TB; MRS: mesophilic bacilli; M17: mesophilic lactococci; YELA: Propionibacteria; VRBA: Coliforms; PCAp: proteolytics and CBA: hemolytic Streptococci.

CHAPTER IV

Variation of intestinal microbiota of dairy cows kept on Alpine pasture or indoor and relationships with microbiota and composition

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INTERPRETIVE SUMMARY

Variation of intestinal microbiota of dairy cows kept on Alpine pasture or indoor and relationships with milk microbiota and composition

By Secchi et al., page 000. This study deals the relationship between intestinal metagenomics, milk metagenomics and milk composition of 4 herds (5-7 Italian Simmental cows/each) sampled during the summer highland pasture, and when moved back to indoor farming. A total of 14 bacterial taxa were obtained in faeces, which appear to be interrelated with the milk microbiome, at least with regarding to some taxa of particular interest for characterizing milk quality and health.

ABSTRACT

Metagenomic approach allowed to obtain new knowledge on intestinal microbiota of the dairy cow, and which is known for having an important role in digestive processes, immune functions and the health of the animals. Less studied are the relationships with the microbiota of the udder and the quality of milk produced. To study this issue, four herds moved to Alpine pasture during summer transhumance and returning to lowland indoor permanent farms during the rest of the year were involved selecting 5-7 cows per herd and sampling them one month after summer transhumance (in July) and one month after returning indoor (in October). Milk was sampled during milking and fecal material was grab directly from the rectum. After DNA extraction and Illumina Miseq sequencing, all fecal samples (n. 52) were also processed by means of an open source pipeline called Quantitative Insights Into Microbial Ecology (Qiime2, version 2020.11; https://qiime2.org).

The relative abundances of 14 intestinal bacterial taxa were analyzed using a model including the fixed effect of herds, farming system (Alpine pasture vs indoor feeding) and their interaction, and the random effect of cow within herd. The intestinal microbiota of dairy cows is based on many different bacterial taxa, each characterized by different proportions among the mail sources of variability. The differences among individual herds are very important for some taxa (*Verrucomicrobia*, *Tenericutes* and *Lachnospiraceae*, in particular), those between different farming systems (Alpine pasture *vs* lowland indoor feeding) affects strongly other taxa (*Methanobacteriaceae*, *Actinobacteria-Bifidobacteriaceae*, *Bacteroidaceae*, *Peptostreptococcaceae*, Other *Clostridia* and *Proteobacteria*), whereas their interaction is quantitatively less important but affect almost all intestinal bacterial taxa (*Tenericutes* and *Verrucomicrobia* are notable exceptions). The individual cow within herd represent a moderate source of variation in the majority of taxa, whereas residual variance is very different for different taxa, being the dominant source of variation in the case of *Rikenellaceae*, *Paraprevotellaceae*, and *Ruminococcaceae*. The very complex interrelationships among different bacterial taxa were analysed through Pearson correlations and multivariate factor

analysis. This allowed to extract 6 latent explanatory factors of intestinal microbiota representing 72% of total variance. One factor (F2-im) could be used to characterize well different herds, whereas F1-im, F3-im, and in part also F5-im and F6-im could be very useful in discriminating farming systems.

Intestinal microbiota appears to be interrelated also with milk microbiota, at least with some of the taxa having particular interest for characterizing quality and health of milk. Five out of eight latent factors obtained from the merging of data from intestinal and milk metagenomics (imm) combined taxa from both type of samples demonstrating the interrelations between cow's intestine and udder. Furthermore, the milk taxa associated with intestinal taxa are known for being of interest for the dairy industry (lactic acid bacteria, other probiotics, spoilage bacteria and pathogenic bacteria). Finally, intestinal bacterial taxa have found to be associated also directly with some quality traits of milk. Four out of eight latent factors obtained merging the dataset of intestinal metagenomics with that of milk quality traits (imq) combined traits from both databases, linking the intestinal bacteria with the milk udder health traits (F4-imq), with B-vitamins content of milk (F5-imq), with cheese yield (F6-imq) and with Lactoferrin (F8-imq). If this study demonstrated the interest of cow's intestinal metagenomics, because of the differences due to farming systems, herds and individual cows and the many interrelationships with milk metagenomics and milk quality, it demonstrated also the need for further research in the field.

Keywords: Fecal metagenomics, MiSeq Illumina, summer transhumance, intestinal-milk relationships.

INTRODUCTION

Metagenomic studies are opening new insight in the complex relationships between animal's microbiota, metabolism regulation, immunological functions and quality and nutritional value of the animal's product. Ruminants, and particularly dairy cows, are the very complex and interesting study subjects because of the relevance of microbial function in different compartments of the animal body and dairy chain, like: the fore-stomachs, the intestine, the udder, the milk produced and, finally, the cheese and other dairy products obtained from milk. Many sectorial studies have been carried out, especially in these last years, on all these microbiota compartments (Rainard, 2017; Frétin et al., 2018; Xin et al., 2019; Parente et al., 2020; Xu et al., 2021; Reuben et al., 2023). But research on relationships between the microbiota present in the different compartments are scarce, as are scarce the studies on relationships between the microbiota of the different compartments and the quality and technological properties of milk and cheese (Ferrocino et al., 2022). Moreover, rare are the holistic research on the effects of different dairy farming systems on microbiota and product quality (Gomes et al., 2020).

In a research project on the comparison between indoor farming and Alpine pasture (Juribello project), we have analyzed the effect of farming system on milk microbiota composition (Carafa et al., 2020), on milk quality traits and technological properties (Saha et al., 2019), and especially on the sources of variation of milk microbiota and its complex relationships with milk characteristics (Secchi et al., 2023a). In this last study we showed the many correlations between bacterial taxa relative abundances in milk and several milk traits. Using the factor analysis, we have also demonstrated that four out of eight latent explanatory factors identified were based on the contemporary variation of microbiological and quality traits of milk. These four latent factors, because of their major loadings, were named: Gelation, Pro-Dairy, Udder health, and Caseins. Another chapter of the same project is dealing with the study of bacterial and yeast microbiota of

rumen liquor and their relationships with rumen fermentation pattern and the previously cited milk microbiota and quality traits.

The effect of farming system (indoor feeding vs Alpine grazing), the major relationships between milk microbiota and chemical composition, and the nature of the major latent factors have been confirmed in a following project (Secchi et al., 2023b). But the objective of this last project (MilkBiota project) was also extended to the study of the microbiological functions in the cow's intestine. So, the aims of this study were: a) to analyze the composition, the effect of farming system (indoor feeding vs Alpine pasture), the sources of variation and the aggregation (latent factors) of the relative abundances of bacterial taxa identified on intestinal material sampled from the rectum of the cows; b) to identify the possible relationships (correlations, latent factors) between the intestinal microbiota and the microbiota of milk produced by the same cows in the same day; c) to search for relationships (correlations, latent factors) between intestinal microbiota and milk quality traits.

Moreover, studies available are generally carried out in single herds (experimental units or commercial farms) and variability/interaction between different herds is not much known. A study of 150 dairy cows from 10 commercial farms across California by Hagey et al., (2019) found that fecal microbiota is significantly affected by farming type, individual farm and diet.

The aims of this study were pursued in 4 different farms to consider the possible interaction with environmental, feeding and management of the herds.

MATERIALS AND METHODS

Experimental design

The experimental design was described in details in our previous study on the variation of milk microbiota and composition during summer transhumance in Alpine pasture (**ALP**) and after the return of the cows to the lowland indoor permanent farm (**PF**) conditions (Secchi et al., 2023b). In short, the experimental design was a 4 × 2 factorial design: 4 herds and two locations/seasons. Each herd was housed in a different PF (Vicenza and Trento provinces, north east Italy, 70-500 m a.s.l.) from autumn to spring, and was moved in a different ALP (Vezzena highland, Trento province, 1380-1700 m a.s.l.) during summer transhumance, to return to the PF in autumn.

At the end of June 2020 Simmental cows were moved from the four PF to their respective ALP, where the cows were free to graze day and night on Alpine pastures in different areas according to grass availability, without a rigid rotation plan. Each farm was also giving a compound feed supplement in the milking parlor according to milk yield. At the end of September, the herds were moved again indoor in the respective PF where cows received meadow and alfalfa hay and compound feeds.

Intestinal material and milk sampling

From 26 mid-lactation healthy cows (5-7 per herd), individual intestinal material and milk samples were collected once at the end of July, four weeks after the beginning of Alpine pasture, and once at the end of October four weeks after cows were moved back to the PF for a total of 52 fecal and 52 milk samples. Details on milk sampling were described in our previous study on the same project (Secchi et al., 2023b).

Approximately 30 g of fecal material were collected from the rectum of each cow by use of individual plastic palpation sleeves, and immediately placed in a sterile 50 mL propylene tube, transported on ice to the laboratory on the same day and immediately stored at −80 °C until use.

Metagenomic analyses

Total DNA extraction from intestinal and milk samples

For total genomic DNA extraction, 4 mL of milk were centrifuged at 4,000 g for 10 min at 4 °C, and the supernatant was discarded. Genomic DNA was extracted from the pellet using the DNeasyPower Food Microbial Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific, USA).

The DNA was extracted from intestinal samples using FastDNA® Spin Kit for Feces (MP Biomedicals, LLC, Santa Ana, CA, USA) according to the manufacturer's instructions with small modifications. Briefly, 0.4 – 0.5 g of thawed intestinal sample were added with the solution provided in vials filled with ceramic beads, ready for the process. The samples were incubated 65 °C for 10 min and then homogenized twice at 6000 rpm for 30 s each with a 30-s pause in between, using FasrPrep-24 ™ Classic Instrument (MP Biomedicals). After homogenization, the suspension was incubated for lysis. Finally, DNA was bound into the spin filter, washed, and eluted according to the manufacturer's instructions. At the end the samples were quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific, USA).

Preparation of the MiSeq library

Amplicon library preparation, quality and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina, USA) were performed at the Sequencing Platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3-V4 region (Baker et al., 2003; Claesson et al., 2010), of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen

dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2020.11, https://qiime2.org). Sequences were quality-filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013); Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA903798 (https://www.ncbi.nlm.nih.gov/sra/PRJNA903798).

Bacterial categories

Due to the modest information on relationships of intestinal microbial taxa with dairy cow metabolism and milk production and quality, all bacterial taxa identified in cow's feces were listed as one group. On the contrary, on the basis of our previous study (Secchi et al., 2023a), also in this work we decided to classify the relative abundances of the principal milk bacterial taxa identified by Qiime2 into four categories: the Lactic Acid Bacteria (LAB) category includes the taxa belonging to

the *Lactobacillales* order (*Lactobacillus*, *Lactococcus* and *Enterococcus*) and *Streptococcus* (Khalid and Department, 2011; Gagnon et al., 2020); the "Other probiotics" category includes all the taxa belonging to the *Propionibacterium* (Rabah et al., 2017) and *Bifidobacterium* genera (Prasanna et al., 2014); the "Spoilage bacteria" category includes all the taxa belonging to the *Clostridiaceae* (Burtscher et al., 2020) and the *Pseudomonas* (Meng et al., 2017); and at the end the "Pathogenic bacteria" category includes all the taxa belonging to the *Staphylococcus* genus (Gebremedhin et al., 2022), and the *Enterobacteriaceae* family (Anand and Griffiths, 2011).

The remaining 30 bacterial taxa were grouped as "other milk bacteria".

Milk composition traits

Each individual milk sample was analyzed for protein, fat, lactose, and urea content with a MilkoScan FT2 infrared analyzer (Foss Electric A/S, Hillerød, Denmark) calibrated according to the following reference methods: ISO 8968-2/IDF 20-2 for protein, ISO 1211/IDF for fat, and ISO 26462/IDF 214 for lactose. Somatic cell counts (SCC) were obtained with a Fossomatic Minor FC counter (Foss Electric A/S) and log-transformed to somatic cell score (SCS) using the formula $SCS = \log_2(SCC/100{,}000)+3$ as proposed by Ali and Shook, (1980)Ali and Shook .

The content of lactoferrin in the milk was determined by commercial enzyme-linked immunosorbent assay (ELISA) test kits from Cloude-Clone, (Cat. SEA780Bo 96 test for Lactoferrin (LTF), organism species: Bos Taurus, CLOUD-CLONE CORP. Houston, USA), according to the manufacturer's recommendations.

The B-vitamins separation was performed with an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) using an Acquity UPLC BEH C18 (1.7 μm, 2.1 mm × 50 mm) column (Waters corporation, Milford, MA, USA) at 40 °C. Methanol and acetonitrile used were LC-MS grade, all the reagent and chromatographic solvents (including formic acid and B-vitamins standards)

were purchased from Sigma-Aldrich (St. Louis, MO, USA); Milli-Q water was used for the chromatography. Details are reported in our previous study (Secchi et al., 2023b).

Statistical analysis

The data regarding intestinal and milk bacterial abundances were $\log_{10}$ transformed. All bacterial and qualitative data were checked to identify and exclude outliers value (outside the interval ± 3 SD of the mean).

Mixed-model analysis of variance

The $\log_{10}$ transformed relative abundances of bacterial taxa identified in intestinal samples were analyzed according to a linear mixed model (RStudio version 1.4.1106) including the fixed effects of Herds (four levels: C, M, P, F), Location (two levels: ALP and PF), their interaction, and the random effect of cow nested in the Herd. It is worth noting that the ALP vs PF contrast reflects not only the effects of location (geographical area, altitude, management, feeding, etc) but also the effect of season (July vs October) and of the lactation stage (mid-lactation vs late-lactation). We used a function to estimate R^2_{GLMM} statistic, *r.squaredGLMM*, included in the *MuMln* package for the R statistical software, and we consider the conditional R-squared because concern variance explained by both fixed and random factors (Nakagawa and Schielzeth, 2013).

Contrasts were estimated to examine the difference between ALP and PF to test for the effect of summer transhumance on the main effects, but also within each herd to explain the interaction between herd and location. A similar model with all effects as random factors was run for quantifying the relative importance of the different herds and locations (and their interaction), of individual animals within herd, and of residual factors non accounted for by the model in the total variance of each trait. The variances of these five sources of variation were expressed as percentage of their sum (total variance).

Correlation analysis and latent explanatory factor analysis

Three datasets were created:

d) Intestinal metagenomics (**im-dataset**): including the relative abundances of the 14 bacterial taxa identified in cow's rectal samples;

e) Intestinal and milk metagenomics (**imm-dataset**): merging the two sub-datasets of the relative abundances data of the 14 bacterial taxa identified in cow's rectal samples and those of the 15 milk microbial traits considered of interest for the dairy sector and consumers according our previous study (Secchi et al., 2023b);

f) Intestinal metagenomics and milk quality (**imq-dataset**): merging the two sub-datasets of the relative abundances of the 14 bacterial taxa identified in cow's rectal samples and the 16 traits related to milk composition, udder health and B-vitamins;

Pearson correlations were carried out among the intestinal metagenomic relative abundances of the im-dataset, between the two sub-datasets included in the imm-dataset, and between those of the imq-dataset.

We also used multivariate factor analysis (FA) to summarize the interrelated measured traits in a small number of unmeasured latent independent explanatory variables (factors). We ran three FA: within dataset a), dataset b), and dataset c), named respectively FN-im, FN-imm, and FN-imq latent factors, where N is the progressive number of factors (N: 1 to 8) within FA. For each FA, first we performed KMO (Kaiser-Meyer-Olkin) and Barlett's tests, which showed that the traits were suitable for FA. The factor analysis was carried out with Varimax rotation in the R environment (R Core Team, 2016) using the psych package (available at CRAN: The Comprehensive R Archive Network) in three steps: (i) extraction of factors such that the minimum number of uncorrelated latent factors explained the greatest proportion of common variance; (ii) factor rotation until each factor was defined by a few variables with high loadings; and (iii) biological interpretation of the factors

based on the strength of the loadings of the variables. The eigenvalues of the factors and the communalities of the variables after rotation were also determined.

A total of 6, 8, and 8 latent explanatory factors were extracted from the analyses of datasets a), b), and c). The loadings of each trait were used for calculating the scores of each latent factor for each sample analyzed. The scores of each factor were analyzed statistically according to the same linear model used for analysing the relative abundances of every bacterial taxon.

RESULTS AND DISCUSSION

Metagenomics of dairy cows intestinal material

The metagenomic approach allowed to select and identify the DNA of 14 major bacterial taxa and to calculate their relative abundances in each fecal material sample. The descriptive statistics of the relative abundances of bacterial taxa, after logarithmic transformation, are presented in Table 1. It is possible to see that the three most important taxa identified were *Bacteroidaceae*, *Bacteroidetes*, and *Ruminococcaceae*, followed by *Rikenellaceae*, *Paraprevotellaceae*, *Lachnospiraceae*, and Other *Clostridia*. Albonico et al. (2020) found similar result, *Firmicutes* and *Bacteroidetes* as the most dominant phyla, and *Ruminococcaceae* and *Lachnospiraceae* were the two most abundant families, followed by *Paraprevotellaceae* and *Bacteroidaceae*. In addition to being some of the prevalent taxa, *Ruminococcaceae* and *Lachnospiraceae* play an important role in starch and fiber digestion (Kim et al., 2014; Mao et al., 2015), over the positive association with the gut health of several mammalian species, such as cats, dogs, horses, laboratory mice, cattle (Raats et al., 2011; Rudi et al., 2012; Suchodolski et al., 2012; Hildebrand et al., 2013; Honneffer et al., 2014; Weese et al., 2015). Ruminococci are important bacterial species for ruminants due to their cellulolytic activity and ability to convert complex polysaccharides into a variety of nutrients in the host (Li et al., 2019); however a higher abundances of Ruminococcaceae sequences was found in the fecal samples of forage-fed animals than grain-fed animals (Callaway et al., 2010; Shanks et al., 2011).

The results of the mixed model carried out on the 14 selected taxa are also summarized in Table 1. It is worth noting that 12 out of the 14 taxa were affected by one or more of the fixed effects included in the model: 9 taxa by herd effect, 3 taxa by location effect (ALP vs PF) and 7 taxa by their interaction (different ALP vs PF effect within each herd). The conditional coefficient of determination of the model (R^2c, Table 1) ranged from a minimum of 0.29 for *Ruminococcaceae* to a maximum of 0.91 for *Verrucomicrobia* taxon.

The R^2c, as expected, is inversely related with the proportion of the residual variance on total variance obtained from the all-random model of analysis. The Figure 1, in fact, shows that *Ruminococcaceae* were characterized by the greater incidence of residual variance, and *Verrucomicrobia* by the smallest one. But Figure 1 shows also that all the major sources of variation analyzed are very different for different bacterial taxa. The peculiarity of *Tenericutes* and *Verrucomicrobia* are to be the taxa with the highest permanent differences among herds, whereas the peculiarity of *Bacteroidaceae*, *Rikenellaceae*, Other *Clostridia*, and *Proteobacteria* are to be those with the lowest permanent differences. This means that a herd (group of cows) having a high (or low) relative abundancy of one of the former two taxa tends to maintain these characteristics both in the ALP and PF. Viceversa, in the case of the latter four taxa, the average values of ALP and PF of the four herds are negligible.

Lastly, the model includes the random effect of individual cow within herd. It can be seen from Figure 1 that 9 out of 14 bacterial taxa identified in fecal material are characterized by a certain variability among the values (average of ALP and PF data) of different cows within herds. This result is much different than that found in the case of the milk microbiota of the same cows (only 4 out of 15 microbial taxa and groups) (Secchi et al., 2023b). In a previous study (Secchi et al., 2023a), with 5 monthly samples per cows, we found and intermediate result (7 out of 15 milk microbial traits). No other data that the authors are aware of is available in the literature.

Effect of Alpine pasture vs indoor feeding on intestinal microbiota and herd interactions

The average difference between the four ALP and corresponding PF was also very variable for different bacterial taxa. It was almost null in the case of the relative abundances of *Paraprevotellaceae* and *Ruminococcaceae*, very modest for *Tenericutes* and *Verrucomicrobia*, and moderate to very large for all the other taxa (Figure 1). For example, we have confirmation the *Bacteroidetes* are higher in ALP, as this is a prevalent phylum in animals with diet richer of plant

fiber, and they have strong ability to degrade crystalline cellulose (Henderson et al., 2015; Houlden et al., 2015; Johnson et al., 2017), while Li et al. (2020) was found less abundant in the summer season. The interaction between herd and location (the fact that the comparison ALP vs PF yields different results for different herds) is generally much less important than the variability caused by the two major factors of variations and is almost null for *Tenericutes* and *Verrucomicrobia*. The fact that interaction is significant in a greater number of taxa than location (Table 1) is due to the fact that the number of degrees of freedom at the numerator is 4 for interaction and 1 for location.

The four degrees of freedom of interaction are corresponding to the differences (contrasts) between ALP and PF for each of the four herd, which are shown in Table 2. On average only the relative abundances of *Methanobacteriaceae* and *Peptostreptococcaceae* taxa were lower and those of *Proteobacteria* taxon were higher in ALP intestinal samples than in PF intestinal samples. Healthy cows have reported to have less than 4% of *Proteobacteria*, while an increase of this relative abundance has been associated with subacute ruminal acidosis (Khafipour et al., 2009; Pitta et al., 2010; Xu et al., 2017). For all these taxa the contrasts calculated within each herd maintained the same sign of the average value and in three out of four herds the contrast was significant (Table 2). In any case the variability among herds was large and the interaction was significant for the former two taxa. Of the other 11 intestinal taxa, 4 taxa (*Paraprevotellaceae*, *Ruminococcaceae*, and Other *Clostridia*, and *Tenericutes*) did not present any significant contrast at herd level, whereas the remaining 7 taxa presented significant contrasts (for one to three herds), having always the same sign.

No information about the effect of farming system tested on several herds, and of its interaction with individual herd is available in the literature, the authors are aware of.

Albonico et al. (2020) investigated about 100 cows from 10 small alpine farms, and explored factors may influence their milk and fecal microbiota, highlighting the evident variation between individual cows. The microbiota of each farm could be influenced by several management choices, such as sources of dietary ingredient, water supply, bedding material, hygiene practices, use of

pastures, milking hygiene. Globally, intestinal microbiota, despite the variability among different herds, appears to be more stable than milk microbiota, both in this same project (Secchi et al., 2023b) and in our previous project (Secchi et al., 2023a), the influence of management types needed further study

Correlations and latent factors explaining intestinal bacterial relative abundances covariance

The peculiar individuality of the major sources of variation of relative abundances of bacterial taxa identified and the different susceptibility to the effect of different farming systems, individual herds and their interactions give us a partial picture of the intestinal microbiota. The next step is the knowledge of the relationships (association, competition or independency) among them. The 91 Pearson correlations calculated among the 14 intestinal bacterial taxa are depicted as heat-map plot in Figure 2. The situation is very complex. Being "relative" abundances (their sum is a constant), it is expected that the increase of some taxa will be compensated by the decrease of others and then that the sign of correlation will be sometimes positive and sometimes negative. Anyway, the intestinal taxa present many correlations, in several cases high correlations, and in no cases a bacterial intestinal taxon was not or negligibly correlated with any other taxa. This means that all intestinal taxa are variably associated with each other and that Pearson correlation are not able to clarify easily the aggregation of bacterial taxa in groups. This is why we performed the factor analysis on the dataset of relative abundances of intestinal material bacterial taxa obtained from the metagenomic approach.

Six latent explanatory factors were able to absorb 72% of the variance-covariance matrix of the 14 intestinal taxa (Table 4). The loadings of the 6 latent factors are listed in Table 3, together with the communality coefficient of each taxon. This last coefficient was >0.50 for all taxa, with the only exception of *Porphyromonadaceae* (0.404), the only taxon not well represented by the 6 latent factors obtained, even though also this taxon was included in one factor. Overall, 6 taxa were included in only one factor, 6 taxa were included in two factors contemporarily, and two taxa (*Lachnospiraceae*

and *Peptostreptococcaceae*) presented a more complex relationship with other taxa, being included in 3 factors each. *Peptostreptococcaceae* and *Lachnospiraceae,* belonging to Firmicutes phylum, play an important role in ruminal fermentation, as well as in other digestion-associated processes (Cortés et al., 2019; Uchiyama et al., 2020). Moreover *Lachnospiraceae* family was correlated with tryptophan metabolism and valine, leucine, and isoleucine degradations in the rumen of beef cattle, so it's associated with feed efficiency (Li and Guan, 2017).

As shown in Table 3, the first latent factor (F1-im) appears to be based on the strong antagonism between *Actinobacteria* - *Bifidobacteriaceae* (loading 0.783) and *Bacteroidaceae* taxon (loading -0.837). This is confirmed by the very strong negative correlation between these two taxa shown in Figure 2. In this first factor, *Methanobacteriaceae*, *Lachnospiraceae* and *Peptostreptococcaceae* also are included with positive moderate loadings, and *Porphyromonadaceae* and *Rikenellaceae* with negative moderate loadings. Seven out of 14 intestinal taxa are than involved in the F1-im, which represents 18.4% of total variance (Table 4). The scores of F1-im are not significantly different in different herds, whereas they present the strongest difference between ALP and PF (Table 4) among all 6 factors. The average value of these scores is in fact much lower in intestinal samples taken during the Alpine pasture in July than in indoor permanent farms in October. The interaction between location and herds was significant but not much relevant (Table 4).

The second latent factors of intestinal microbiota of dairy cows (F2-im) included 6 bacterial taxa (4 with positive loadings and 2 negative). Also in this case two antagonist taxa showed the highest loadings (Table 3): *Tenericutes* (0.785) and *Verrucomicrobia* (-0.735). They represented 15.6% of all variance (Table 4) and, differently from F1-im, the scores of F2-im were strongly affected by the herds, and more modestly by farming system and interaction.

The third latent factor (F3-im), representing 12.7% of total variance, appears to be based on a taxon with a strong positive loading (*Bacteroidetes*, 0.824), and on two taxa with negative loadings (*Methanobacteriaceae*, -0.620, and *Peptostreptococcaceae*, -0.606). The scores of F3-im were

affected more by farming system than by individual herds and interaction (Table 4). The cows sampled showed a higher average score during summer transhumance to Alpine pasture, than during indoor rearing.

The fourth intestinal latent factor (F4-im) was also based on three taxa: one negative (Ruminococcaceae, -0.785) and two positive (*Rikenellaceae*, 0.640, and *Paraprevotellaceae*, 0.514), representing 9.8% of total variance. In this case, only interaction was significant, with only one herd showing a significant difference between ALP and HF.

The fifth intestinal latent factor (F5-im) is a mono-taxon factor based only on *Proteobacteria* taxon (-0.859) and representing 7,9% of total variance. The statistical analysis of the scores of this factor, as that of the relative abundancy of *Proteobacteria* taxon (but with opposite sign), showed a strong effect of farming system, with lower scores during summer transhumance than indoor rearing. The *Proteobacteria* could then be considered a taxon substantially independent from all the others.

Lastly, the sixth intestinal latent factor (F6-im), representing 7.6% of total variance, appears to be based on two positive and two negative loadings, the only one >0.50 being Other *Clostridia* (0.689).

Summarizing the results obtained, one latent factor (F2-im) seems very useful for characterizing the intestinal microbiota of different herds, independently from summer transhumance, all the factors, except F4-im, are affected by farming systems, whereas the interaction between herds and farming system seems to be less relevant than in the case of statistical analyses of individual bacterial taxa (Table 1 and 2).

Relationships between intestinal and milk microbiota

After having characterized the intestinal microbiota of dairy cows, the second major aim of this study is the analysis of possible interrelationships between intestinal microbiota and milk microbiota of samples from the same cows taken the same day.

The milk microbiota, analyzed according the metagenomic approach with the same methods and in the same laboratory used for intestinal microbiota, was the object of a previous study, where all the details are illustrated (Secchi et al., 2023b). In short, 41 bacterial taxa were identified in milk samples. Eleven of these taxa have been identified in a previous study on a different project (Secchi et al., 2023a) as interesting for characterizing the quality and technological properties of milk and classified in four groups of taxa: Lactic acid bacteria (LAB), Other probiotics, Spoilage bacteria, and Pathogenic bacteria. The remaining 30 taxa were grouped as Other bacteria. The list of the 41 bacterial taxa identified in milk, with the average and standard deviation of their relative abundances, and their classification in 5 groups are reported in Table 5. The major sources of variation, and the effects of herds, farming system and interaction were described and discussed in the previous study and are not the aims of this one. Here, as said before, the interest is on relationships between intestinal and milk microbiota. The Figure 3 depicts, as heat-map plot, the Pearson correlations between the relative abundances of the 14 intestinal bacterial taxa and the those of the 11 milk taxa and 4 groups of taxa having a direct interest for milk quality and dairy industry. The first impression is that these two microbiota are not independent but variably associated. Many of the 210 Pearson correlation coefficients summarized are moderate to high, both positively or negatively. Again the interpretation of results cannot be left to this level, but require the simplification of this complex picture searching for possible latent explanatory factors.

The joint factor analysis of the two dataset of intestinal and milk relative abundances yielded 8 latent factors explaining, all together, 71.2% of total variance (Table 7). The loadings of each latent factor obtained and the communality coefficients of each bacterial trait analyzed are listed in Table 6.

The first factor obtained from intestinal and milk microbiota (F1-imm) is a “intestinal factor”, which substantially mimics what obtained with the F1-im factor yielded by the analysis of intestinal taxa alone, the loadings of common taxa are obviously different but the signs are the same. The major

difference is that in the joint intestinal+milk factor analysis the F1-imm includes 8 intestinal taxa instead of 7 and represents 13.1% of total variance instead of 18.4%.

Being based on 14+15=29 bacterial traits instead of only 14, it is expected that the factors obtained in the joint factor analysis be more in terms of number of factors (8 *vs* 6), each representing a smaller proportion of variance (like in the case of F1-imm vs F1-im) and that the significance level achieved be greater, because of the much larger number of degrees of freedom.

Even though the majority of factors and contrasts included in the model resulted significant, the F1-imm (Table 7) respect to the corresponding values of the F1-im (Table 4) showed a similar ranking of importance, confirming that F1-imm is especially important for differentiating the two farming systems, with samples from Alpine pastures characterized by lower scores than those from indoor rearing.

The second factor obtained from the joint intestinal+milk analysis (F2-imm) is a different story. This is a mixed intestinal-milk factor. It includes 5 intestinal bacterial taxa (4 of which in common with the F2-im, Table 3) and 5 milk taxa and groups of taxa (Table 6). This F2-imm seems very interesting from the point of view of dairy industry because it is positively associated to LAB group (and particularly *Lactococcus* taxon) and to Pathogenic bacteria (and particularly to *Enterobacteriaceae* taxon), and negatively to the *Streptococcus* taxon. This F2-imm represents 11.90% of total variance and its scores are affected contemporarily by herds, farming system and their interaction (Table 7).

The third joint factor (F3-imm) is mainly a milk factor, being strongly and positively based on Other probiotics group (and especially on *Propionibacterium* taxon) and on *Lactobacillus* taxon, but including moderate negative loadings with milk *Lactococcus* taxon and also with intestinal *Lachnospiraceae* taxon (Table 6). Also F3-imm, like F2-imm, represents a relevant proportion (11.3%) of total variance and is affected by herd, farming system and interaction (Table 7), beyond being of interest for dairy industry.

The fourth joint latent factor (F4-imm) is again a mixed intestinal-milk factor. Three of the four intestinal taxa of this joint factor (Table 6) are in common (same sign, smaller loadings) with the taxa included in the F2-im (Table 3). The four milk bacterial traits included in this factor, all positively, are *Clostridiaceae* taxon (strong loading), Spoilage group, *Bifidobacterium* taxon, and Other LAB taxa (Table 6). This interesting factor represents 9.5% of total variance and is affected mainly by interaction between herd and farming system (Table 7).

Similarly to F3-imm, the fifth joint factor (F5-imm) is mainly based on milk microbiota but includes also an intestinal bacterial taxon (*Proteobacteria*, negatively). The milk bacterial traits included (Table 6) are, strongly and negatively, the Pathogenic group (*Staphylococcus* taxon), and positively and moderately the LAB group (*Lactococcus* taxon). Similarly to F4-imm, its quota of total variance is 9.1% of total variance but is not directly affected by the herd or farming system, but only by some interaction (Table 7).

The sixth joint factor (F6-imm) is based moderately on only 3 intestinal taxa (*Bacteroidaceae* and *Rikenellaceae* taxon, positively, and, *Lachnospiraceae* negatively), and on 3 milk bacterial traits: 2 positively (LAB group and *Enterococcus* taxon) and *Enterobacteriaceae* negatively. This factor, representing 6.5% of total variance, is affected especially by herd effect and interaction and slightly by the farming system (Table 7).

The last two joint factors, the seventh (F7-imm) and the eighth (F8-imm), represents respectively only 5.8% and 4.1% of total variance, are affected mainly by interaction (the seventh). Both are based mainly on milk bacterial traits and then are not much interesting for the objective of this study.

In summary, 5 out of 8 latent factors combine the relative abundances of bacterial intestinal and milk taxa, demonstrating many interrelationships between the microbiota living in the cow's intestine (the intestinal samples were grabbed directly from the rectum) and those present in the udder or contaminating milk during milking, even though only some *Clostridia* were present in both type

of samples. Moreover, the milk taxa associated with some intestinal taxa in these latent factors are known for having a direct interest in milk quality and/or its technological properties.

Relationships between intestinal microbiota and milk composition and udder health traits

After having demonstrated the many relationships existing between the intestinal microbiota of the cow with the milk microbiota, the next step is to search for relationships between the intestinal microbiota and the quality traits measured on milk.

The milk quality traits comprised 8 milk composition traits, 4 udder health traits and 4 B-vitamins contents of milk. These 16 milk traits are listed in Table 8 together with their mean values and standard deviations. All these traits have been illustrated and discussed in the previous study of this project on milk quality and microbiota (Secchi et al., 2023b). The correlations with each other traits were shown to be relevant in the case of those representing milk composition (except milk urea content). Of the 4 traits related to udder content, as usual, lactose and SCS were negatively correlated and presented opposite sign for the correlations with all other traits, whereas milk pH was not much correlated with any other trait. Lastly, the milk contents of B-vitamins were correlated with each other (Folic acid-vit B_9 excluded) but scarcely correlated with other milk traits.

In this study we have calculated the Pearson correlations between the relative abundances of the 14 intestinal bacterial taxa and the 16 milk quality traits. The resulting 224 values are represented in the heat-map plot of Figure 4. It is possible to see that the correlations are more ordered in blocs respect to those of the Figure 3. Anyway, also in this case, we carried out a factor analysis on this third dataset and the result was again a set of 8 latent explanatory factors representing 71.4% of total variance (Table 10).

Two out of 8 FN-imq factors were based only on intestinal microbiota (Table 9): the F5-imq and the F7-imq. These two factors do not strictly mimic any of the FN-im factors obtained analyzing the intestinal taxa alone. The F5-imq includes, with the same sign, 7 intestinal taxa present in F1-im,

F5-im and F6-im, represented 9.5% of total variance and is significantly affected by all fixed factors included in the statistical model (Table 10). The F7-imq includes, but with different signs, 5 intestinal taxa present in F3-im, F4-im and F6-im, represented 6.1% of total variance and was marginally affected by the fixed factors included in statistical analysis (Table 10). Therefore, these two FN-imq factors are of no interest for the third aim of this study (relationships between intestinal microbiota and milk quality).

F1-imq, F2-imq, F3-imq, and F4-imq are of a certain interest. The first two are based mostly on milk quality traits which reproduce the latent explanatory factors of milk composition often named "caseins" and "cheese yield" (Secchi et al., 2023a; b). The only connection to the intestinal metagenomics is a moderate link to the *Porphyromonadaceae* taxon (negative) for the first factor and to the *Proteobacteria* (positive) for the second one. F1-imq represents 12.2% of total variance and, as expected, is strongly affected by farming system and also by its interaction with individual herds, while F2-imq represents 10.7% of total variance and seems to have only a slight influence of the herd (Table 10).

The F3-imq is similar to F5-imq and F7-imq, been mainly based on intestinal microbiota except a moderate positive link to the milk pH, and includes, with the same sign, 7 intestinal taxa present in F2-im and F4-im (Table 3). It represents 9.7% of total variance and was affected substantially only by the effect of herd (Table 10).

The F4-imq is substantially based on udder health traits, with the addition of folic acid content of milk and of the relative abundancy of only one intestinal taxa: the *Bacteroidetes* (Table 9). The meaning of the inclusion of folic acid contained in milk (with positive loading) and of the relative abundancy of *Bacteroidetes* (negative) is not very clear and further research are needed on this issues. This latent factor represents 9.6% of total variance and seems not affected by the effects included in the model (Table 10).

The remaining 2 latent factors (F6-imq and F8-imq) are even more interesting, combining together more intestinal metagenomic information with milk quality traits.

The F6-imq is based on the other three B-vitamins (thiamine, riboflavin and nicotinic acid) on one side and on three intestinal bacterial taxa (all included in F1-im, but with opposite sign): *Methanobacteriaceae* (negatively) and *Bacteroidaceae* and *Porphyromonadaceae* (positively) on the other side (Table 9). This latent factor explains 8.6% of total variance and is affected mainly by the farming system. It is well known that some B-vitamins are produced in the intestine of humans and animals, and also in the pre-stomachs of ruminants (Najjar and Barrett, 1945; Hill, 1997) and they are important for human health (Graulet and Girard, 2017)

Finally, the F8-imq represents only 5.0% of total variance and is not much affected by experimental sources of variation. The interest of this factor is that this is the only one based on lactoferrin (negatively) and SCS (positively). In some way it seems to represent another factor related, but negatively, to the udder health of the cow. It is worth noting that this factor includes three intestinal taxa (all represented in F1-im); the *Bacteroidaceae* (negatively), and the *Actinobacteria-Bifidobacteriaceae* and the *Lachnospiraceae* (positively). The meanings of these associations are mainly to be studied in future research.

CONCLUSIONS

The intestinal microbiota of dairy cows is based on many different bacterial taxa, each characterized by different proportions among the main sources of variability. The differences among individual herds are very important for some taxa (*Verrucomicrobia*, *Tenericutes* and *Lachnospiraceae*, in particular), those between different farming systems (Alpine pasture *vs* lowland indoor feeding) affect strongly other taxa (*Methanobacteriaceae*, *Actinobacteria-Bifidobacteriaceae*, *Bacteroidaceae*, *Peptostreptococcaceae*, Other *Clostridia* and *Proteobacteria*), whereas their interaction is quantitatively less important but affects almost all intestinal bacterial taxa (*Tenericutes* and *Verrucomicrobia* are notable exceptions). The individual cow within herd represents a moderate source of variation in the majority of taxa, whereas residual variance is very different for different taxa, being the dominant source of variation in the case of *Rikenellaceae*, *Paraprevotellaceae*, and *Ruminococcaceae*. The very complex interrelationships among different bacterial taxa allowed to extract 6 latent explanatory factors of intestinal microbiota representing 72% of total variance. One factor (F2-im) could be used to characterize well different herds, whereas F1-im, F3-im, and in part also F5-im and F6-im could be very useful in discriminating farming systems.

Intestinal microbiota appears to be interrelated also with milk microbiota, at least with some of the taxa having particular interest for characterizing quality and health of milk. Five out of eight latent factors obtained from the merging of data from intestinal and milk metagenomics (imm) combined taxa from both type of samples demonstrating the interrelations between cow's intestine and udder. Furthermore, the milk taxa associated with intestinal taxa are known for being of interest for the dairy industry (lactic acid bacteria, other probiotics, spoilage bacteria and pathogenic bacteria). Finally, intestinal bacterial taxa have found to be associated also directly with some quality traits of milk. Four out of eight latent factors obtained merging the dataset of intestinal metagenomics with that of milk quality traits (imq) combined traits from both databases, linking the intestinal bacteria with the milk udder health traits (F4-imq), with B-vitamins content of milk (F6-imq), with cheese

yield (F2-imq), and with lactoferrin (F8-imq). If this study demonstrated the interest of cow's intestinal metagenomics, because of the differences due to farming systems, herds and individual cows and the many interrelationships with milk metagenomics and milk quality, it demonstrated also the need for further research in the field.

Conflicts of interest

None.

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TABLES AND FIGURES

Table 1. Descriptive statistics (mean ± standard deviation), and statistical analysis (F-value and significance levels) of the Herds, Location (ALP vs PF) and interaction between Herd and Location of the log_{10} relative abundances of intestinal bacterial taxa of cows from 4 permanent farms moved in June to four Alpine temporary summer pasture (ALP) and moved back to permanent farm (PF) at the end of September.

Traits	Samples N	Descriptive statistics:		Herd	ALP *vs* PF	Interaction	R^2c	RMSE
		Mean	±SD					
Methanobacteriaceae	50	0.074	0.086	9.6 ***	15.7 ***	3.7 *	0.67	0.050
Actinobacteria – Bifidobact.	51	0.304	0.287	1.6	2.1	1.2	0.45	0.221
Bacteroidaceae	51	1.279	0.104	5.2 **	1.3	5.5 **	0.76	0.052
Porphyromonadaceae	50	0.514	0.091	0.5	0.1	3.1 *	0.59	0.061
Rikenellaceae	51	0.923	0.060	1.9	2.0	3.9 *	0.42	0.048
Paraprevotellaceae	51	0.870	0.111	8.0 ***	3.9	3.2 *	0.42	0.089
Bacteroidetes	51	1.251	0.074	5.7 **	0.7	2.4	0.66	0.045
Lachnospiraceae	51	0.895	0.149	3.9 *	0.4	3.2 *	0.71	0.083
Peptostreptococcaceae	49	0.623	0.298	18.7 ***	22.3 ***	8.8 ***	0.77	0.145
Ruminococcaceae	51	1.306	0.048	6.4 **	3.0	2.2	0.29	0.042
Other Clostridia	50	0.906	0.075	2.7	1.0	1.4	0.45	0.058
Proteobacteria	50	0.310	0.123	1.5	15.6 ***	3.0	0.54	0.087
Tenericutes	51	0.494	0.171	12.1 ***	1.6	0.4	0.64	0.105
Verrucomicrobia	51	0.341	0.210	24.3 ***	1.5	0.6	0.91	0.062

*P<0.05; **P<0.01; ***P<0.001

RMSE= root mean square error. R^2c = conditional R^2.

Table 2. Contrast estimates and their significance levels (average and for each farm) during the summer transhumance on Alpine pastures (mid-July) respect to indoor farming (mid-october) after transhumance for the log_{10} relative abundances of intestinal bacterial taxa

Traits	Difference of ALP respect PF:				
	Overall effect	Single herds			
		C	M	P	F
Methanobacteriaceae	-0.11***	-0.11***	-0.05	-0.18***	-0.11**
Actinobacteria Bifidobact.	-0.31	-0.18	-0.31*	-0.49***	-0.27
Bacteroidaceae	0.13	0.04	0.11**	0.18***	0.20***
Porphyromonadaceae	0.08	-0.01	0.12**	0.12**	0.08
Rikenellaceae	0.04	-0.04	0.04	0.07*	0.09*
Paraprevotellaceae	0.02	-0.10	-0.02	0.08	0.10
Bacteroidetes	0.06	0.02	0.04	0.12***	0.08*
Lachnospiraceae	-0.11	0.03	-0.22***	-0.15**	-0.05
Peptostreptococcaceae	-0.36***	-0.37***	-0.01	-0.57***	-0.50***
Ruminococcaceae	-0.01	0.04	-0.04	0.01	-0.03
Other Clostridia	-0.08	-0.03	-0.10	-0.05	-0.12
Proteobacteria	0.13***	0.20***	0.02	0.11*	0.20**
Tenericutes	0.06	0.07	0.01	0.07	0.10
Verrucomicrobia	-0.08	-0.05	-0.11**	-0.07	-0.11*

*P<0.05; **P<0.01; ***P<0.001

Table 3. Latent explanatory factors of the intestinal microbiota (im): loadings of each factor and communality coefficient of each trait included in the factor analysis ($\chi^2 = 38.8$; 22 degrees of freedom; P=0.015).

	Latent explanatory factors:						Communality
	F1-im	F2-im	F3-im	F4-im	F5-im	F6-im	
Methanobacteriaceae	0.555*		-0.620*				0.789
Actinobacteria Bifidobact.	0.783*						0.731
Bacteroidaceae	-0.837*					-0.429	>0.900
Porphyromonadaceae	-0.473						0.404
Rikenellaceae	-0.385			0.640*			0.666
Paraprevotellaceae		0.463		0.514*			0.568
Bacteroidetes			0.824*			-0.314	0.855
Lachnospiraceae	0.444	0.521*				0.418	0.683
Peptostreptococcaceae	0.513*	-0.496	-0.606*				>0.900
Ruminococcaceae				-0.765*			0.707
Other Clostridia						0.689*	0.646
Proteobacteria		0.337			-0.859*		>0.900
Tenericutes		0.785*					0.689
Verrucomicrobia		-0.735*					0.622

*High loadings, >0.50.

Table 4. Latent explanatory factors of the intestinal microbiota (im): percentage fraction of total variance explained by each factor, significance of the fixed effects of Herd, Location (ALP *vs* PF) and their interaction, contrast between ALP and PF as effect and as interaction within each herd, and root mean square error (RMSE).

	Latent explanatory factors:					
	F1-im	F2-im	F3-im	F4-im	F5-im	F6-im
Explained variance:						
- individual, % total	18.4%	15.6%	12.7%	9.8%	7.9%	7.6%
- cumulative, %/total	18.4%	34.0%	46.7%	56.5%	64.4%	72.0%
Fixed effects:						
Herd, *F-value*	2.30	37.33 ***	3.33 *	1.77	0.65	0.83
ALP vs PF, *F-value*	54.42 ***	6.41 *	9.27 **	0.22	7.97 *	7.13 *
ALP vs PF, *contrast*	-1.19***	0.31*	0.59**	0.11	-0.40*	-0.50*
Interaction, *F-value*	2.47	4.12 *	2.22	3.72 *	1.33	1.75
Herd C, *contrast*	-0.63	0.40	0.58	-1.14*	-0.86**	0.04
Herd M, *contrast*	-0.93**	-0.35	-0.07	0.68	-0.18	-0.70
Herd P, *contrast*	-1.76**	0.33	1.30**	0.26	-0.16	-0.25
Herd F, *contrast*	-1.44***	0.85**	0.55	0.63	-0.42	-1.11*
R^2c	0.69	0.80	0.55	0.26	0.39	0.50
RMSE	0.54	0.42	0.65	0.80	0.48	0.63

*P<0.05; **P<0.01; ***P<0.001
RMSE= root mean square error. R^2c = conditional R^2.

Table 5. Descriptive statistics (mean ± standard deviation) of the log10 relative abundances of milk bacterial taxa with known dairy (LAB), other probiotics, spoilage and pathogenic activities, and of other bacteria found in the milk of cows.

Traits	Samples N	Descriptive statistics: Mean	±SD
Lactic acid bacteria (LAB):	50	1.240	0.478
Lactobacillus	49	0.222	0.374
Lactococcus	50	0.880	0.672
Enterococcus	51	0.227	0.305
Streptococcus	51	0.330	0.353
Other LAB	51	0.203	0.243
Other probiotics:	49	0.248	0.394
Propionibacterium	50	0.169	0.398
Bifidobacterium	50	0.095	0.119
Spoilage bacteria:	51	0.399	0.256
Clostridiaceae	49	0.181	0.215
Pseudomonas	50	0.232	0.195
Pathogenic bacteria:	51	0.748	0.517
Staphylococcus	51	0.528	0.577
Enterobacteriaceae	51	0.291	0.363
Other bacteria:	50	1.660	0.260
Corynebacterium	51	0.327	0.424
Dermabacteraceae	50	0.172	0.275
Intrasporangiaceae	50	0.122	0.229
Microbacteriaceae	50	0.340	0.277
Micrococcaceae	51	0.503	0.418
Propionicimonas	50	0.085	0.165
Other *Actinobacteria*	50	0.265	0.230
Porphyromonas	49	0.003	0.009
Flavobacteriaceae	51	0.238	0.282
Chryseobacterium	50	0.480	0.391
Wautersiella	49	0.100	0.221
Chitinophagaceae	49	0.140	0.274
Other *bacteroidetes*	51	0.212	0.196
Cyanobacteria	50	0.044	0.105
Solibacillus	50	0.189	0.310
Aerococcus	50	0.063	0.131
Trichococcus	50	0.074	0.121
Turicibacter	50	0.220	0.270
Peptostreptococcaceae	49	0.401	0.389
Ruminococceae	50	0.091	0.139
Tissierellaceae	49	0.020	0.054
Other *Firmicutes*	50	0.213	0.259
Agrobacterium	50	0.118	0.224

Paracoccus	50	0.189	0.352
Sphingomonadaceae	50	0.079	0.103
Comamonadaceae	49	0.190	0.175
Acinetobacter	51	0.939	0.469
Enhydrobacter	51	0.242	0.327
Xanthomonadaceae	50	0.210	0.239
Other *Proteobacteria*	51	0.363	0.279

Table 6. Latent explanatory factors of the intestinal and milk microbiota (imm): loadings of each factor and communality coefficient of each trait included in the factor analysis (χ^2 = 529.2; 202 degrees of freedom; P<0.0001).

	Latent explanatory factors:								Communality
	F1-imm	F2-imm	F3-imm	F4-imm	F5-imm	F6-imm	F7-imm	F8-imm	
Intestinal bacterial taxa:									
Methanobacteriaceae	0.862*								0.772
Actinobacteria Bifidobact.	0.558*							0.342	0.598
Bacteroidaceae	-0.638*					0.334			0.689
Porphyromonadaceae	-0.440								0.403
Rikenellaceae						0.400			0.399
Paraprevotellaceae		-0.531*							0.502
Bacteroidetes	-0.623*			-0.305					0.568
Lachnospiraceae		-0.389	-0.477	0.312		-0.341			0.704
Peptostreptococcaceae	0.898*								0.870
Ruminococcaceae		0.590*							0.359
Other Clostridia	0.417								0.380
Proteobacteria	-0.422				-0.384				0.465
Tenericutes		-0.600*		0.310					0.665
Verrucomicrobia		0.710*		-0.320					0.714
Milk bacterial taxa:									
Lactic acid bacteria (LAB):		0.590*			0.511*	0.305	-0.400		0.879
Lactobacillus			0.820*						0.719
Lactococcus		0.596*	-0.375		0.537*				>0.900
Enterococcus						0.804*			0.718
Streptococcus		-0.398							0.295
Other LAB				0.746*					0.657
Other probiotics:			0.913*						>0.900
Propionibacterium			0.929*						>0.900
Bifidobacterium				0.331				0.832*	>0.900
Spoilage bacteria:				0.722*			0.633*		>0.900
Clostridiaceae				0.874*					>0.900
Pseudomonas							0.890*		>0.900
Pathogenic bacteria:		0.323			-0.877*				>0.900
Staphylococcus					-0.928*				>0.900
Enterobacteriaceae		0.746*				-0.436			>0.900

*High loadings, >0.50.

Table 7. Latent explanatory factors of the intestinal and milk microbiota (imm): percentage fraction of total variance explained by each factor, significance of the fixed effects of Herd, Location (ALP *vs* PF) and their interaction, contrast between ALP and PF as effect and as interaction within each herd, and root mean square error (RMSE).

	Latent explanatory factors:							
	F1-imm	F2-imm	F3-imm	F4-imm	F5-imm	F6-imm	F7-imm	F8-imm
Explained variance:								
- individual, % total	13.10%	11.90%	11.30%	9.50%	9.10%	6.50%	5.80%	4.10%
- cumulative, %/total	13.10%	24.90%	36.20%	45.70%	54.80%	61.30%	67.00%	71.20%
Fixed effects:								
Herd, *F-value*	12.62***	31.79***	42.92***	9.27***	0.82	41.49***	4.69**	1.75
ALP vs PF, *F-value*	94.79***	12.13**	46.31***	0.61	3.63	7.47*	6.47*	3.29
ALP vs PF, *contrast*	-1.26***	-0.50**	0.65***	0.13	-0.41	0.27*	0.42*	-0.30
Interaction, *F-value*	12.96***	11.37***	90.88***	15.03***	6.86**	17.33***	10.94***	0.62
Herd C, *contrast*	-1.16***	0.52	-0.10	1.82***	-1.60**	-0.64**	-0.11	-0.41
Herd M, *contrast*	-0.02	-1.25***	3.34***	0.19	-0.46	0.66**	0.26	-0.53
Herd P, *contrast*	-2.16***	0.13	-0.64**	-0.39	0.97*	1.20***	-0.55	-0.36
Herd F, *contrast*	-1.69***	-1.38***	-0.01	-1.11**	-0.55	-0.14	2.10***	0.10
R^2c	0.78	0.74	0.89	0.61	0.35	0.88	0.56	0.18
RMSE	0.45	0.50	0.33	0.57	0.75	0.33	0.57	0.58

*P<0.05; **P<0.01; ***P<0.001
RMSE= root mean square error. R^2c = conditional R^2.

Table 8. Descriptive statistics (mean ± standard deviation) of milk composition, udder health traits, and B-vitamins of milk of cows.

Traits	Samples N	Descriptive statistics: Mean	±SD
Milk composition:			
Total solids, %	50	12.66	1.37
Milk fat, %	50	3.63	1.37
Non-fat solids, %	50	9.14	0.53
Milk Protein, %	50	3.57	0.42
Milk casein, %	50	2.85	0.29
Fat/Protein ratio, %	50	1.01	0.36
Casein number, %	50	79.95	3.48
Milk Urea, mg/dL	50	20.92	7.93
Udder health traits:			
pH	51	6.45	0.14
SCS, unit	51	3.45	2.01
Milk lactose, %	50	4.83	0.34
Lactoferrin (mg/L)	50	181	152
Vitamins, (µg/L):			
Thiamine (B_1)	51	187	119
Riboflavin (B_2)	51	2,225	1,302
Nicotinic Acid (B_3)	50	2.72	2.43
Folic Acid (B_9)	49	0.37	0.43

SCS= somatic cell score.

Table 9. Latent explanatory factors of the intestinal microbiota and milk quality traits (imq): loadings of each factor and communality coefficient of each trait included in the factor analysis ($\chi^2 = 577.9$; 182 degrees of freedom; P<0.0001).

	Latent explanatory factors:								Communality
	F1-imq	F2-imq	F3-imq	F4-imq	F5-imq	F6-imq	F7-imq	F8-imq	
Intestinal bacterial taxa:									
Methanobacteriaceae					0.838*	-0.365			>0.900
Actinobacteria Bifidobact.					0.503*			0.367	0.591
Bacteroidaceae					-0.378	0.335		-0.657*	>0.900
Porphyromonadaceae	-0.314					0.321			0.415
Rikenellaceae							0.781*		0.733
Paraprevotellaceae			0.381				0.514*		0.614
Bacteroidetes				-0.351	-0.596*		0.341		0.668
Lachnospiraceae			0.556*					0.352	0.608
Peptostreptococcaceae			-0.314		0.726*				0.778
Ruminococcaceae			-0.360				-0.584*		0.539
Other Clostridia					0.322		-0.397		0.384
Proteobacteria		0.364	0.507*		-0.443				0.634
Tenericutes			0.851*						0.858
Verrucomicrobia			-0.820*						0.803
Milk composition:									
Total solids, %	0.417	0.848*							>0.900
Milk fat, %		0.926*							>0.900
Non-fat solids, %	0.876*			-0.360					>0.900
Milk Protein, %	0.847*			0.348					>0.900
Fat/Protein ratio, %		0.940*							>0.900
Milk casein, %	0.930*								>0.900
Casein number, %				-0.757*					0.844
Milk Urea, mg/dL	0.475								0.283
Udder health traits:									
pH	-0.347		0.349	0.418					0.537
SCS, unit				0.524*				0.307	0.521
Milk lactose, %		-0.379		-0.807*					>0.900
Lactoferrin (μg/mL)								-0.585*	0.537
B-vitamins, (μg/L):									
Thiamine (B_1)						0.912*			>0.900
Riboflavin (B_2)						0.878*			0.827
Nicotinic Acid (B_3)						0.485			0.336
Folic Acid (B_9)				0.701*					0.539

*High loadings, >0.50.

Table 10. Latent explanatory factors of the intestinal microbiota and milk quality traits (imq): percentage fraction of total variance explained by each factor, significance of the fixed effects of Herd, Location (ALP *vs* PF) and their interaction, contrast between ALP and PF as effect and as interaction within each herd, and root mean square error (RMSE).

	Latent explanatory factors:							
	F1-imq	F2-imq	F3-imq	F4-imq	F5-imq	F6-imq	F7-imq	F8-imq
Explained variance:								
- individual, % total	12.20%	10.70%	9.70%	9.60%	9.50%	8.60%	6.10%	5.00%
- cumulative, %/total	12.20%	22.90%	32.60%	42.20%	51.70%	60.20%	66.40%	71.40%
Fixed effects:								
Herd, *F-value*	2.87	2.98*	53.27***	0.83	8.04***	0.05	1.02	1.63
ALP vs PF, *F-value*	21.80***	1.59	5.41*	0.68	24.41***	8.90**	2.43	8.84*
ALP vs PF, *contrast*	-0.96***	-0.27	0.28*	-0.15	-0.83***	0.69**	0.33	-0.49*
Interaction, *F-value*	3.30*	1.36	3.31*	0.75	7.45**	3.43*	6.13**	3.39*
Herd C, *contrast*	-1.12*	-0.08	0.70**	0.15	-1.00**	0.70	-1.11*	0.20
Herd M, *contrast*	-1.13*	-0.96*	0.03	-0.56	0.47	1.47**	0.33	-0.22
Herd P, *contrast*	0.10	-0.27	-0.19	-0.13	-1.65***	1.18*	0.70	-0.70
Herd F, *contrast*	-1.69**	0.23	0.58*	-0.05	-1.14**	-0.58	1.41**	-1.26**
R^2c	0.51	0.26	0.82	0.34	0.63	0.36	0.39	0.57
RMSE	0.71	0.75	0.42	0.61	0.58	0.81	0.74	0.54

*P<0.05; **P<0.01; ***P<0.001
RMSE= root mean square error. R^2c = conditional R^2.

Figure 1. Sources of the variation (expressed as % of total variance) in individual fecal bacterial taxa relative abundances and their categories (in bold): effects of the Herds (dark blue), ALP vs PF (red), their interaction (orange), individual cow nested in the Herd (green), and the residual variability (light blue).

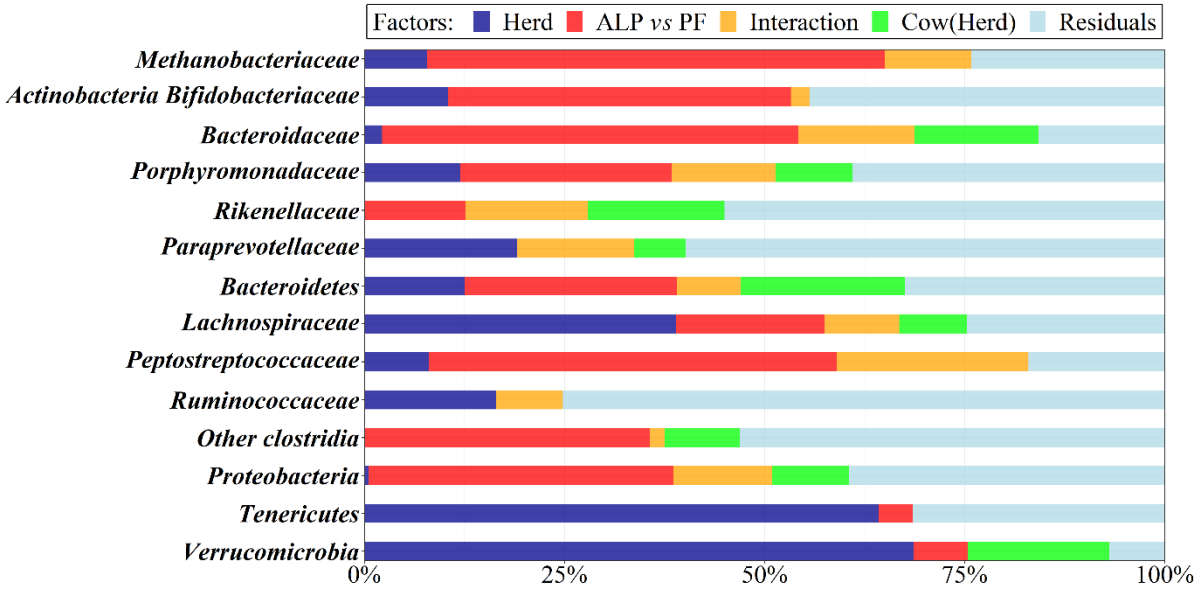


Figure 2. Heat map plot of the correlations among the relative abundances of intestinal bacterial taxa.

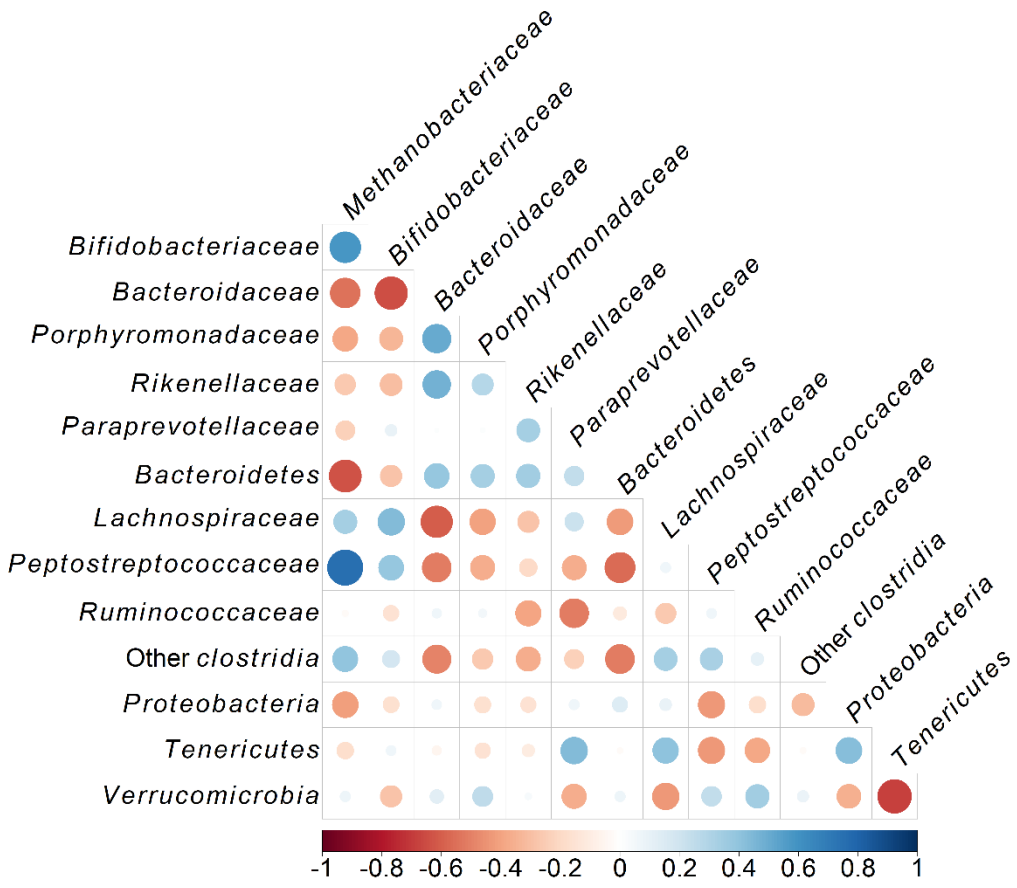


Figure 3. Heat map plot of the correlations among the intestinal bacterial taxa and the milk bacterial groups and taxa included in the factor analysis.

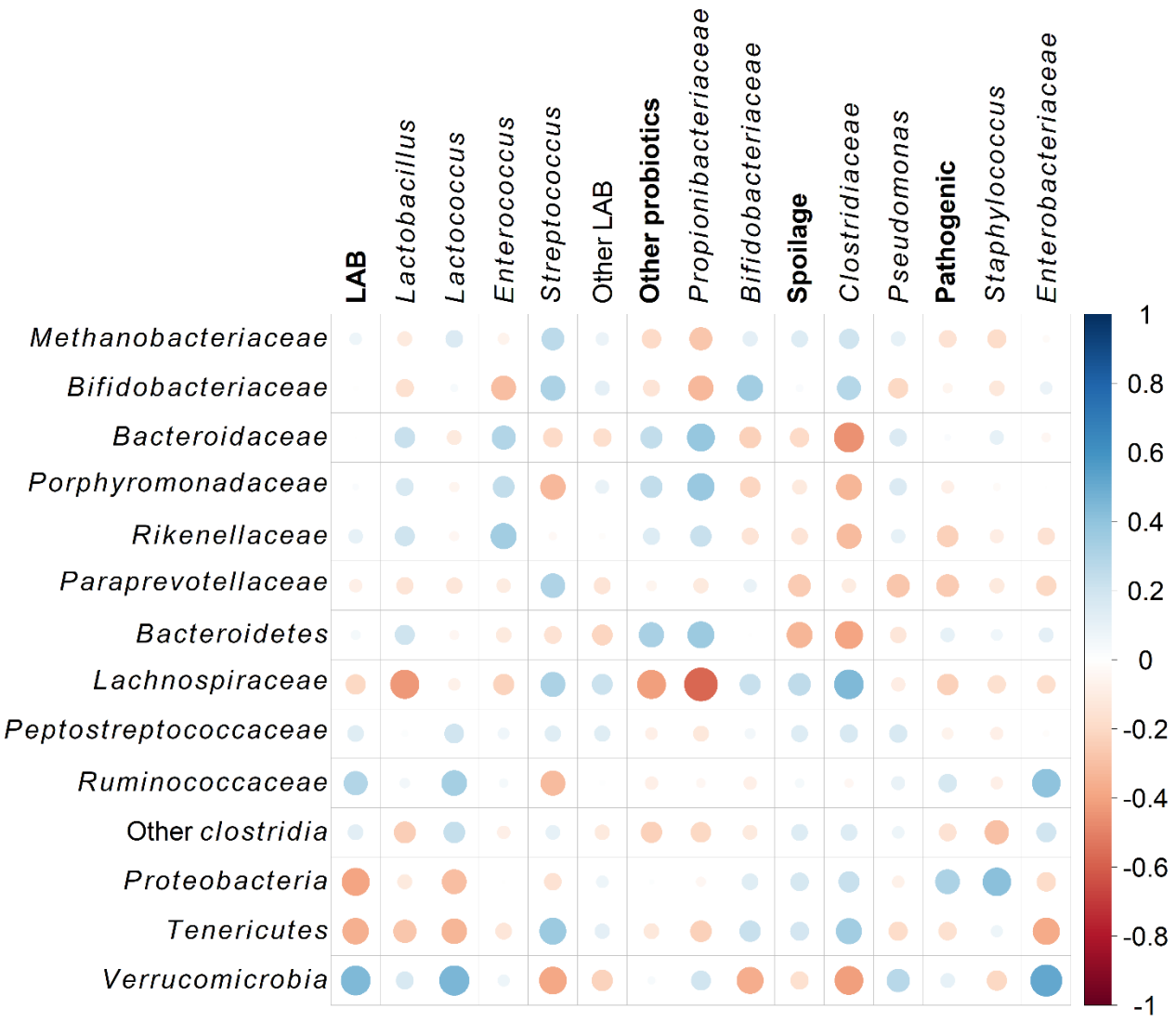
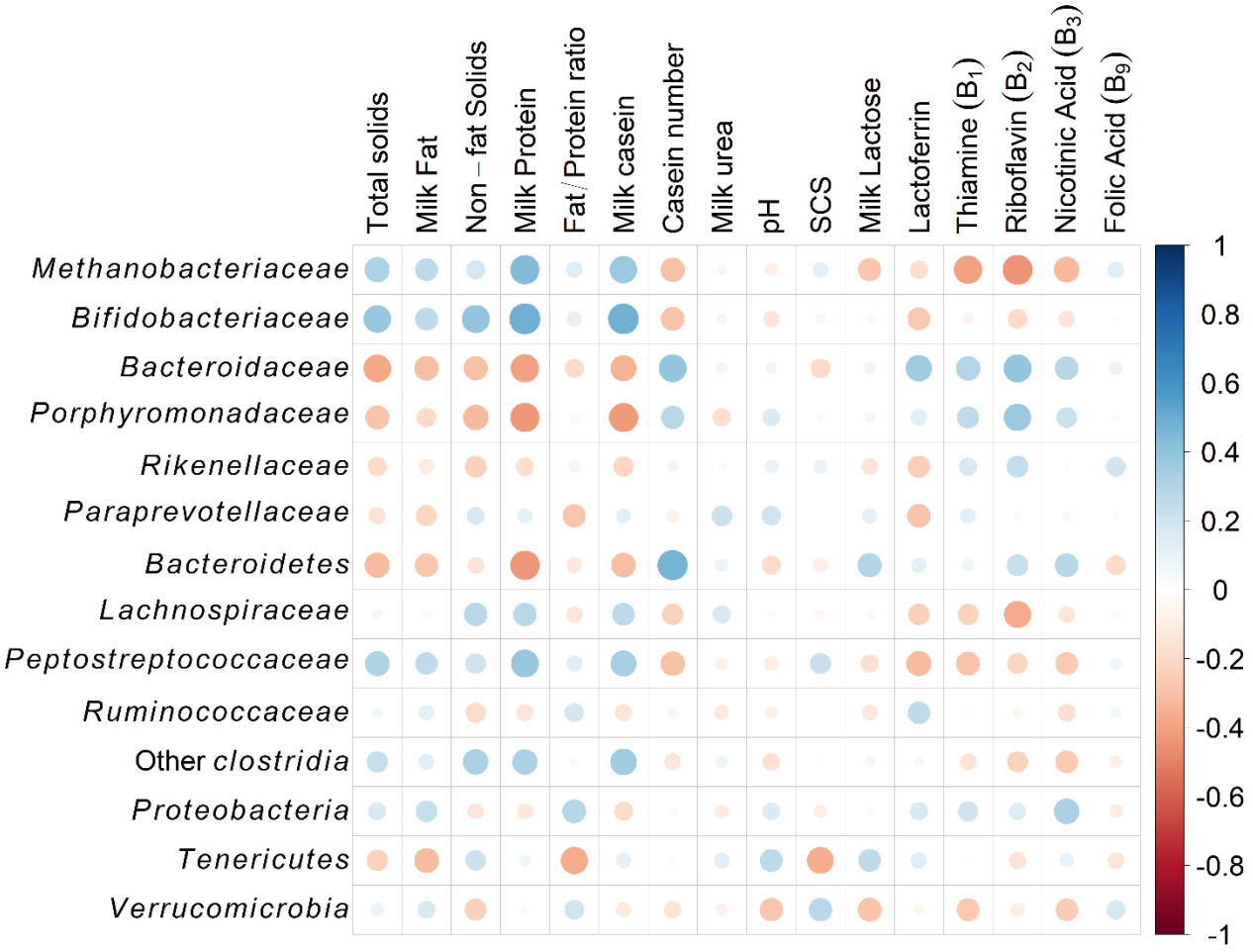


Figure 4. Heat map plot of the correlations between the relative abundances of the intestinal bacterial taxa and milk chemical traits.



GENERAL CONCLUSION AND FUTURE PERSPECTIVES

In the last decades the livestock sector in mountain areas experienced a relevant evolution. The number of traditional, small and low input farms has been drastically decreased, while a trend towards modern farms, oriented to high production and less labour has been remarked. These changes, however, have economic, social and environmental consequences that need to be quantified. Metagenomic analysis of different biological matrices has also gained much interest in recent years to better understand the microbial composition and the resulting functional and phylogenetic aspect. The latter applied to the microbiota of milk, and of rumen and intestinal contents appears to be a powerful tool for studying the complex relationship among farming and feeding system, individual cow, the complexity of the rumen environment, milk quality and cheese-making value, and for human and animal health. In fact, studies generally focus on the microbiome of dairy ruminants, but focusing on how the milk microbiome changes when it becomes a food product, instead of investigating, for example, influences on the context of animal health and physiology. Another aspect about on which previous hypotheses are being re-evaluated is the fact that the milk microbiota is not given only by a mere contamination at the time of secretion, but several authors have described the outcome of pathways by the microorganisms from the intestine to the udder. All this researches are the beginning of new perspectives and discoveries. Scientific knowledge should be assembled to better understand these complex relationships and this Doctoral thesis has been realised within this general framework.

The PhD thesis consists of four main parts. In particular, the first study contribution clearly shows the extent to which the ruminal microbiome is influenced by summer transhumance to summer pasture, with a modest carryover effect once the animals have returned to the lowland permanent farm. Given the complexity of the interactions between microbiome, the trait considered and the environment, the use of the multivariate approach aided the understanding of the strong relationship highlighted between ruminal fermentations and the ruminal microbiota.

The second part discusses the effect of transhumance of lactating cows on temporary summer farms comparing with a control group that remained at the permanent farm throughout the trial, milk metagenomics. It shows that there is a very significant effect of pasture on the positive influence of different useful taxa, and decreases the abundances of spoilage bacteria. Compared with the ruminal samples, no carryover effects were evident in the milk microbiome when the cows return indoors in autumn.

In the third contribution, we confirmed that milk produced during alpine grazing is very different in terms of composition, udder health, B-Vitamin content and microbiological characteristics in respect to the milk obtained indoor by feeding strategy. We consider the single animal samples, but in this study we also showed the difference among farms in the same area.

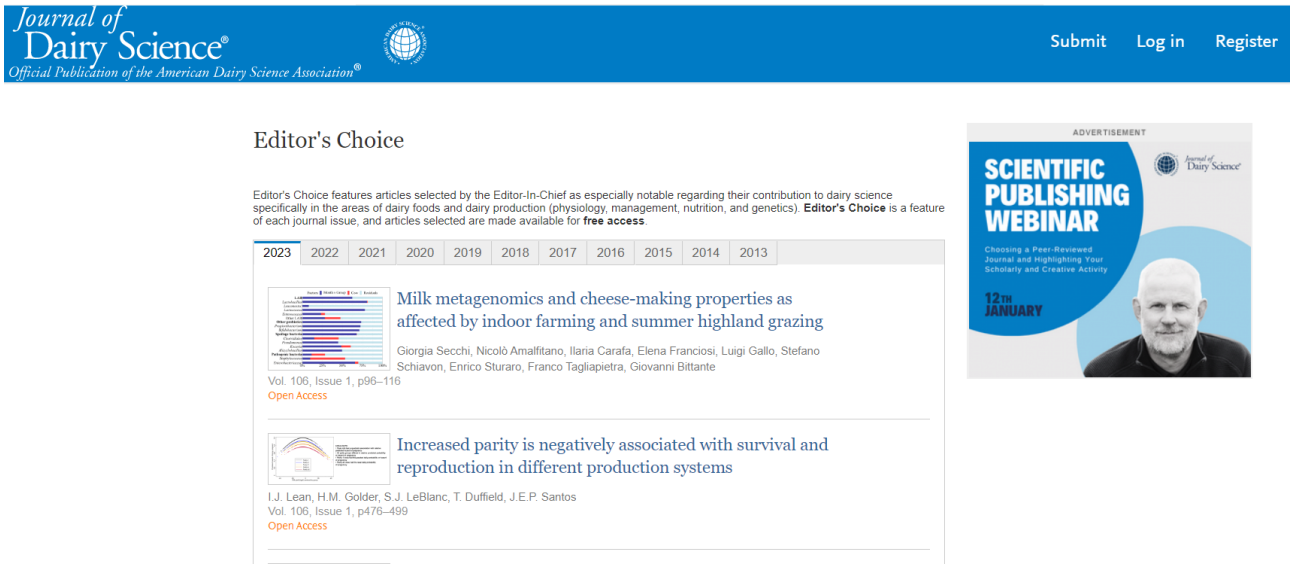
The last contribution highlighted the very complex interrelations among the different intestinal taxa, but also with milk microbiota. This might be of interest for the dairy industry mainly for the lactic acid bacteria, other probiotics, spoilage and pathogenic bacteria. In addition, we found other connection with some milk quality traits. In general, this study has shown interest regarding gut metagenomics, but opened the field for the need for deeper detailed future studies.

The results of this research show a great potential interest in the dairy farming, with particular interest for the summer farming to Alpine highland pasture. Especially it has laid the foundation for expanding future research with this multidisciplinary approach. The most original aspects to be investigated more in the future are, for example, understanding the interactions between the rumen and fecal microbiome, or even more in detail how the ruminal microbiome influencing the udder microbiome and consequently the milk and dairy products derived from it. Lastly, the extension of metagenomic studies to cheese production on one side and the enlargement of microbial populations studied to yeasts seem to be particularly promising.

LIST OF PUBLICATIONS

Secchi, G., N. Amalfitano, I. Carafa, E. Franciosi, L. Gallo, S. Schiavon, E. Sturaro, F. Tagliapietra, and G. Bittante. 2023. Milk metagenomics and cheese-making properties as affected by indoor farming and summer highland grazing. J. Dairy Sci. 106:96–116. doi:10.3168/jds.2022-22449.

Articles Selected as "Editor's Choice" by the Editor-In-Chief of the Journal of Dairy Science



To be submitted in Journal of Dairy Science

- **Secchi, G.**, N. Amalfitano, I. Carafa, E. Franciosi, L. Gallo, S. Schiavon, E. Sturaro, F. Tagliapietra, and G. Bittante. 2023. Rumen fluid metagenomics in relation to milk quality ad affected by indoor farming and summer highland grazing.
- **Secchi, G.**, N. Amalfitano, E. Sturaro, D. Perenzoni, U. Vrhovsek, G. Bittante, and E. Franciosi. 2023. Variation of microbiota, chemical composition and B-vitamins in milk from Alpine pasture and indoor dairy cows.

- **Secchi, G.**, N. Amalfitano, E. Sturaro, E. Franciosi G. Bittante, and. 2023. Variation of intestinal microbiota of dairy cows kept on Alpine pasture or indoor and relationships with microbiota and composition

ATTENDED CONFERENCES

- **G. Secchi**, N. Amalfitano, S. Pegolo, A. Cecchinato, M.L. Dettori, M. Pazzola, G.M. Vacca and G. Bittante. Quantifying and genotyping protein fractions in milk of different goat breeds by RP-HPLC. In: EAAP – 71st Annual Meeting, Virtual Meeting 2020. 1-4 December, 2020 (oral communication)

- **Secchi, G.**, Amalfitano, N., Mancini, A., Bittante, G., Franciosi, E. Milk microbiota investigation during and after summer Alpine transhumance and relation with cheese. In: EAAP 72nd Annual Meeting 2021, Davos, Switzerland. August 30 –September 3, 2021. (oral communication)

- **Giorgia Secchi**, Nicolò Amalfitano, Ilaria Carafa, Kieran Michael Tuohy, Elena Franciosi, Giovanni Bittante. Metagenomics of milk before, during and after summer transhumance to highland pasture in relation to human health and cheese making properties. In: ASPA 24th congress; Padova, September 21-24 2021 (oral communication)

- **G. Secchi**, N. Amalfitano, S. Pegolo, A. Cecchinato, M.L. Dettori, M. Pazzola, G.M. Vacca and G. Bittante. Detailed protein fraction profile of goat milk of six breeds. In: EAAP 73rd Annual Meeting 2022, Porto, Portugal. September 5 – 9 2022. (oral communication)

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