Alternatives for the redesign of beef cattle production: dietary protein, forage intake and feed efficiency

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Universitat de Lleida

TESI DOCTORAL

Alternatives for the redesign of beef cattle production: dietary protein, forage intake and feed efficiency

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CERTIFIQUEN:

Que la present memòria de tesi titulada “ALTERNATIVES FOR THE REDESIGN OF BEEF CATTLE PRODUCTION: DIETARY PROTEIN, FORAGE INTAKE AND FEED EFFICIENCY” elaborada per SANDRA COSTA ROURA, ha estat realitzada sota la seva direcció i reuneix les condicions exigides per optar al Grau de Doctora, pel que consideren que és apta per la seva presentació.

Lleida, a 17 de maig de 2021

Els directors de tesi,

Joaquim Balcells Terés

Daniel Villalba Mata

A can Espluga hi havia tres mainades: en Cisco, la Maria i la Pilar. Dels tres germans, només el noi va anar a l'escola, perquè havia de fer la mili.

La Pilar és la meva iaia.

Aquesta tesi va dedicada a totes les dones a qui, fins no fa tant temps, se'ls va negar la oportunitat d'estudiar.

Perquè fórem, som; perquè som, serem.

Voldria agrair als meus directors, Dani i Quim, i a en Gabi, per haver batallat amb mi durant el llarg procés que representa fer una tesi doctoral.

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The present thesis aimed at identifying and assessing strategies to redesign beef cattle intensive fattening systems in order to increase their profitability and decrease their environmental burden. Moreover, the potential role of ruminal microbiota in the outcome of the tested strategies was evaluated, focusing on their link with ruminal microbiota robustness, defined as the community's ability to cope with disturbances. First, the impact of reducing dietary crude protein from 14 % to 12 % (on a dry matter basis) was assessed, observing that it did not have major impacts on either dry matter intake or animal performance and that it significantly reduced nitrogen waste. Second, the effects of improving forage quality were evaluated and it was seen that replacing barley straw by oats haylage as forage source did not modify dry matter intake, performance or nutrient apparent digestibility, while vetch haylage feeding reduced concentrate intake, slaughter body weight and nutrient apparent digestibility. Both reducing dietary protein and providing high-quality haylage as forage source did increase ruminal microbiota alpha diversity and network complexity, suggesting that these dietary strategies can enhance rumen microbial community robustness. Third, the potential relationship between animals' feed utilization efficiency, and ruminal microbiota composition and associative patterns was explored, revealing increased nutrient apparent digestibility rates and a fermentation pattern oriented towards the production of propionate in high-efficiency animals. Beside, alpha diversity and genera network complexity increased with time in low-efficiency bulls, highlighting a possible trade-off between feed efficiency and ruminal microbiota robustness. In conclusion, moderate restriction in dietary protein supply, increasing forage quality and intake and improving feed efficiency were all proved to be feasible options to redesign intensive beef cattle production. The potential link between ruminal microbiota robustness, animal health and system's economic profitability deserves to be further studied.

En la presente tesis se identificaron y evaluaron estrategias para rediseñar los sistemas de engorde intensivo de terneros, con el objetivo de mejorar sus resultados económicos y disminuir el impacto medioambiental. Además, se determinó su vínculo con la microbiota ruminal, haciendo hincapié en el concepto de robustez, definida como la capacidad de una comunidad de hacer frente a perturbaciones. En primer lugar, se evaluó el impacto de una reducción del contenido de proteína en la dieta del 14 % al 12 % (sobre materia seca): la disminución de la ingestión proteica no penalizó ni la ingestión de materia seca ni el rendimiento productivo de los animales, y redujo la excreción de nitrógeno. En segundo lugar, se estudiaron los efectos de la administración de un forraje de calidad: la substitución de la paja de cebada por henolaje de avena no modificó la ingestión de materia seca ni el rendimiento productivo de los animales, pero la administración de henolaje de veza redujo la ingestión de concentrado y el peso vivo al sacrificio. Tanto la reducción del contenido proteico de la dieta como la administración de un forraje de calidad aumentaron la alfa diversidad y la complejidad de la red de interacciones de la microbiota ruminal, por lo que dichas estrategias podrían mejorar la robustez de la comunidad microbiana. En tercer lugar, se exploró la relación entre la eficiencia alimentaria de los terneros y su microbiota ruminal, observando mayores coeficientes de digestibilidad y un patrón de fermentación orientado a la producción de ácido propiónico en los animales más eficientes. Además, la alfa diversidad y la complejidad de la red de interacciones entre microbios incrementaron con el tiempo solamente en los animales menos eficientes, revelando una posible correlación negativa entre eficiencia alimentaria y robustez de la microbiota ruminal. En conclusión, la restricción moderada de la ingestión proteica, el aumento de la calidad e ingestión de forraje y el incremento de la eficiencia alimentaria son estrategias viables para rediseñar el sistema de engorde intensivo de terneros. En futuros estudios, sería interesante analizar el posible vínculo entre robustez ruminal, salud animal y resultados económicos de la actividad ganadera.

La present tesi ha servit per identificar i avaluar estratègies per tal de redissenyar els sistemes d'engreix intensiu de vedells, amb l'objectiu de millorar-ne els resultats econòmics i disminuir-ne l'impacte mediambiental. A més, se n'ha determinat el vincle amb la microbiota ruminal, fent èmfasi en el concepte de rusticitat, definida com la capacitat d'una comunitat de fer front a pertorbacions. En primer lloc, es va avaluar l'impacte d'una reducció del contingut de proteïna de la dieta del 14 % al 12 % (sobre matèria seca): la disminució de la ingestió proteica no va penalitzar ni la ingestió de matèria seca ni el rendiment productiu dels animals, i va reduir l'excreció de nitrogen. En segon lloc, es van estudiar els efectes de l'administració d'un farratge de qualitat: la substitució de la palla d'ordi per fenolatge de civada no va modificar la ingestió de matèria seca ni el rendiment productiu dels animals, però l'administració de fenolatge de veça va reduir la ingestió de concentrat i el pes viu al sacrifici. Tant la reducció del contingut proteic de la dieta com l'administració d'un farratge de qualitat van augmentar l'alfa diversitat i la complexitat de la xarxa d'interaccions de la microbiota ruminal, així doncs, aquestes estratègies podrien millorar la rusticitat de la comunitat microbiana. En tercer lloc, es va explorar la relació entre l'eficiència alimentària dels vedells i la seva microbiota ruminal: es van observar majors coeficients de digestibilitat i un patró de fermentació orientat cap a la producció d'àcid propiònic en els animals més eficients, manifestant una possible correlació negativa entre l'eficiència alimentària i la rusticitat de la microbiota ruminal. En conclusió, la restricció moderada de la ingestió proteica, l'augment de la qualitat i la ingestió de farratge i l'increment de l'eficiència alimentària són estratègies viables per tal de redissenyar el sistema d'engreix intensiu de vedells. En futurs estudis, seria interessant analitzar el possible vincle entre rusticitat de la microbiota ruminal, salut animal i resultats econòmics de l'activitat ramadera.

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GENERAL DISCUSSION

INTRODUCTION

In the European Union, Spain is the fourth largest beef cattle producer, only overcome by France, Germany and England (Figure 1). The economic importance of beef cattle industry is evident in Spain as it represents the 15 % of the total livestock production; indeed, in 2019, more than 2.5 million bovines were slaughtered, producing 700.000 tons of meat with an economic value of 3000 million euros (MAPA, 2020).

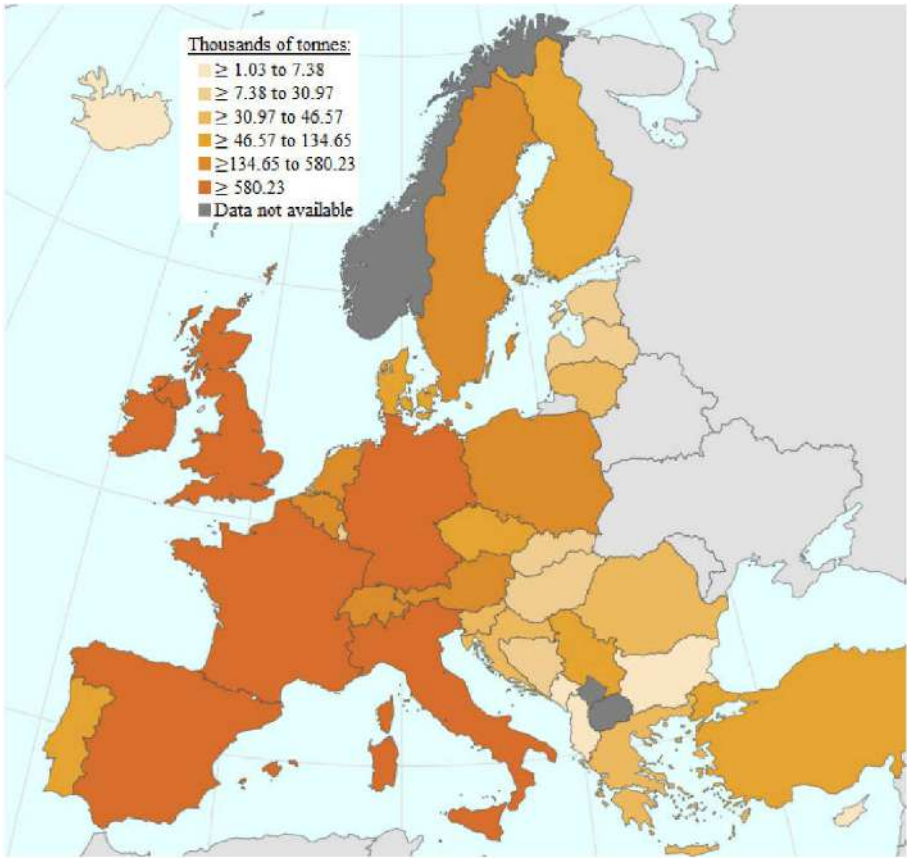

Figure 1 Distribution of beef production on European regions in 2019.

(Eurostat, 2021).

Contrarily to the north-European regions, climate conditions in Spain do not sustain a significant forage production, leading to a lack of high-quality forages at affordable prices. In such conditions, together with an increase in consumer meat demands, beef cattle production has undergone intensification, evolving to an industrial production system in which animals are kept indoors and are fed cereal-based concentrates, with a minimum supply of a low-quality forage (i.e., cereal straw) as fiber source.

Despite the high productivity of intensive livestock rearing systems, they also entail important negative impacts (Dumont et al., 2014).

(i) If animal feed is specifically cultivated for that purpose, livestock production competes directly with human food supply.

(ii) Manure management in industrial systems may hinder the current efforts to reduce greenhouse gas emissions and to preserve biodiversity, water and soil fertility.

(iii) Chemical drugs that are commonly used in intensive systems to limit animal disease and production losses have led to the dumping of pharmaceutical residues and metabolites into the environment and to the spread of antibiotic resistance.

In that sense, (Dumont et al., 2013) have proposed five principles for the redesign of intensive production systems that can aid livestock industry to reduce its indisputable negative effects (Figure 2).

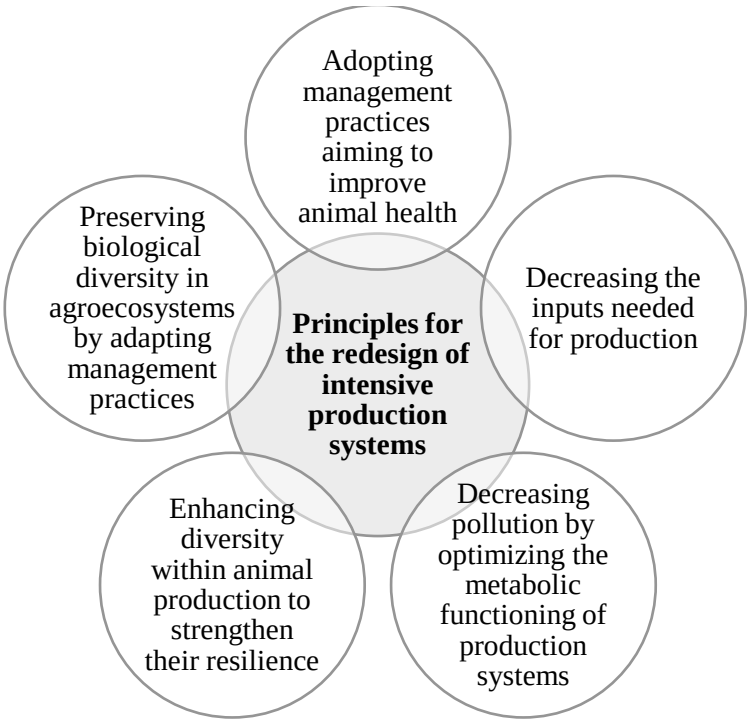

Figure 2 Principles for the redesign of intensive production systems.

Adapted from Dumont et al. (2013).

Regarding those five basic principles, the present thesis has studied three different strategies that can be applied to beef cattle intensive fattening systems in order to reduce their inputs and pollution.

(i) Dietary protein restriction has emerged as a feasible option to save protein feed resources and to reduce nitrogen excretion, as previous studies have shown in a wide range of ruminants including cattle (He et al., 2018), growing goats (Zhang et al., 2020), as well as lambs (Santos et al., 2015).

(ii) Increasing dietary forage-to-concentrate ratio is a well-trust strategy to ensure the supply of a sufficient amount of physically effective fiber, which is highly needed in ruminant diets to stimulate chewing activity, saliva production and rumen motility, decreasing the risk of ruminal pH drop and improving overall animal health (Humer et al., 2018). Moreover, forage cropping does provide some relevant ecosystem services, e.g., they preserve soil carbon, promote soil health, control erosion, reduce invasion of undesirable species, improve water quality, maintain biodiversity, provide wildlife habitat, etc. (Guyader et al., 2016).

(iii) Improving animal feed efficiency, understood as decreasing their residual feed intake (i.e., the difference between observed feed intake and the expected requirement to support both maintenance of body weight and production), can be a useful strategy to increase producer profitability and simultaneously lower the environmental impact of cattle industry. More efficient animals are able to attain a certain level of production with a lower feed intake than less efficient animals, which may be explained by an increased dry matter digestibility due to enhanced intestinal nutrients absorption (Kenny et al., 2018).

Besides the benefits that those three strategies may provide to beef cattle industry, their potential effects on such a key element of ruminant nutrition as it is their ruminal microbiota should not be understated. Despite recent studies have evaluated the effects on rumen

microbial community of a dietary protein restriction (Lv et al., 2020; Zhou et al., 2019), of an increase in forage-to-concentrate ratio (Belanche et al., 2019; Wang et al., 2020) and of an improvement in animal feed efficiency (Delgado et al., 2019; Perea et al., 2017), most of these works characterized ruminal microbiota only in terms of populations richness and evenness (i.e., community alpha diversity), which may not be informative enough. In fact, certain studies have pointed out that, when the immediate environment of a community or the community itself are altered, biotic interactions are the first to be affected and thus can modify the community functioning even before the species disappear (Valiente-Banuet et al., 2015). Moreover, current evidences show that both microbial alpha diversity (Van Elsas et al., 2012; Wittebolle et al., 2009) and network complexity (Karimi et al., 2016; Zappelini et al., 2015) are key drivers of community robustness, understood as its ability to cope with disturbing events. Therefore, assessing how populations interact with each other is of vital importance in order to determine whole community state and functioning (Heleno et al., 2012; Landi et al., 2018).

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OBJECTIVES

General goal

The main objective of this thesis was to identify and assess strategies to redesign beef cattle intensive fattening systems in order to increase their profitability and decrease their environmental impact. Moreover, we focused on evaluating the potential role of ruminal microbiota in the outcome of the tested strategies.

Specific goals

(1) To assess the impact of reducing dietary crude protein from 14 % to 12 % (on a dry matter basis) on animal performance and ruminal microbiota composition and associative patterns.

(2) To evaluate the effects of improving forage quality on animal's intake, performance and ruminal microbiota composition and associative patterns.

(3) To determine the potential relationship between animals' feed utilization efficiency, and ruminal microbiota composition and associative patterns.

This thesis has been accomplished in Animal Science Department at University of Lleida. It has been financed by Ministerio de Ciencia, Innovación y Universidades (FPU 2016/03761) and developed in the frame of two research projects funded by the European Union H2020 program (GenTORE project nº727213) and Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RTA-14-038-C02).

CHAPTER I

Ruminal microbiota robustness: unwrapping the black box

Abstract

Despite its central role in ruminants' nutrition, little is known about ruminal microbiota robustness, understood as its ability to cope with disturbances. The aim of the present review is to offer a comprehensive description of microbial robustness, as well as its potential drivers. First, we define the concept of disturbance (any discrete event that disrupts the structure of a community, and changes either the resource availability or the physical environment). Second, we talk about community temporal stability (the ability to keep its structure constant over time, when no disturbances occur), resistance (the ability to remain unchanged in the face of a disturbance), resilience (the ability to return to its initial structure following a disturbance) and functional redundancy (the ability to recover its initial function despite compositional changes), all considered to be key properties of robust microbial communities. Then, we overview the main biological features attributed to community robustness, which are microbial diversity and network complexity, and the current methodology to properly assess them. Finally, we approach the impact on ruminal microbiota of the most common nutrition practices in ruminants' production (e.g., diet and feeding management, plant secondary compounds, antibiotics and alternative control agents), giving a practical insight on what must and must not be done in order to enhance microbial robustness in rumen.

1. Introduction

Rumen microbial fermentation is of central importance in ruminants' nutrition as it provides energy, in the form of volatile fatty acids, and protein, in the form of microbial cell protein, to meet animal requirements (Hungate, 1966).

The study of ruminal microbiota started with the development of culture techniques suitable for strictly anaerobic bacteria; however, microbiologists soon realized that the cultivable isolates constituted only 8 % of the total bacterial community in rumen (Weimer, 2015). Contrarily, the onset of high throughput sequencing techniques has provided an opportunity to fully appreciate the wide variety of microbial species inhabiting the rumen (Li et al., 2016; Myer et al., 2016). Over the last years, literature has described that, although a core microbiome across ruminant hosts does exist (Jami and Mizrahi, 2012; Petri et al., 2013), ruminal microbiota composition and function can be modulated by a broad range of factors, with an special emphasis on those related to feeding practices, e.g. early life feeding management (Saro et al., 2018), high-concentrate acidogenic diets (Costa-Roura et al., 2020a; Plaizier et al., 2017), dietary protein restriction (Costa-Roura et al., 2020b; He et al., 2018), antibiotic treatments (Ji et al., 2018), or dietary additives (Tian et al., 2019; Zhang et al., 2020). However, the impact of such dietary factors on ruminal microbiota robustness, understood as the ability of a microbial community to cope with disturbances, remains largely unknown.

The aim of the present review is to define the concept of robustness in microbial communities and to describe the current methodology to assess it. Moreover, the impact of the most common feeding practices in ruminant nutrition on their microbiota robustness is reviewed and assessed.

2. Defining ecosystem disturbance

Disturbance is a key component of all ecosystems, affecting every level of biological organization (Fraterrigo and Rusak, 2008).

The concept of *disturbance* has received several definitions over the last few decades (Svensson et al., 2007): Grime (1977) defined it as a partial or total destruction of biomass,

and Sousa (Sousa, 1984) extended this definition by adding that disturbance also creates opportunities for new individuals to become established. Pickett and White (1985) proposed a more general definition, stating that disturbance could be defined as any relatively discrete event that disrupts the structure of an ecosystem, community, or population, and changes either the resource availability or the physical environment. Therefore, disturbances are causal events that can either alter the immediate environment and have potential impact upon a community, or directly alter it (Shade et al., 2012); depending on the magnitude of the disturbance, organisms may be killed or displaced, consumable resources (e.g., living space and nutrients) may be depleted, and habitat structure may be degraded or destroyed (Lake, 2000).

Beside their destabilizing effects, disturbances are believed to have a crucial role in maintaining community robustness: the *intermediate disturbance hypothesis* (Connell, 1978) predicts maximum diversity at intermediate levels of disturbance frequency (Figure 3). Given that disturbances can differ in their origin (biotic vs abiotic, natural vs anthropogenic) and can occur at various spatial and temporal scales, extensive research is needed to accurately classify them (Table 1).

Microorganisms exist across the most diverse environments and form fundamental bases of every ecosystem; for this reason, recent advances in the field of microbial ecology have outlined the importance of studying those disturbances that affect them (Shade et al., 2012). Improving our understanding of microbial communities' robustness will be crucial to better understand and predict how microbes behave in disturbed environments.

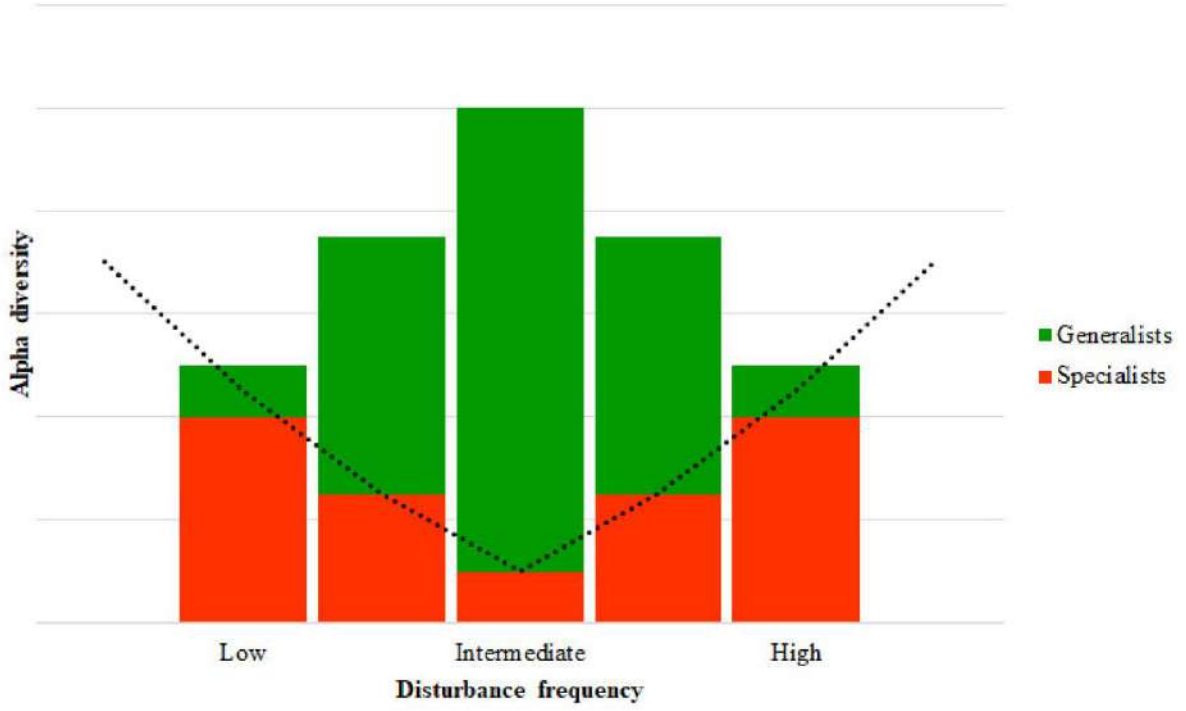

Figure 3 Conceptual representation of the intermediate disturbance hypothesis. *The relationship between alpha diversity and disturbance frequency can be understood as follows: at low frequency of disturbance, strong competitors exclude weaker species and communities are dominated by few members; intermediate frequency of disturbance increase rates of mortality, giving an opportunity to inferior species to proliferate; finally, high frequency of disturbance may cause excessive rates of mortality and slow-growth species will tend to decrease and disappear. Such trade-off between alpha diversity and rate of disturbance is probably rooted in the processes that drive microbial community assembly. At intermediate rates of disturbance, environmental conditions are unstable and community assembly is driven by stochastic processes: all species have equal fitness and chances to succeed; thus, specialized traits are not advantageous to taxa and generalists proliferate, increasing alpha diversity. Contrarily, at low and high rates of disturbance, environmental conditions are recurrent and deterministic processes drive community assembly: the more adapted species are selected, so specialists dominate the community and alpha diversity does decrease. Adapted from Santillan et al. (2019), Sriswasdi et al. (2017) and Svensson et al. (2007).*

Table 1 Disturbance classification, temporal pattern and community's response to them.

Adapted from Bengtsson (2002), Lake (2000) and Shade et al. (2012).

Disturbance classification	Temporal pattern	Community response to disturbance	Examples
Pulses	Short term and sharply delineated disturbances.	Relatively common natural phenomena. Most communities affected by such type of disturbances are already adapted to survive to them or to recolonize the damaged areas.	Floods, fires, windthrows.
Presses	Disturbances that may arise sharply and then reach a constant level that is maintained, evolving to become a chronic stress.	Usually of anthropogenic origin. Most species have not yet been able to respond evolutionary to them and, after presses, communities can either reach an alternative stable state or continuously decrease.	Intensive grazing systems, monoculture agriculture.
Ramps	Disturbance's strength steadily increases or declines over time, therefore, they may either not have an endpoint or reach an asymptote after an extended period.	Environmental conditions get progressively worse and, consequently, communities' response to ramps is also gradual and they rarely become stable again.	Droughts, spread of exotic organisms.
Large infrequent disturbances	Uncommon events with an extremely low frequency.	Most communities are unlikely to be adapted to them.	Hurricanes, volcano eruptions.

3. Defining microbial community robustness

The concept of robustness is widely used in the scientific literature, although there is considerable confusion about its meaning (Levin and Lubchenco, 2008). In general, *robustness* is used as a comprehensive term that describes the extent to which a microbial community exhibits: (i) temporal stability, (ii) resistance, (iii) resilience and (iv) functional redundancy (Robinson et al., 2010).

(i) *Temporal stability* is the ability of a community to keep its structure constant over time, when no disturbances occur (Tilman, 1999).

(ii) *Resistance* is the ability of a community to remain unchanged in the face of a disturbance and, presumably, it is rooted in the high degree of metabolic flexibility and physiological tolerance of certain microbial populations to changing environmental conditions (Allison and Martiny, 2009).

(iii) *Resilience* is the ability of a community to return to its initial structure following a disturbance. Resilience is thought to be a common feature of microbial communities due to some of the intrinsic characteristics of their members: (i) many microbes have fast growth rates so, if their abundance is suppressed by a disturbance, they have the potential to recover quickly; (ii) many microbes have a high degree of physiological flexibility so, even if their relative abundances decrease initially, these taxa may acclimate to the new environmental conditions over time and eventually return to their original abundance; and finally, (iii) the rapid genetic evolution (through mutations and horizontal gene exchange) can also allow microbial taxa to adapt to new environmental conditions and recover from disturbances (Allison and Martiny, 2009).

(iv) *Functional redundancy* is the ability of a community to recover its initial function despite compositional changes (Moya and Ferrer, 2016). There are two main reasons why changes in microbial composition may not affect ecosystem process rates: (i) the

new community might contain taxa that are functionally redundant with the taxa in the old community; and (ii) taxa in the new community may function differently but result in the same process rate when combined at the community level (Allison and Martiny, 2009).

When a microbial community is not robust enough to cope with a certain disturbance, it may evolve to an alternative equilibrium or alternative stable state: literature has for long supported the idea that communities can be found in one of several possible alternative stable states, which is usually referred to as *stability landscape* (Holling, 1973). When alternative stable states occur, state variables that characterize a community (i.e., species composition or function) can persist in one of a number of different possible configurations that constitute different equilibrium points that are locally stable (Beisner et al., 2003). In 2011, the stability landscape concept was first applied in the field of human gut microbiota leading to the definition of the term *enterotype*, which refers to the stratification of gut microbiota into different clusters of microbes of similar nature that can be associated with specific host phenotypes (Arumugam et al., 2011). Few years later, this concept was extended to ruminal microbiota and referred to as *ruminotype* (Kittelmann et al., 2014).

Stability landscape concept is gaining interest in the study of ruminal microbiota, as the existence of alternative stable states or ruminotypes may provide explanation to the immense variability observed within and among individual microbial communities (Shade et al., 2012). Different factors such as environment (Shaani et al., 2018), early-life events (Abecia et al., 2014), diet (Tapio et al., 2017), host genetics (F. Li et al., 2019), or even stochastic forces (Furman et al., 2020) can drive ruminal microbiota assembly and ultimately determine the dominance of one alternative state over the others. Community alternative stable states can be differentiated by the enrichment of certain taxonomic or functional groups and not necessarily imply complete exclusion between them: different taxa and function rates coexist within the

rumen microbiota but the balance among them shifts in each stable state. In that sense, as microbial community states may have diverse functionalities, they could also be connected to many host attributes (Moraïs and Mizrahi, 2019); for example, in the case of ruminal microbiota, available literature agrees in the existence of three ruminotypes that are associated with differences in animals' methane yield (Kittelmann et al., 2014; Ramayo-Caldas et al., 2020).

4. Biological features attributed to robust microbial communities

Stability of microbial communities in the face of disturbances is influenced by individual, population and community biological features that contribute to overall community robustness. Individuals that are able to overcome a specific disturbance can increase persistence of their population and eventually promote a more stable community. In the present review, only those community properties that enhance its robustness are discussed; however, for more information about individual and population biological characteristics inherent to robust communities, readers are related to Shade et al. (2012).

4.1 Microbial diversity and its temporal succession

The first evidence of the positive correlation between ecosystem diversity and stability was obtained in grassland field communities: authors gathered information about species richness and community biomass through time and found that diversity within an ecosystem tends to exhibit a positive correlation with plant community stability, as it tends to decrease the coefficient of variability in community biomass (Tilman and Downing, 1994).

The positive effect of diversity on community robustness was later demonstrated in microbial communities. Van Elsas et al. (2012) reported a negative correlation between soil microbial richness and survival of invading species, demonstrating that community richness is crucial

for preventing the spread of bacterial invaders. Likewise, Wittebolle et al. (2009) used microbial microcosms to study denitrifying bacteria and showed that any community should have an even distribution among its members if it is to respond rapidly to disturbances; contrarily, uneven communities depend too strongly on their dominant species and, in consequence, their stability is endangered by environmental fluctuations.

Community diversity, in terms of both species' richness and evenness, enhances ecosystem's robustness probably due to its buffering effect against disturbances, phenomenon referred to as *Insurance Hypothesis* (Yachi and Loreau, 1999): the ability of a community to buffer disturbances, loss in species and species invasions is dependent on (i) the functional redundancy of its members, and (ii) the ability of its members to respond differentially to disturbances. On the one hand, increasing community richness increases the odds for functionally redundant and disturbance-resistant species exist and, on the other hand, increasing community evenness increases the odds that such species can proliferate when disturbances occur (McCann, 2000; Shade et al., 2012; Wittebolle et al., 2009).

When trying to predict community robustness based on its species diversity, it is important to be aware of the possibility that community's composition may be sensitive to time, phenomenon known as the *species-time relationship* (Adler and Lauenroth, 2003; Preston, 1960) (Figure 4). Such patterns of temporal succession of species composition and abundance have been observed, not only in animal and plant communities (Korhonen et al., 2010; Swenson et al., 2012), but also in microbial communities (Shade et al., 2013). Gut microbiota constitutes a clear example of a microbial community in which consistent temporal variability takes place, as previous studies have evidenced in a wide range of hosts including humans (Koenig et al., 2011), ruminants (Jami et al., 2013), crustaceans (Xiong et al., 2019), as well as insects (Anderson et al., 2018).

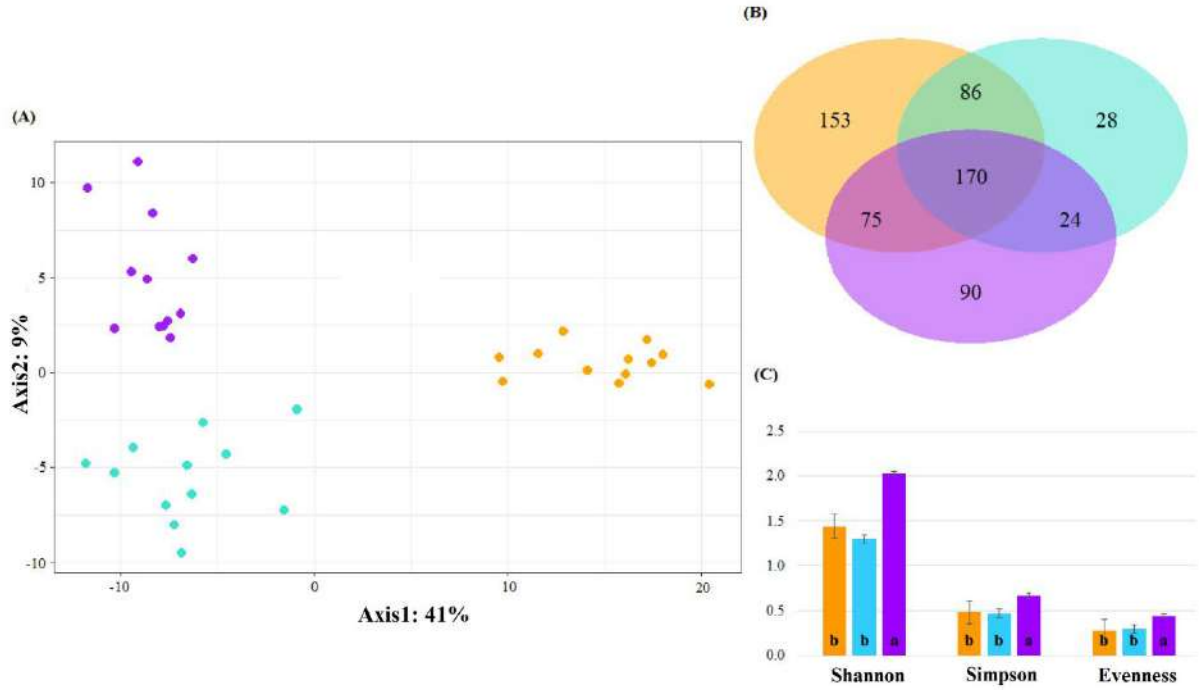

Figure 4 Visualization of species-time relationship in the ruminal microbiota.

Obtained in 3 months old (orange), 5 months old (turquoise) and 9 months old (purple) fattening bulls (own data). (A) Graphical representation of a multivariate analysis (PLS-DA) on bacterial and archaeal taxa in ruminal fluid. Each point represents a different animal and a greater distance between two points infers a higher dissimilarity between them. Samples are clearly clustered by age group, highlighting the existence of shifts in microbiota composition related with time. PERMANOVA test results confirmed that the foreseen graphical differences were significant (P<0.001). (B) Venn diagram showing the numbers of bacterial and archaeal taxa that are shared or unshared by age groups, depending on overlaps. Even though a core ruminal microbiota exists across age groups, there are unique taxa that changes with time. (C) Histogram showing the evolution of microbial alpha diversity with time; the age-dependent increase in ruminal microbiota alpha diversity has already been observed elsewhere (Dill-McFarland et al., 2019; Jami et al., 2013).

4.2 Microbial network complexity

Despite its generalized use, microbial diversity may not necessarily be informative or sensitive enough as an indicator of community state in response to disturbances (Karimi et al., 2017). In fact, when a disturbance occurs, biotic interactions are the first to be affected and thus can alter the community functioning even before the species disappear (Valiente-Banuet et al., 2015). For this reason, some studies have outlined the importance of studying how populations interact with each other in order to determine whole community state and functioning (Heleno et al., 2012; Landi et al., 2018).

It is widely known that individuals establish symbiotic interactions that exist in a continuum from beneficial to antagonistic associations: mutualism is an association in which both individuals derive benefit from one another; commensalism includes relationships in which one partner derives benefit from the other and the other partner neither is harmed nor benefits from the association; and parasitism is an association where one partner, the parasite, lives on or in another partner, the host, and causes harm, as the parasite obtains all or part of their necessary nutrients at the expense of the host. Other antagonistic interactions include competition for a common resource and predation of one individual upon another (Little et al., 2008). All these relationships existing between individuals in a given community can be represented in networks, providing a single holistic vision of communities that integrates both direct and indirect effects of disturbances on diversity, taxonomic composition and relationships between populations (Karimi et al., 2017).

For long, it has been hypothesized that network complexity may affect community robustness (MacArthur, 1955) and, more recently, some studies have shown that, when an environmental disturbance occurs, microbial network complexity is reduced (Karimi et al., 2016; Zappelini et al., 2015). In relation to that, Karimi et al. (2017) and Tylianakis et al. (2010) have

reviewed certain attributes of network structure that may be useful to evaluate community robustness.

(i) The *number of nodes* is the number of connected taxa within the network.

(ii) The *number of edges* refers to the amount of links established between nodes and *connectance* is the number of potential links that are actually realized. A greater number of edges can stabilize the rate of ecosystem processes through time under fluctuating environmental conditions thanks to the ecological redundancy of links, phenomenon that can also be explained by the already mentioned Insurance Hypothesis.

(iii) *Nestedness* is the tendency of nodes to interact with subsets of the interaction partners of better-connected nodes, in other words, a network is considered to be nested when the species interacting with specialists are a proper subset of the species interacting with generalists. Nestedness is an important feature of robust communities in that specialists are usually the first species to go extinct from a network but, if it is nested, the remaining species will still have generalists to interact with.

(iv) *Pattern of interaction strength* is also believed to affect community stability, more specifically, the presence of many weak links within a network serves to limit energy flow in a potentially strong consumer-resource interaction and, therefore, to inhibit runaway consumption that destabilizes the community dynamics (McCann, 2000).

(v) *Modularity* is the measure in which networks are compartmentalized into subsets in which species interact frequently with one another, but little with other species outside the compartment. Modularity increases community robustness because disturbances will spread more slowly through a modular network, so compartmentalized communities will deteriorate more gradually than randomly connected ones (Wilmers, 2007).

(vi) *Node degree* is the number of interactions established per node and its distribution is an important parameter determining community robustness: if interactions are not evenly distributed over nodes within the network and a few well-connected species concentrates most of the existing links, such community will be robust to random loss of nodes but very fragile to the elimination of the most connected ones (Albert and Barabási, 2000).

5. Measuring microbial community robustness

In the present section, the methods for alpha diversity estimation and network inference that are most commonly applied to high throughput sequencing data are briefly explained. For more information about the analysis pipeline that is generally followed in microbial ecology studies, readers are related to the review by Di Bella et al. (2013).

5.1 Alpha diversity estimation

Alpha diversity measures the variability of taxa in a particular community or, in other words, the diversity of species within a sample (Whittaker, 1960).

The simplest way to assess community alpha diversity is to list the species present. *Species richness* is the number of species on that list and is often used as the first pass estimate of alpha diversity for a community.

Despite its simplicity, species richness estimation presents some limitations. On the one hand, species richness is strongly dependent on sample size: when comparing samples of different size, the potentially observed differences in species richness may just be consequence of one community being sampled more intensively than the other. To overcome such problem, *rarefaction techniques* are commonly applied to approximate the number of species expected in a random sample of individuals taken from a sample collection, enabling to compare

observed species richness among communities that have been unequally sampled (Hughes et al., 2001; Kim et al., 2017). On the other hand, species richness is not informative about *species evenness*, i.e., the relative abundance of the different species making up a community. In that sense, a wide range of diversity indices that include both species richness and evenness information have been developed and, amongst them, Shannon-Weaver index and Simpson index are the most often used. *Shannon-Weaver index* (Shannon and Weaver, 1949) is based on the weighted geometric mean of the proportional abundances of the species and it measures the entropy of a system: if every individual that constitutes a community belongs to a different species, such community would have minimal redundancy and maximum entropy (Bent and Forney, 2008). *Simpson index* (Simpson, 1949) gives the probability of any two individuals drawn at random from an infinitely large community belonging to different species.

5.2 Network inference

The inference of interaction networks has enabled a movement from reductionist approaches, focusing on individuals in isolation, to more holistic approaches, focusing on interactions among members of the community (Layeghifard et al., 2017).

Network inference is the prediction of association networks from presence/absence or abundance data and it is based on the simple principle that it is possible to predict taxa relationships under the premise that strongly nonrandom distribution patterns are mostly due to ecological reasons. In that sense, when two taxa co-occur or show a similar abundance pattern over multiple samples, a positive relationship is assumed; contrarily, when they show mutual exclusion, a negative correlation is supposed (Faust and Raes, 2012). Even though the idea behind network inference may seem straightforward, the mathematical procedure to put it into practice presents some issues, mainly because the large number of possible interactions

between taxa exceeds by far the number of independent samples from which the interactions can be inferred (De Smet and Marchal, 2010). Currently, there is a wide range of network inference tools available and choosing the most appropriate one is not obvious; along the following lines, the most common methods for network modelling are summarized (Faust and Raes, 2012; Jiang et al., 2019; Layeghifard et al., 2017).

Network inference methods can be classified into two groups: (i) those that can predict associations between two taxa (i.e., pairwise associations) and (ii) those that can predict more complex relationships.

Pairwise relationships are most often inferred using either dissimilarity-based methods or correlation-based methods. *Dissimilarity-based methods* consist of a few steps: first, a distance-dissimilarity matrix is built using any distance metric (e.g., Jaccard index or Bray-Curtis index), then, the significance of pairwise dissimilarity scores is evaluated through a permutation test, and all significant pairwise connections are aggregated to construct a network. For its part, *correlation-based methods* aim to detect significant pairwise interactions between taxa using a correlation coefficient, such as Pearson (parametric) or Spearman (non-parametric).

Complex relationships, in which one taxon is influenced by multiple other taxa, are usually predicted using regression-based methods or probabilistic graphical models. *Regression-based methods* use regression analysis to infer the abundance of one taxon from the combined abundance of the other taxa. In *probabilistic graphical models*, the conditional dependences between random variables are depicted as a graph in which the edges correspond to direct probabilistic interactions between the variables.

5.3 Dealing with compositionality of microbial abundance datasets

Microbial abundance data are considered to be compositional, as they offer quantitative descriptions of the parts of some whole, conveying relative information: the value of each taxon is not informative by itself and the relevant information is contained in the ratios between them. Such microbial abundance datasets present some relevant characteristics that may cause certain problems in their downstream analysis (Rivera Pinto, 2018): (i) the total number of counts per sample is highly variable along samples; (ii) the total number of counts per sample is constrained by the maximum number of sequence reads of the DNA sequencer; and (iii) abundance matrices typically contain a large proportion of zeros.

If the compositional nature of microbial abundance data is ignored, three important issues may arise: (i) spurious correlations, (ii) subcompositional incoherence and (iii) the increase of type I error (Rivera Pinto, 2018). To avoid such pitfalls, some authors have put forward statistical methods to analyze microbial compositional data more consistently; for a detailed description of such alternative methods readers are related to the reviews by Calle (2019) and Gloor et al. (2017).

6. Practical insights on enhancing ruminal microbiota robustness: dos and don’ts

After reviewing the state-of-the-art literature, this section aims to provide some of the current strategies to enhance ruminal microbiota robustness, mainly focused on: (i) increasing microbial alpha biodiversity and (ii) increasing microbial network complexity. Along the following lines, authors will try to disentangle the effects of the most common feeding practices in ruminant production on rumen microbial community, with an eye on their positive or negative impact on microbiota robustness.

6.1 Diet and feeding management

Diet is one of the major factors shaping rumen microbial community and practices like diet formulation, feed intake level as well as early life feeding management can potentially modify ruminal microbiota and its robustness to face disturbances.

The influence of dietary protein or energy limitation on ruminal microbiota robustness is not clear-cut. Regarding to their impact on microbial alpha diversity, the available literature is far from being conclusive: some studies reported no changes at all, while other studies described increased diversity levels due to both nutrients' restriction (Table 2). However, when it comes to network complexity, the consulted works do agree: Costa-Roura et al. (2020b) observed that dietary protein reduction increased network complexity, in terms of number of nodes, edges and betweenness centrality; similarly, Park et al. (2020) reported that the number of nodes and edges exclusive to one treatment was higher when dietary energy was limited.

Improving rumen microbial robustness through increasing dietary forage-to-concentrate ratio seems to be a reliable option: most of the revised studies agree in the fact that including a higher proportion of forage in ruminants diet have positive effects on both ruminal microbiota alpha-diversity (Table 2) and network complexity (Belanche et al., 2019a; Costa-Roura et al., 2020a).

The potential impact of feed intake level on ruminal community robustness must be revised with caution. As it can be seen in Table 2, different levels of voluntary feed intake did not seem to have an impact on microbial alpha diversity; on the contrary, starvation periods caused a dramatic decrease of both microbial richness and evenness. In that sense, microbial alpha diversity levels have been observed to follow a quadratic progression, reporting the highest values in animals fed the 96% of their nutrient requirements (Wang et al., 2017).

Early life events may modulate the microbial community structure and functioning, with lasting effects into adult ruminant life (Yáñez-Ruiz et al., 2015); in that sense, it seems

reasonable that early life feeding management may also have an impact on rumen microbial robustness. It has been observed that natural rearing allows the establishment of a more diverse ruminal microbiota than artificial milk feeding (Table 2). Interestingly, it has also been observed that the supplementation with solid feed during the milk feeding period (either natural or based on milk replacer) impairs microbial alpha diversity in the undeveloped rumen (Table 2). Considering that supplementation with solid feed has also been reported to increase microbial community network complexity (Lv et al., 2019), further studies are needed to shed light into that question.

Table 2 Effects of different feeding practices on ruminal microbial alpha diversity.

Feeding practice	Animals (age, body weight)	Treatments[1]	Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)	Reference
Dietary protein limitation	Holstein bulls (118 d, 165 kg)	CTR: 140 g CP/kg DM LP: 120 g CP/kg DM	No effects on richness. Shannon and Simpson indexes reached higher values at an earlier age in LP than in CTR.	Costa-Roura et al. (2020b)
	Chinese Hu lambs (60 d, 15 kg)	HP: 157 g CP/kg DM LP: 118 g CP/kg DM	Richness and Simpson index tended to be lower in LP. No effects on Shannon index.	X. Lv et al. (2020)
	Simmental steers (350 kg)	HCP: 136 g CP/kg DM LCP: 111 g CP/kg DM	Increased richness in LP. No effects on Shannon index.	Zhou et al. (2019)
Dietary energy limitation	Holstein cows (primiparous, 590 kg)	HE: 1.72 Mcal/kg DM LE: 1.52 Mcal/kg DM	No effects on richness, Shannon or Simpson indexes.	Park et al. (2020)
	Chinese Hu lambs (60 d, 15 kg)	HE: 2.61 Mcal/kg DM LE: 2.07 Mcal/kg DM	Increased richness, Shannon and Simpson indexes in LE.	X. Lv et al. (2020)
	Holstein bulls (17 m, 493 kg)	HE: 2.79 Mcal/kg DM ME: 2.61 Mcal/kg DM LE: 2.42 Mcal/kg DM	Increased richness and Shannon index in LE.	H. Wang et al. (2019)
	Holstein heifers (7 m, 268 kg)	HE: 2.60 Mcal/kg DM ME: 2.41 Mcal/kg DM LE: 2.23 Mcal/kg DM	No effects on bacterial richness or Shannon index.	Bi et al. (2018)

Table 2 Continued.

Feeding practice	Animals (age, body weight)	Treatments[1]	Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)	Reference
Increasing F/C ratio	Aberdale ewes (69 kg)	CON: hay *ad libitum* plus concentrate supplementation PAS: pasture grazing	Increased bacterial richness and Shannon index in PAS. Increased archaeal richness in PAS, but no effects on Shannon or Simpson indexes.	Belanche et al. (2019a)
	Red deers (3 yr, 150 kg)	A1: 80 F/20 C A2: 70 F/30 C A3: 60 F/40 C A4: 50 F/50 C	Increased richness, Shannon and Simpson indexes in A1 and A2.	Qian et al. (2018)
	Holstein cows (3 yr, 563 kg)	HF: 70 F/30 C HC: 30 F/70 C	Increased richness, Shannon and Simpson indexes in HF.	Wang et al. (2020)
	Holstein heifers (8-10 m, 263 kg)	C20: 80 F/20 C C40: 60 F/40 C C60: 40 F/60 C C80: 20 F/80 C	Increased bacterial and archaeal richness in response to increasing F/C ratio. Increased bacterial Shannon index in response to increasing F/C ratio. No effects on archaeal Shannon index.	Zhang et al. (2017)
	Holstein bulls (119 d, 164 kg)	CTR: 87 F/13 C, barley straw as forage source OATS: 87 F/13 C, oats haylage as forage source VETCH: 84 F/16 C, vetch haylage as forage source	Increased richness, Shannon and Simpson indexes in response to improving forage quality.	Costa-Roura et al. (2020a)

Table 2 Continued.

Feeding practice	**Animals (age, body weight)**	**Treatments[1]**	**Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)**	**Reference**
Increasing F/C ratio	Crossbreed sheep (2 yr, 88 kg)	H: 90 F/10 C M: 70 F/30 C L: 50 F/50 C	Increased richness in response to increasing F/C ratio. No effects on Shannon index.	R. Li et al. (2019)
	Castrated crossbreed goats (3 yr, 30 kg)	HAY: All-forage diet. HG: 35 F/65 C	Increased richness and Shannon index in HAY. Increased Simpson index in HG.	Zhang et al. (2019)
	Castrated crossbreed goats (1 yr, 25 kg)	AF: All-forage diet. CF: Complete feed diet.	No effects on richness or Shannon index.	Liu et al. (2017)
	Tibetan sheep (1 yr, 21 kg)	HS1: All-forage diet. HS2: 85 F/15 C HS3: 70 F/30 C HS4: 55 F/45 C HS5:40 F/60 C	Increased richness and Shannon index in HS1, HS2 and HS3 respect to HS4 and HS5.	Liu et al. (2019)

Table 2 Continued.

Feeding practice	**Animals (age, body weight)**	**Treatments[1]**	**Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)**	**Reference**
Feed intake level	Tan sheep (135 d, 36 kg)	I: 84% nutrient requirements II: 96% nutrient requirements III: 108% nutrient requirements IV: 120% nutrient requirements	Richness and Shannon index followed a quadratic progression with the highest values in treatment II. No effects on Simpson index.	Wang et al. (2017)
	Holstein cows (multiparous, 584 kg)	LFI: 21.19 kg DM/day VFI MFI: 23.30 kg DM/day VFI HFI: 25.76 kg DM/day VFI	No effects on richness, Shannon or Simpson indexes.	Li et al. (2020)
	Jiulong yaks (3 yr, 238 kg)	NFP: normal feeding period SP: starvation period RFP: refeeding period	SP caused a drop in richness and Shannon index that re-increased during RFP.	Zou et al. (2019)

Table 2 Continued.

Feeding practice	Animals (age, body weight)	Treatments[1]	Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)	Reference
Early-life feeding management	Crossbreed lambs (newborn, 4 kg)	AA: colostrum alternative plus artificial milk feeding NA: ewe colostrum plus artificial milk feeding NN: natural rearing	Increased richness and Shannon index in NN. No long-term effects.	Belanche et al. (2019b)
	Hu lambs (5 d, 4 kg)	STA: milk replacer plus *ad libitum* starter pellets S-ALF: milk replacer plus *ad libitum* starter pellets and chopped alfalfa	No effects on richness and Shannon index.	Yang et al. (2018)
	Hu lambs (newborn, 4 kg)	C: milk replacer with no supplementation S: milk replacer plus starter	Decrease in richness, Shannon and Simpson index in S.	F. Lv et al. (2020)
	Beef calves (130 d, 177 kg)	CON: natural rearing with no supplementation PCON: natural rearing plus concentrate supplementation	Decrease in richness and Shannon index in PCON. No effects on Simpson index.	Lourenco et al. (2019)
	Haimen kids (20 d, 5 kg)	MRO: milk replacer only MRC: milk replacer plus concentrate MRA: milk replacer plus concentrate plus alfalfa pellets	Decrease in richness in MRC and MRA. No effects on Shannon index.	Lv et al. (2019)

[1] C: concentrate; CP: crude protein; DM: dry matter; F: forage; VFI: voluntary feed intake.

6.2 Plant secondary compounds

Tannins, essential oils and saponins are plant secondary compounds that have risen great interest due to their primary role on ruminants' digestion and performance, and also on meat and dairy products quality (Vasta and Luciano, 2011). A summary of their effects on rumen microbial community and robustness is presented along the following lines.

The influence of tannins supplementation on rumen microbial community robustness is inconsistent: generally, no significant effects on alpha biodiversity are observed, but an increase in Shannon index levels has been also reported (Table 3).

The administration of essential oils to ruminants does not have an impact on ruminal microbiota alpha biodiversity (Table 3) but it seems to reduce microbial network complexity, in terms of number of nodes, edges and unique correlations (Patra et al., 2019).

Finally, while saponins supplementation increased microbial richness when animals were fed concentrate plus low-quality forage, no effects of such compounds were observed either on Shannon and Simpson indexes (Table 3) or on microbial network complexity (Popova et al., 2019).

Table 3 Effects of different plant secondary compounds on ruminal microbial alpha diversity.

Plant secondary compound	Animals (age, body weight)	Treatments[1]	Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)	Reference
Tannins	Simmental steers (350 kg)	CTR: No supplementation TA: 16.9 g tannic acid/kg DM	No effects on richness. Increased Shannon index in TA.	Zhou et al. (2019)
	Holstein cows (584 kg)	CTR: No supplementation TA: 2 g chestnut and quebracho tannins blend/ kg DM	Richness tended to be lower in TA. No effects on Shannon index.	Díaz Carrasco et al. (2017)
Essential oils	Suffolk sheep (121 d, 33 kg)	CTR: No supplementation PBLC-L: 80 mg menthol-rich PBLC/d PBLC-H: 160 mg menthol-rich PBLC/d	No effects on richness, Shannon or Simpson indexes.	Patra et al. (2019)
	Holstein cows (pluriparous)	CTR: No supplementation EO: 1 g essential oils blend/d, containing thymol, guaiacol, eugenol, vanillin, salicylaldehyde and limonene	No effects on richness or Shannon index.	Schären et al. (2017)
Saponins	Holstein bulls (150 d, 150 kg)	AH: Concentrate plus alfalfa hay AHS: AH plus 9 g camellia seed saponins/d SH: Concentrate plus soybean hulls SHS: SH plus 9 g camellia seed saponins/d	Increased richness in SHS compared to SH. Similar richness between AH and AHS. No effects on Shannon index.	B. Wang et al. (2019)
	Holstein cows (658 kg)	CTR: No supplementation TEA: 0.77% tea saponin	No effects on richness, Shannon or Simpson indexes.	Popova et al. (2019)

[1] DM, dry matter; PBLC, plant bioactive lipid compounds.

6.3 Antibiotics and alternative control agents

Since the ban of in-feed antibiotics as growth promoters by the EU Regulation 1831/2003 for environmental and hygienic reasons, probiotics and prebiotics administration has emerged as a useful tool for antimicrobial control (Uyeno et al., 2015). The impact of both old and new strategies on ruminal microbiota is assessed below.

Available literature does agree in the fact that antibiotic administration decrease rumen microbiota alpha biodiversity, in terms of microbial richness and evenness (Table 4).

Probiotics are live microorganisms that stimulate the growth of beneficial microbes in the rumen (Mamuad et al., 2019). The effects of probiotics on rumen microbial community usually depend on the microorganism provided: neither *Saccharomyces cerevisiae* nor *Propionibacterium acidipropionici* administration had any effect on microbial richness or evenness; however, animals treated with both *Bacillus amyloliquefaciens* and *Bacillus pumilus* had a more diverse ruminal microbiota (Table 4).

Prebiotics are non-digestible sugars that provide substrate for beneficial microbes inducing their growth and activity (Mamuad et al., 2019). Regarding the supplementation with inulin as a prebiotic, it seems possible to increase ruminal microbiota alpha biodiversity when animals are fed high-concentrate diets; contrarily, if animals are forage-fed, the administration of inulin may negatively impact microbial richness and Shannon index (Table 4).

Table 4 Effects of antibiotics and alternative control agents on ruminal microbial alpha diversity.

Antibiotics and alternative control agents	**Animals (age, body weight)**	**Treatments**	**Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)**	**Reference**
Antibiotics	Crossbred steers (fattening)	NA: non-antibiotic treatment AB: hormone-implanted cattle fed a beta-agonist (ractopamine) and antibiotics (monensin and tylosin) as feed additives	Decreased richness, Shannon and Simpson indexes in AB.	Thomas et al. (2017)
	Holstein cows (pluriparous)	CTR: non-antibiotic treatment MO: administration of 335 mg monensin/d	Decreased richness and Shannon index in MO.	Schären et al. (2017)
Prebiotics	Crossbred steers (320 kg)	LCD: low-concentrate diet LCIN: LCD plus 2% inulin supplementation HCD: high-concentrate diet HCIN: HCD plus 2% inulin supplementation	Decreased richness and Shannon index in LCIN compared to LCD; no effects on Simpson index. Increased richness and Shannon and Simpson indexes in HCIN compared to HCD.	Tian et al. (2019)

Table 4 Continued.

Antibiotics and alternative control agents	**Animals (age, body weight)**	**Treatments**	**Effects on alpha diversity metrics (OTU richness, Shannon and Simpson indexes)**	**Reference**
Probiotics	Holstein steers (504 kg)	CON: no-probiotic treatment YEA: administration of a feed additive containing *Saccharomyces cerevisiae* and other active ingredients from yeast cell wall	No effects on Shannon index.	Ogunade et al. (2019)
	Crossbred steers (434 kg)	CTR: no-probiotic treatment P169: administration of *Propionibacterium acidipropionici* strain P169 (10^{11} cfu)	No effects on richness, Shannon and Simpson indexes.	Azad et al. (2017)
	Jintang black male goats (80 d)	CTR: no-probiotic treatment BA: administration of *Bacillus amyloliquefaciens* fszne-06 (10^9 cfu) BP: administration of *Bacillus pumilus* fszne-09 (10^9 cfu)	Increased richness, Shannon and Simpson indexes in the probiotic-treated groups.	Zhang et al. (2020)
	Romane lambs (fattening)	CTR: no-probiotic treatment SUP: administration of a combination of live yeast *Saccharomyces cerevisiae* CNCM I-1077 ($3x10^9$ cfu) and selected yeast metabolites.	No effects on richness or Shannon index.	Chaucheyras-Durand et al. (2019)

7. Conclusions

Understanding ruminal microbiota robustness is of great importance to predict community's response to disturbances. Microbiota robustness depends on its resistance, resilience and functional redundancy and it can be assessed via alpha diversity metrics and network complexity inference. In that sense, although alpha diversity is commonly reported, network complexity information is still missing in most of ruminal microbiota literature. Further studies providing a more holistic vision of the ruminal microbiota are needed to fully comprehend its composition and functioning.

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CHAPTER II

Effects of protein restriction on performance, ruminal fermentation and microbial community composition in Holstein bulls fed high-concentrate diets

Abstract

The aim of this work was to study the impact of a crude protein (CP) restriction on performance, ruminal fermentation and microbial community composition in fattening Holstein bulls (from 118 to 331 d of age and from 165 to 522 kg body weight [BW]) fed high-concentrate diets. Twenty animals were assigned to two dietary treatments: concentrate CP was formulated either based on the levels used commercially (CTR: 140 g CP/kg dry matter [DM]) or reducing them (LP: 120 g CP/kg DM). Concentrate was supplemented with barley straw and both were supplied *ad libitum.* Animal BW and concentrate intake were automatically recorded on a daily basis. Feces, urine and ruminal fluid were sampled twice, during the growing period (160 d of age and 225 kg BW) and during the finishing period (280 d of age and 444 kg BW), for digestibility, ruminal fermentation and microbial population characterization. No differences in BW or cumulated concentrate intake were found between treatments, despite the fact that average daily gain was lower in LP group at the beginning of the growing period ($P < 0.001$). Crude protein limitation did not penalize dry matter ($P = 0.654$) or organic matter ($P = 0.526$) apparent digestibility, but it did affect CP apparent digestibility during the finishing period ($P = 0.042$). Nitrogen (N) excretion was greater in CTR animals ($P = 0.017$). Regardless of treatment ($P = 0.511$), ruminal ammonia-N concentration was low (4.36 ± 1.01 mg/L). Even though 135 OTUs (out of 489) were shared between treatments and periods (gathering 98.7 % of analyzed sequences), ruminal microbial community composition was different between periods ($P = 0.003$) and also between diets in either growing ($P < 0.001$) or finishing ($P = 0.046$) bulls. Bacteroidetes, Firmicutes and Actinobacteria were the three dominant phyla and *Prevotella ruminicola* was the most abundant species. Ruminal microbial biodiversity was low but increased with age ($P = 0.002$ for Shannon index and $P = 0.035$ for Simpson index), as well as, ruminal microbial heterogeneity. Crude protein limitation increased functional interdependency among

microbial genera, so LP-fed bulls were found to have a more complex microbiota community structure than CTR-fed bulls. No relevant correlations between microbial genera and ruminal fermentation parameters were detected.

1. Introduction

Ammonia produced by livestock is a main pollutant that contributes to eutrophication, soil acidity and aerosol formation, impairing atmospheric visibility and human health (Hristov et al., 2011). Moreover, animals and their waste also emit methane and nitrous oxide gases, which are contributors to global warming.

Ruminants are inefficient dietary nitrogen (N) utilizers and, in a common beef cattle fattening system, from 10 to 20 % of N intake is retained, from 30 to 50 % is excreted in feces and from 40 to 70 % is excreted in urine (Cole and Todd, 2008). Available research data indicate that diet has strong effects on ammonia emissions from excreted manure and, in beef cattle, a minimum requirement of 150-160 g crude protein (CP)/ kg dry matter (DM) for growing Holstein bulls is generally accepted (NRC, 2000).

Lowering dietary CP content may compromise animal performance, which would be undesirable for most producers. However, this is not always the case. Using high-concentrate diets, a significant CP reduction from 170 g/kg to 140 g/kg (on a DM basis) did not alter the average daily gain (ADG) in crossbred heifers (Devant et al., 2000). Similar results were obtained when decreasing CP from 135 g/kg to 119 g/kg (on a DM basis) in Holstein heifers receiving 70/30 roughage to concentrate diets (Zhang et al., 2017). Going further, Erickson and Klopfenstein (2001) were able to reduce CP inputs by 10 % in crossbred animals maintaining ADG. Therefore, young cattle fed high-concentrate diets seem to be able to adapt to a protein supply reduction, maintaining growth performance and reducing N waste.

However, it remains unclear if such adaptation is accomplished throughout animal metabolism itself or throughout its ruminal symbiotic microbiota.

Ammonia is the main N source for microbes and a minimum level of ammonia-N concentration in the rumen (50 mg/L) has been defined to fulfil microbial N requirements (Satter and Slyter, 1974). However, several authors are critical with the definition of a constant threshold level and microbial ammonia-N requirements may be dependent on fermentable organic matter (OM) availability (Song and Kennelly, 1990) and/or presence of preformed protein (Broudiscou and Jouany, 1995). Other authors outline a differential ammonia-N level to attain either the maximum microbial protein yield or DM degradation (Balcells et al., 1993).

Intensive research has been done to describe the relationship between CP availability and microbial yield (Hoover and Stokes, 1991); however, much less research has been conducted to explore the impact of CP availability on rumen microbiota. Chanthakhoun et al. (2012) demonstrated an increase in total bacteria counts with CP availability, whereas Yang et al. (2016) could not confirm such findings.

Increasing CP supply raised proteolytic bacteria abundance (*Butyrivibrio fibrisolvens* and *Prevotella ruminicola*) in crossbred beef steers fed total mixed ration (Wang et al., 2017), though such effect was not that clear on other microbial populations: the abundance of cellulolytic bacteria (*Ruminococcus albus, Ruminoccocus flavefaciens* and *Fibrobacter succinogenes*) significantly increased with dietary CP supply (Wang et al., 2017), while other authors could not detect such shift (Yang et al., 2016). Protozoa and fungi counts remained unchanged with increasing levels of CP supply but archaea counts were found to be superior (Chanthakhoun et al., 2012).

Most of the existing studies are focused on titers and/or properties of singular microbes in response to specific challenges (i.e. dietary treatments), leaving microbiota interactions

unexplored. Considering rumen ecosystem complexity, microbial interactions may explain relevant aspects of rumen functioning, so searching for new tools to reveal the impact of nutritional challenges on the whole ecosystem under real conditions is of great interest. In this sense, some studies of rumen microbiota across different ruminant species (Kittelmann et al., 2013) and diets (Kumar et al., 2015) have demonstrated that rumen microbiota profile and function are determined by factors still unexplored (Henderson et al., 2015).

Bearing in mind that the possibility of reducing N supply without impairing productive results could be feasible, this assay was built with a double objective: assess the impact of reducing dietary CP from 140 g/kg to 120 g/kg (on a DM basis) on animal performance and to analyze rumen microbiota adaptation to such shortage, in Holstein bulls raised under commercial intensive feeding system.

2. Materials and methods

2.1 Animals, diets and housing

This experiment was conducted at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* (Ivars d'Urgell, Spain, 41°41'50"N, 0°58'53"E) between January and August 2017. All procedures were carried out under Project License CEEA 01-07/16 and approved by the in-house Ethics Committee for Animal Experiments at the University of Lleida. Care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purpose.

Twenty Holstein bulls (mean ± standard error: 118 ± 1 d of age) were group-housed in two outdoor paved and covered pens equipped with two feedbunks each: an individual one for concentrate and a common one for straw. According to body weight (BW), animals were assigned to two experimental treatments, consuming two corn-based concentrates and barley

straw (Table 5). Experimental concentrates were formulated to reach two levels of CP: one with 140 g/kg (on a DM basis), which was considered as control (CTR) because it represented the conventional CP level used by beef cattle producers in the north-east region of Spain (*Cooperativa d'Ivars d'Urgell, SCCP*), and the other one with 120 g/kg (on a DM basis), which was considered as a low protein (LP) concentrate. Feedbunks were filled once daily at 08:00h and animals had free access to drinking water. One animal in the LP group was excluded from the trial due to a respiratory illness.

2.2 Measurements and sampling

The experimental period included the whole fattening phase. The first 150 d of the trial were considered to be the growing period, beginning when animals were 118 ± 1 d of age and 165 ± 3 kg of BW and ending when they were 268 ± 1 d of age and 435 ± 8 kg of BW. After that, the finishing period started and it continued until animals reached the commercial slaughter weight (331 ± 1 d of age and 522 ± 9 kg of BW).

Daily animal BW and concentrate consumption were automatically controlled by a feed station. Briefly, it was equipped with a feedbunk and an individual tunnel-type feeder both provided with a scale. When a calf entered the feeder, it was identified, its BW was registered and concentrate intake was obtained by difference between initial and final feedbunk weight. Both concentrate and straw, which was the only source of roughage, were supplied *ad libitum* in a free-choice system.

Feces, urine and ruminal fluid samples were taken on days 37, 38, 42 and 43 of the trial, corresponding to growing period (GRO) (160 ± 1 d of age and 225 ± 5 kg of BW), and on days 160, 161, 162 and 163 of the trial, corresponding to finishing period (FIN) (280 ± 1 d of age and 444 ± 9 kg of BW) for digestibility, ruminal fermentation and microbial population characterization.

Table 5 Ingredients and chemical average composition of concentrates and straw.

ADF: acid detergent fiber; CP: crude protein; DDGS: distillers dried grains and solubles; DM: dry matter; EE: ether extract; FM: fresh matter; NDF: neutral detergent fiber; OM: organic matter; PDIN and PDIE: protein digestible in the small intestine allowed by protein and energy, respectively; UFC: forage unit for meat production.

Item	Control concentrate	Low protein concentrate	Straw
Ingredient composition, g/kg FM			
Raw corn	431.2	527.1	
Bran	204.5	248.8	
Raw barley	99.5	21.0	
Corn DDGS	65.0	0.0	
Wet ear of corn silage	69.8	49.8	
Palm kernel meal	30.0	73.5	
Palm kernel oil	23.6	21.1	
Calcium carbonate	26.9	18.5	
Sugarcane molasses	18.4	17.2	
Soybean hunks	15.6	10.0	
Sodium bicarbonate	7.8	7.5	
White salt	3.5	3.8	
Urea	2.5	0.0	
Vitamin/mineral premix	2.0	2.0	
Chemical composition, g/kg DM			
DM, g/kg FM	856.2	862.1	857.2
OM	939.9	943.3	842.9
CP	140.7	117.7	71.9
EE	51.4	72.0	14.2
NDF	202.5	207.1	754.5
ADF	70.7	78.5	281.8
Nutrient composition			
UFC , UFC/kg DM	1.00	0.97	0.36
PDIN, g/kg DM	89.75	79.80	40.83
PDIE, g/kg DM	89.90	86.20	52.61
PDIN/UFC	90.29	82.64	113.42
PDIE/UFC	90.44	88.58	146.14

Fecal excretion and straw intake were calculated based on concentrate intake and by a double marker system using chromium oxide (Cr_2O_3) as external marker and acid insoluble ash (AIA) as internal marker. Chromium oxide was mixed with concentrate (150 mg/kg in GRO and 90 mg/kg in FIN) and administered during 15 d. Fecal samples (approximately 50 g, one sample per animal and day, obtained between 9:00 – 13:00 a.m. during the four last days of chromium oxide administration) were collected using rectal stimulation and stored at -20 ºC until marker determination (both Cr_2O_3 and AIA) and proximate chemical analysis. After thawing, fecal samples from each animal were pooled and mixed to produce one grab sample per collection period. During sampling days, concentrate and straw samples were also collected and stored at 5 ºC until marker and proximate analysis.

Urine samples (10 mL, one sample per animal and collection period [GRO and FIN], obtained between 9:00 – 13:00 a.m.) were taken by prepuce stimulation. Samples were strained to remove hair and debris, immediately frozen in dry ice and stored at -80 ºC until N and creatinine analysis.

Ruminal fluid samples (one sample per animal and collection period [GRO and FIN], obtained between 9:00 – 13:00 a.m.) were collected using an oral stomach tube connected to a vacuum pump. Each sample was obtained through two sequential collections: firstly, ruminal fluid (approximately 200 mL) was collected and discarded to avoid sample's contamination with saliva that possibly got into the tube during its introduction through the animal's mouth and esophagus. After that, ruminal fluid (approximately 200 mL) was collected again, strained through a cheese-cloth and its pH recorded (Testo 205, Testo AG, Germany). Then, 15 mL of ruminal fluid was immediately frozen with dry ice and stored at -80 ºC for subsequent DNA extraction and molecular analysis. The remaining ruminal fluid was sampled for ammonia-N (2 mL over 0.8 mL of 0.5 N HCl) and volatile fatty acids (VFA) concentration (4 mL over 1 mL solution of 0.4 M ortho-phosphoric acid and 0.02 M 4-

methylvaleric acid as internal standard, in distilled water). Samples were immediately frozen with dry ice and stored at -20 ºC until analysis.

2.3 Chemical analysis

Feed and feces DM (index nº 934.01), ash (index nº 942.05) and ether extract (EE) (index nº 2003.05) contents were determined according to the AOAC methods (AOAC, 2006), as well as N content (index nº 990.03) in feed, feces and urine.

Neutral detergent fiber and acid detergent fiber analyses in feed were carried out following the sequential procedure of Van Soest et al. (1991), with the Ankom200/220 fiber analyzer (Ankom Technology, USA). Neutral-detergent fiber was assayed with a heat stable amylase.

Chromium in feed and feces was analyzed as follows. Samples (0.5 g) were calcined (550 ºC, 2 h) and digested with 3 mL HCl (1:1) in a sand bath (60 ºC, until dry). The residue was then dissolved with 3 mL HCl (1:1), filtered and washed with 50 mL of hot distilled water. Chromium concentration was quantified by inductively coupled plasma mass spectrometry (7700x, Agilent Tecnologies, USA).

Acid insoluble ash was analyzed according to a standard procedure (BOE, 1995) based on the method of Shrivastava and Talapatra (1962). Briefly, the residue of ash content determination was introduced in an Erlenmeyer flask and then hydrolyzed with 75 mL of 3N HCl and boiled for 15 min. The sample was then filtered through ash-free filter paper (cat nº 1004 150, Whatman) and washed the residue with 50 mL of hot distilled water. The filter with the residue was dried (103 ºC, 2 h) and then ashed (550 ºC, 3 h) in a tared crucible. The crucible and its content were cooled in a desiccator at room temperature and weighed to calculate the AIA content.

Creatinine was determined using ultra high performance liquid chromatography coupled with mass spectrometry, using an adaptation of Boudra et al. (2012). The chromatographic system

was an Acquity module (Waters Corporation, USA) and peak separation was performed with an Acquity UPLC® BEH Amide 1.7 µm column (150*2.1 mm, Waters Corporation, USA) using a gradient solvent system (solvent A: acetonitrile with 1 mL/L formic acid; solvent B: methanol-water solution 1:49 with 1 mL/L formic acid). The gradient conditions were as follows: the initial percentage of solvent B was 20 %, which was raised to 40 % between min 1 and 2 and then kept constant for 2 more min. After that, it was lowered to 20 % in 0.01 min and finally kept constant for the last 1.49 min of the analyses. The initial flow rate was 0.4 mL/min, which was kept constant for 2 min, then raised to 0.5 mL/min in 0.1 min and kept constant for 1.9 min. After that, it was lowered to 0.4 mL/min in 0.01 min and kept constant for the last 1.49 min of the analysis. The injection volume was 5 µL and the auto sampler and column temperature were maintained at 10 ºC and 30 ºC respectively. Finally, mass spectrometric analyses were performed on an Acquity TQD triple quadrupole tandem mass spectrometer (Waters Corporation, USA).

Ammonia-N concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (13800 g, 30 min).

Volatile fatty acids concentration and molar VFA profile were determined by gas chromatography according to the technique proposed by Jouany (1982), using a capillary column (GS-BR-SWAX 30m x 0.25 mm D.I. x 0.25 µm, Bruker, USA).

2.4 Extraction and sequencing of DNA

DNA extraction was carried out on freeze-dried ruminal fluid (the initial sample amount was 60 mg) through physical disruption (1 min) using a bead beater (Mini-bead beater 1, BioSpec Products, USA) and subsequent DNA purification with the QIAamp DNA Stool Mini Kit (ID: 51504; QIAGEN N.V., Germany), with the modifications of greater temperature (95 ºC) to

improve cell lysis and greater elution time (3 min). DNA was amplified by using the following primer set:

Forward = 5':

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse = 5':

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

which targets the V3 and V4 regions of the microbial 16S rRNA. Sequencing was conducted on an Illumina MiSeq 2x300 platform. Sequencing of 16S RNA genes was performed by Era7 Bioinformatics (Spain).

2.5 Bioinformatics

Sample reads were assembled by using FLASh software (Fast Length Adjustment of Short reads (Magoč and Salzberg, 2011) and quality filtered using FastQC software (Babraham Bioinformatics, Babraham Institute, U.K.). Operational Taxonomic Units (OTUs) were picked using BLAST software (Basic Local Alignment Search Tool, National Center for Biotechnology Information [NCBI], USA) with a 98 % similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to the *Taxonomy* database by NCBI (USA), according to the Lowest Common Ancestor method.

2.6 Calculations and statistical analysis

Fecal excretion *(FcE)* was calculated using chromium concentration in concentrate and feces, as follows (Eq. 1):

$$(Eq.1)\ Fecal\ excretion\ = \frac{[Cr]c * CI}{[Cr]fc}$$

Where *[Cr]c* and *[Cr]fc* are chromium concentrations in concentrate and feces, respectively, and *CI* is concentrate intake.

Straw intake (*FrI)* was calculated using the estimated fecal excretion (Eq. 1) and the AIA concentration in both feed and feces, as follows (Eq. 2):

$$(Eq.2)\ Straw\ intake = \frac{[AIA]fc * FcE - [AIA]c * CI}{[AIA]fr}$$

Where *[AIA]fc*, *[AIA]c* and *[AIA]fr* are AIA concentration in feces, concentrate and straw, respectively.

Nutrient total tract apparent digestibility was calculated using registered concentrate intake, estimated straw intake (Eq. 2), estimated fecal excretion (Eq. 1) and nutrient concentration in both feed and feces, as follows (Eq. 3):

$$(Eq.3)\ Apparent\ digestibility\ (\%) = \frac{([N]c * CI + [N]fr * FrI) - ([N]fc * FcE)}{([N]c * CI + [N]fr * FrI)}$$

Where *[N]c, [N]fr, [N]fc* are nutrient concentrations in concentrate, straw and feces, respectively.

Urine daily volume was determined assuming a creatinine constant urinary output of 883 µmol per kg of metabolic weight and day (Chen et al., 1992).

Performance, intake, apparent digestibility, ruminal fermentation parameters, N balance, microbial abundance and microbial biodiversity data were analyzed with a mixed model; including treatment (CTR *vs* LP), period (GRO *vs* FIN) and their interaction as fixed effects and animal as random effect, to account for repeated measurements (R Core Team, 2018; lme4 package). Comparisons among groups were performed by the Tukey's method (R Core Team, 2018; emmeans package); additionally, only for microbial taxa abundance, false discovery rate was addressed using Benjamini-Hochberg statistical test. Individual sample out of three standard deviations of the mean were discarded and not included to the statistical

analysis. Significant effects were declared at $P < 0.05$ and tendency to difference at P between 0.05 and 0.10.

Sequence data were normalized and biodiversity indexes were calculated (R Core Team, 2018; Vegan package). To determine the proportion of shared and unshared OTUs, a Venn diagram was performed (R Core Team, 2018; VennDiagram package). To determine the impact of both treatment and period on the microbial community structure, a permutational multivariate analysis of variance (Adonis) was conducted based on the Bray-Curtis dissimilarity index and calculating statistical significance after 5000 random permutations (R Core Team, 2018; Vegan package). A partial least squares-discriminant analysis (PLS-DA) on community structure data was performed for graphical interpretation (R Core Team, 2018; mixOmics package). Correlation analyses were performed to decipher the interactions within rumen microbial community as well as between rumen microbial community and fermentation patterns. Spearman correlation analysis was performed between all microbial genera and between all microbial genera and rumen fermentation parameters (pH, ammonia-N, VFA, acetate, propionate and butyrate concentrations). Only those genera present in more than 50 % of the individuals and only correlations coefficients larger than 0.6 and P-values (adjusted in the case of within genera correlations) below 0.05 were further included in the correlation network. Microbial correlation network was generated using igraph package (R Core Team, 2018). Microbial network complexity was described in terms of number of nodes (genera), number of edges (positive or negative correlations) and betweenness (measure of centrality in a graph based on shortest paths).

3. Results

3.1 Animal's performance through the whole experimental period

Data on animal performance evolution through the whole fattening process is showed in Figure 5: animals began with 165 (± 3) kg of BW and they finished when they reached the commercial slaughter weight (522 ± 9 kg). During the first month of the experimental period, CTR animals presented a higher ADG than LP ones (Figure 5A; $P < 0.001$); however, live weight tended to equilibrate throughout the experimental time-course (only at day 90 CTR animals tended to weigh more than LP animals; Figure 5A; $P = 0.075$). No differences in cumulated concentrate intake were found (Figure 5B).

3.2 Intake, apparent digestibility and N balance

In Table 6, data on DM intake (both concentrate and forage) are presented. Animals increased voluntary intake with age although such increase was more pronounced in LP animals than in CTR ones. Increased DM intake was mainly explained by concentrate intake (from 5.29 to 8.17 kg) although straw ingestion also incremented significantly (from 0.77 to 1.37 kg).

Data on DM, OM and CP apparent digestibility are shown in Table 6. No differences in DM or OM apparent digestibility were detected, either between treatments or between periods. Crude protein apparent digestibility was impaired in LP-fed animals during the finishing period, but not in the growing.

Data on N balance is also presented in Table 6. As expected, N intake and N excretion were higher in CTR-fed bulls than in LP-fed ones.

3.3 Ruminal fermentation

Data on ruminal fermentation are presented in Table 7. Ruminal pH decreased with period and ammonia-N concentration was higher in finishing than in growing bulls. No differences were detected between experimental diets although numerical variations in mean values registered in the growing period were consistent; in this sense, it is necessary to remark the notorious residual variation observed in ammonia-N concentrations (C.V. = 143 %).

Ruminal VFA concentration increased with bulls age and it was higher in CTR animals than in LP ones. Acetate proportion did not vary among treatments. Comparing to the rest of the treatments, LP-fed bulls presented a lower propionate proportion, which led to a significant treatment-by-period interaction. Butyrate proportion was higher in CTR-fed animals than in LP ones in the growing period but not in the finishing one.

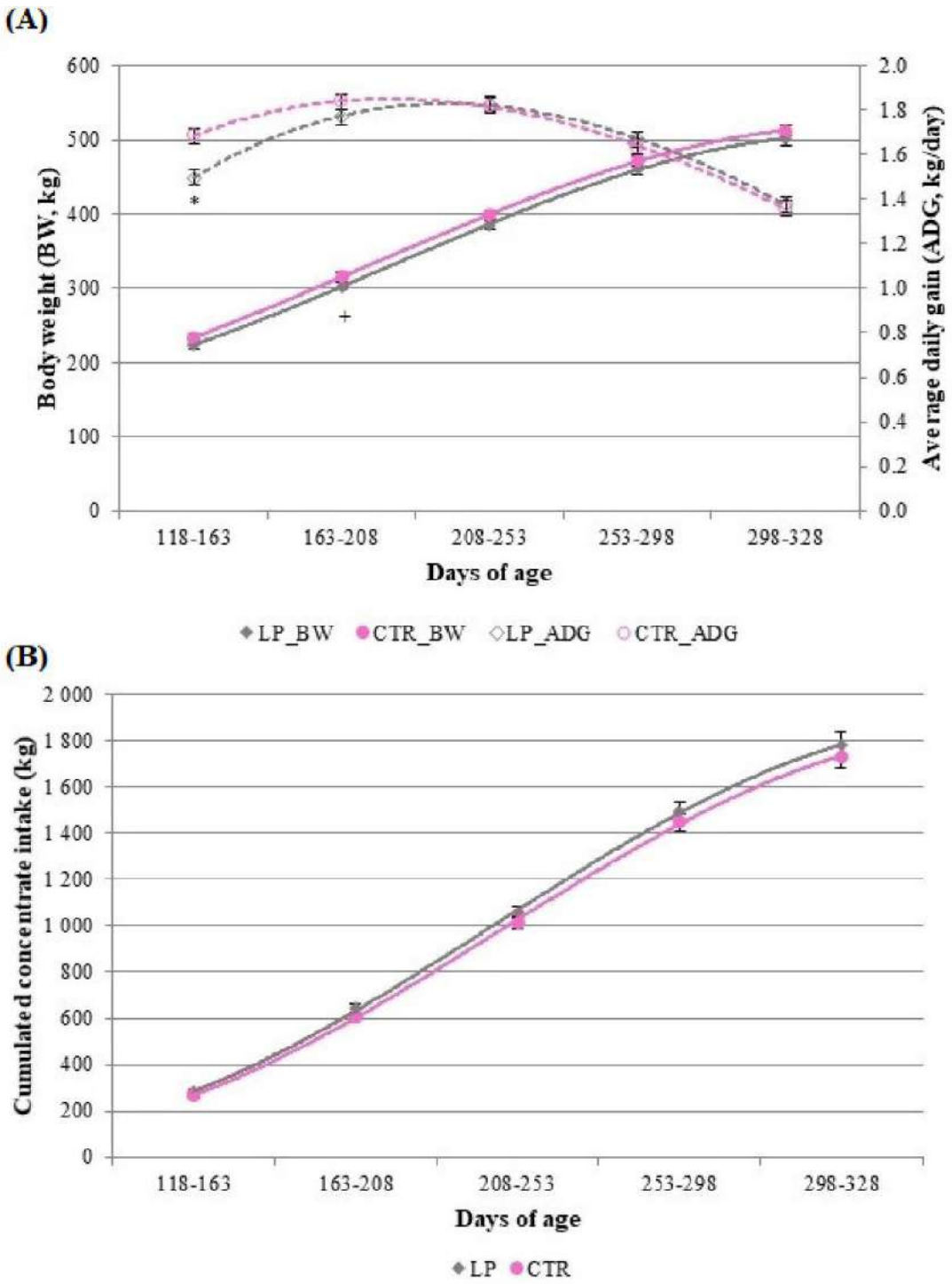

Figure 5 Performance and concentrate intake evolution.

*(A) Body weight and average daily gain and (B) cumulated concentrate intake obtained in intensively reared Holstein bulls (from 118 to 328 d of age). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. * means that a statistical difference was found ($P < 0.05$) and + means that a statistical tendency was found ($P < 0.10$).*

Table 6 Dry matter intake, nutrient apparent digestibility and nitrogen balance.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment, period and their interaction (TxP) effects are shown. CP: crude protein; DM: dry matter; N: nitrogen; OM: organic matter. Mean values within a row with unlike superscript letters differ (P < 0.05).

Item	GRO		FIN		SEM	P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	10	10	8				
Intake								
DM, kg/d	6.46^{b}	5.65^{b}	9.45^{a}	9.69^{a}	0.345	0.460	<0.001	0.049
Concentrate DM	5.71^{b}	4.87^{b}	8.08^{a}	8.25^{a}	0.266	0.328	<0.001	0.002
Straw DM	0.76	0.78	1.36	1.37	0.228	0.937	0.009	0.973
Apparent digestibility coefficient								
DM	0.737	0.745	0.744	0.722	0.014	0.654	0.448	0.183
OM	0.747	0.750	0.753	0.729	0.015	0.526	0.549	0.254
CP	0.717^{ab}	0.711^{ab}	0.725^{a}	0.673^{b}	0.012	0.042	0.099	0.014
N balance								
N intake, g/d	135.00^{c}	97.63^{d}	200.97^{a}	176.79^{b}	6.173	<0.001	<0.001	0.105
N excretion, g/d	66.68^{b}	47.06^{c}	94.54^{a}	85.47^{a}	5.001	0.017	<0.001	0.172
Feces	38.02^{b}	28.17^{c}	55.35^{a}	57.50^{a}	2.542	0.175	<0.001	0.006
Urine	28.65^{ab}	18.88^{b}	39.19^{a}	30.42^{ab}	3.663	0.010	0.002	0.882

Table 7 Ruminal fermentation parameters.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment, period and their interaction (TxP) effects are shown. A/P: acetate/propionate; N: nitrogen; VFA: volatile fatty acids. Mean values within a row with unlike superscript letters differ (P < 0.05).

Item	GRO		FIN		SEM	P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	10	9	9				
Ruminal fermentation parameters								
pH	7.29^{a}	7.28^{a}	6.45^{b}	6.90^{ab}	0.124	0.080	<0.001	0.065
Ammonia-N, mg/L	4.15	0.58	6.30	7.06	1.985	0.511	0.026	0.235
VFA, mmol/L	66.96^{ab}	57.81^{b}	91.27^{a}	60.98^{b}	7.796	0.033	0.050	0.123
VFA, mol/100 mol								
Acetate	46.30	47.79	45.51	45.94	0.769	0.289	0.044	0.392
Propionate	43.59^{a}	39.46^{b}	42.87^{a}	42.73^{a}	0.783	0.022	0.077	0.009
Butyrate	6.42^{c}	9.27^{a}	7.57^{b}	7.03^{bc}	0.228	<0.001	0.019	<0.001
Ratio A/P	1.07^{b}	1.22^{a}	1.07^{b}	1.08^{b}	0.039	0.086	0.042	0.051

3.4 Ruminal microbiota profile

3.4.1 Data set features

After sequencing and normalization procedures, an average of 22658 sequences per sample were obtained, resulting in 860996 sequences from the 38 samples. A total 489 OTUs were obtained at the 98 % sequence similarity cut-off levels with 114 ± 5 (range: 56 to 169) as the mean number of OTUs per sample. Good's coverage value was 99.8 % suggesting that more than 99 % bacterial and archaeal phylotypes were identified. Unclassified mean rates of OTUs at family and genus level were 0.52 % (0.06 – 4.08 %) and 0.81 % (0.15 – 5.18 %), respectively. Operational taxonomic units were assigned to 2 kingdoms, 12 phyla, 24 classes, 45 orders, 92 families and 214 genera. Shared taxa by all individuals in each treatment and sampling period were deemed to be core bacterial/archaeal communities. Out of a total number of 489 OTUs, 135 (27.6 %) were shared by all experimental groups (Figure 6A) and were unchanged across treatments and periods. At a sequence level, the proportion of sequences belonging to the common OTUs was as high as 98.7 %. Composition of the core bacterial and archaeal community is exhibited in Figure 6B and almost 80 % of these taxa belonged to *Prevotella* genus.

3.4.2 Microbial community structure and diversity

Phyla and genera abundance are presented in Table 8. Bacteroidetes and Firmicutes were the predominant bacterial phyla in ruminal fluid. Bacteroidetes abundance decreased in the finishing period only in CTR-fed animals; while Firmicutes abundance followed the opposite pattern. Actinobacteria, Euryarchaeota, Proteobacteria and Fibrobacteres abundance were not affected either by treatment or period.

Prevotella, the most abundant genus in ruminal fluid, decreased with age in CTR-fed animals but not in LP ones. Differences in *Roseburia* and *Olsenella*, which were more prevalent in finishing than in growing animals, were not related to treatment. *Selenomonas* was more abundant in GRO than in FIN. *Bifidobacterium* titers were clearly greater in GRO than in FIN, especially for LP-fed bulls. *Agathobacter* presence rose as animals aged, in a more intense manner in CTR-fed animals. *Sharpea* was richer in finishing animals fed CTR diets: those differences could be explained both by treatment and period effects. *Ruminococcus* was more relevant in older animals but it was not altered by treatment. Presence of *Pseudobutyrivibrio* was found to be independent of diet and bulls age.

Shannon and Simpson biodiversity indexes data are also presented in Table 8: their values were similar across treatments and sampling periods except from growing bulls fed CTR diets, which exhibited significantly lower values. Richness values were proved to be independent of the experimental conditions.

Adonis test indicated that differences in bacterial communities were significant between periods ($P = 0.003$) and also between diets in either GRO ($P < 0.001$) and FIN ($P = 0.046$), as it can be deduced by the graphical representation of PLS-DA (Figure 7).

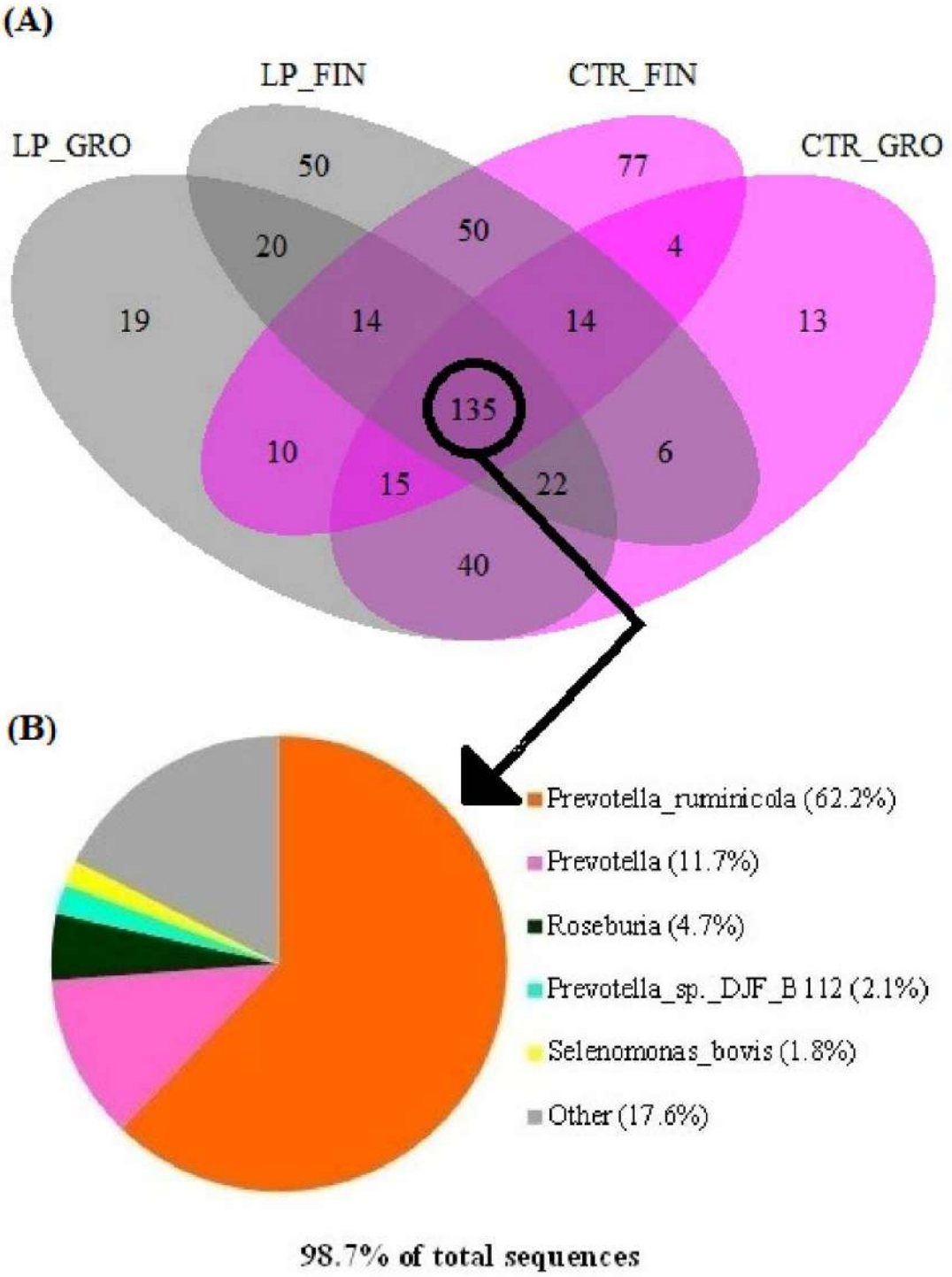

Figure 6 Distribution of bacterial and archaeal OTUs in ruminal fluid.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw.

Table 8 Main phyla and genera abundance in ruminal fluid microbiota and microbial alpha diversity.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Standard error of the mean (SEM) and significance of treatment, period and their interaction (TxP) effects are shown. Mean values within a row with unlike superscript letters differ (P < 0.05).

Item	GRO		FIN		SEM	Adjusted P-value		
	CTR	LP	CTR	LP		Treatment	Period	TxP
n	10	9	10	9				
Phyla abundance, %								
Bacteroidetes	85.60^{a}	82.05^{a}	66.20^{b}	79.11^{ab}	2.529	0.302	0.002	0.016
Firmicutes	11.48^{b}	10.99^{b}	26.20^{a}	14.30^{b}	1.845	0.051	0.001	0.016
Actinobacteria	2.37	6.42	5.78	5.42	0.881	0.166	0.438	0.083
Euryarchaeota	0.19	0.15	0.85	0.39	0.212	0.459	0.166	0.512
Proteobacteria	0.19	0.30	0.33	0.68	0.113	0.205	0.098	0.459
Fibrobacteres	0.12	0.04	0.48	0.02	0.215	0.459	0.544	0.512
Genera abundance, %								
Prevotella	85.56^{a}	81.74^{a}	66.16^{b}	79.02^{ab}	2.549	0.428	0.006	0.036
Roseburia	2.77^{b}	2.36^{b}	8.27^{a}	5.39^{ab}	0.712	0.236	0.002	0.386
Olsenella	0.90^{b}	0.80^{b}	3.16^{a}	2.93^{a}	0.235	0.617	<0.001	0.854
Selenomonas	2.53^{a}	1.59^{ab}	1.43^{b}	1.48^{ab}	0.197	0.319	0.037	0.105
Bifidobacterium	0.98^{b}	5.18^{a}	0.25^{b}	0.52^{b}	0.689	0.085	0.019	0.104
Agathobacter	0.88^{b}	1.15^{b}	2.59^{a}	1.57^{ab}	0.211	0.472	0.001	0.023
Sharpea	0.95^{b}	0.06^{b}	3.99^{a}	0.21^{b}	0.543	0.006	0.082	0.129
Ruminococcus	0.63^{b}	0.29^{b}	2.60^{a}	1.23^{ab}	0.379	0.346	0.006	0.355
Pseudobutyrivibrio	0.37	1.21	1.89	0.78	0.665	0.917	0.492	0.388
Microbial alpha diversity								
Richness	96.40	113.89	110.40	117.44	9.380	0.237	0.299	0.532
Shannon index	1.03^{b}	1.73^{a}	1.92^{a}	1.66^{a}	0.135	0.157	0.002	0.001
Simpson index	0.34^{b}	0.65^{a}	0.63^{a}	0.55^{a}	0.046	0.030	0.035	<0.001

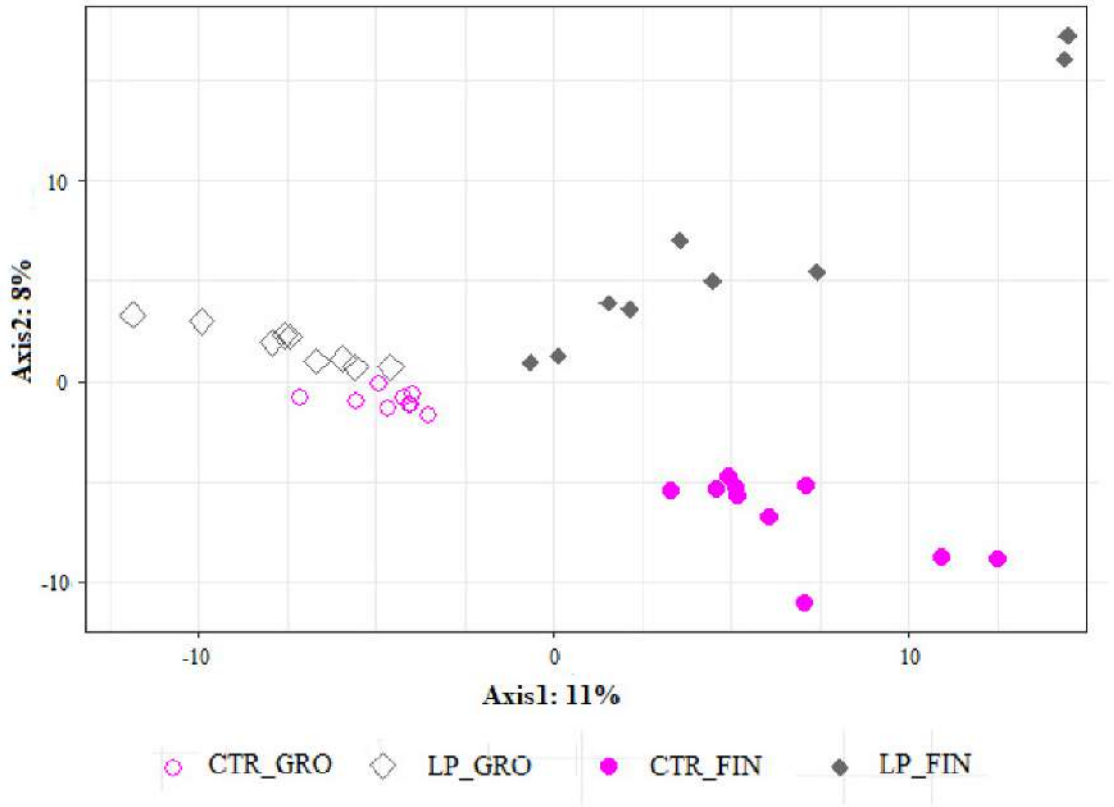

Figure 7 Partial Least Squares-Discriminant Analysis of ruminal fluid microbiota. *Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 160 d of age and 225 kg of body weight) and finishing (FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Each point represents a different sample and a greater distance between two points infers a higher dissimilarity between them.*

3.4.3 Microbial network and interactions with ruminal parameters

Microbial networks were performed to test how bacteria and archaea interact between them (Figure 8). Relating to the degree of interaction, we studied the number of taxa (nodes) that establish significant interactions (edges) with other taxa. In GRO, animals fed a CTR diet, in comparison to a LP diet, had a lower microbial network complexity in terms of nodes (11 in CTR vs 26 in LP) and edges (7 in CTR vs 25 in LP). These differences became even more pronounced in FIN, again in terms of nodes (9 in CTR vs 22 in LP) and edges (8 in CTR vs

40 in LP). Thereafter, we investigated microbial taxa that act as main information gateways in networks. Control networks had no nodes lying on paths that connected other nodes, so betweenness centrality values were 0. Contrarily, LP networks had mean betweenness centrality values of 0.23 in GRO and 1.82 in FIN. The genus with the highest centrality value in GRO was *Pseudomonas*, lying in 6 interconnecting paths; in FIN, the most central genus was *Microbacterium*, being part of 23 interconnecting paths.

Correlations between genera abundance and fermentation parameters (Supplementary Table 1; Supplementary Table 2) varied greatly with period in the case of LP animals: in GRO, 26 genera were found to establish 60 significant correlations with fermentation parameters studied; while in FIN, only 16 genera established 12 significant correlations. Contrarily, in CTR animals, the number of significant correlations and genera involved remained stable, regardless of period (32 genera involved in 20 significant correlations in GRO and 28 genera involved in 20 significant correlations in FIN). When comparing CTR and LP-fed animals, only the abundance of few genera showed consistent correlations with fermentation parameters under both dietary treatments in at least one period: *Prevotella* positively correlated with propionate and some Firmicutes (*Pseudobutyrivibrio, Roseburia, Ruminococcus)* showed positive correlations with acetate and negative with propionate. However, most correlations between microbes and ruminal fermentation parameters were detected under only one dietary treatment and period.

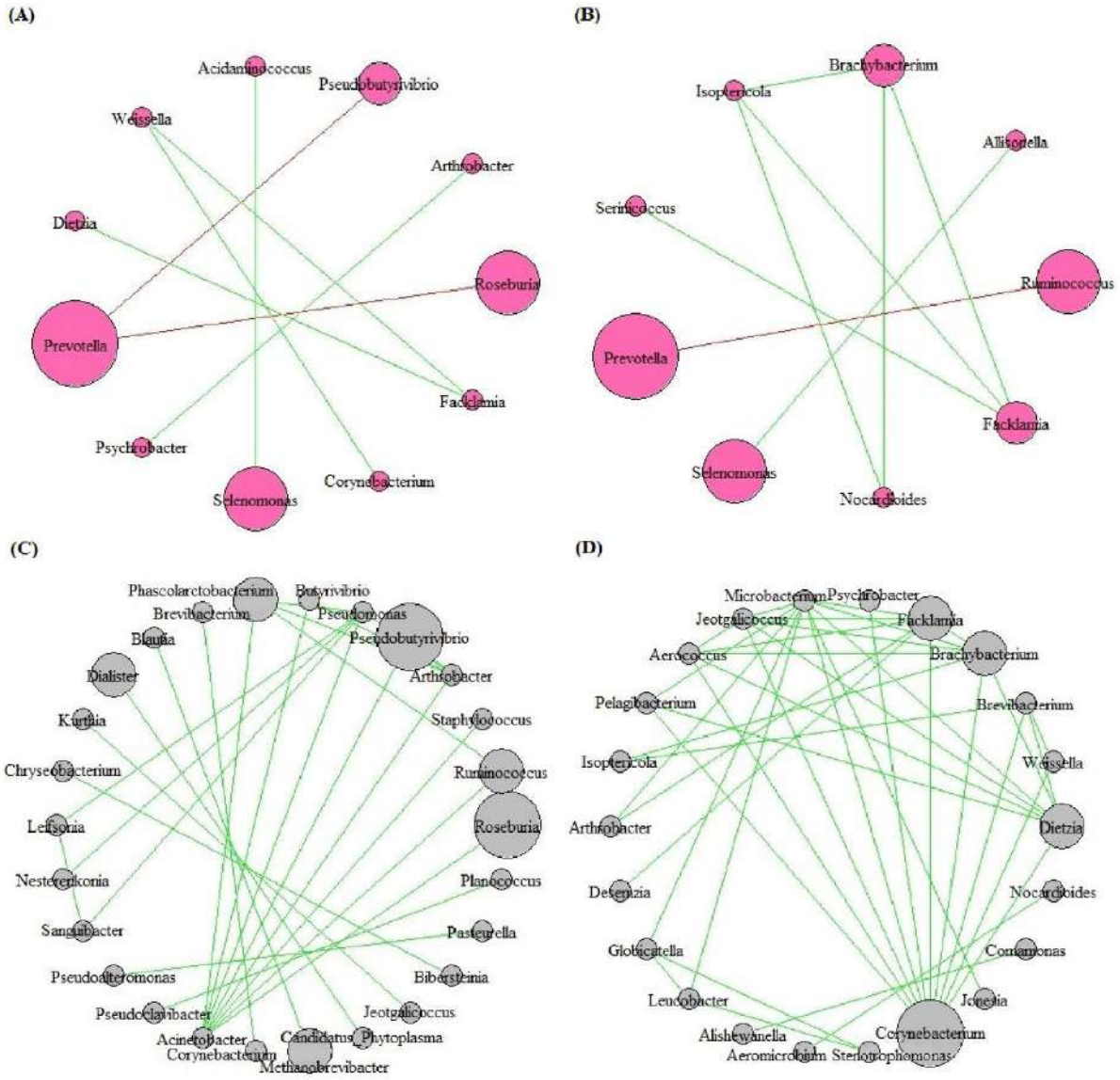

Figure 8 Microbial networks in the rumen of fattening cattle.

Obtained in intensively reared Holstein bulls (A-C, GRO: 160 d of age and 225 kg of body weight; B-D, FIN: 280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (A-B, CTR: 140 g/kg crude protein on a dry matter basis) or low protein (C-D, LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Networks were generated based on those genera which significantly correlated ($r > 0.60$ and $P < 0.05$). Green and red arrows indicate positive and negative correlations, respectively. Node size is proportional to genera abundance.

4. Discussion

4.1 Performance, intake, digestibility, ruminal fermentation and nitrogen balance

Feeding low CP diets optimizes diet cost and reduces N waste. In relation to the latter, animals fed low CP diet excreted less N than those fed CTR diets (1040 mg/kg $BW^{0.75}$ for CTR animals vs 850 mg/kg BW^{075} for LP animals). However, the question that remains is: does such challenge compromise bulls' performance? It is true that, initially (118 – 163 d of age), a low but significant reduction in ADG was observed in LP-fed animals; however, this group recovered its growth rate or grew even faster than CTR-fed animals. The reduced growth in young animals caused by a dietary protein restriction has already been reported in the existing literature (Segers et al., 2014), as well as the lack of effect of protein restriction on finishing animal performance (Dong et al., 2017).

No relevant discrepancies were stated between our reported ADG or DM intake and that from available literature (Verdú et al., 2017), in intensively reared Holstein bulls consuming corn-based concentrate and straw *ad libitum* in a free choice system.

Nutrient apparent digestibility rates were similar to those reported elsewhere (Devant et al., 2000) with animals fed high-concentrate diets. Crude protein was the only nutrient whose apparent digestibility was negatively affected by dietary CP limitation, as the consulted literature has already described, either in growing (Devant et al., 2000) or in finishing animals (He et al., 2018).

In theory, animals receiving high-concentrate diets would have an intense VFA production that would induce a critically low ruminal pH, leading to subacute or acute ruminal acidosis. However, that was not the case, and recorded ruminal pH values in the present study were in agreement with those proposed by other authors working in similar conditions (Rotger et al., 2006); suggesting that young ruminants may be able to adapt to specific challenges, such as

high-concentrate diets. Hypothetically, the young rumen seems to be malleable enough to sustain extreme fermentation conditions and the role of ruminal microbial community in such process may be crucial.

In ruminants, ammonia-N concentration in rumen is commonly used to check the adequacy of N supply for microbial growth. On average, ruminal ammonia-N concentration was very low, especially in growing bulls fed the LP diet. The optimal ruminal ammonia-N concentration for maximal microbial growth has been fixed at 50 mg/L (Satter and Slyter, 1974). However, the existence of a threshold level for maximal microbial growth has long been questioned because N requirements may vary in function of ruminal fermentation conditions (Balcells, 1990). It has been recognized that the necessary ammonia-N concentration to fulfill microbial requirements is lower when high-concentrate diets are employed (Ludden and Cecava, 1995; Devant et al., 2000, 2001) and, in such situation, microbes may preferentially utilize alternative N sources such as soluble amino acids or preformed peptides (Williams and Cockburn, 1991). In a similar sense, Russell et al. (1983) showed that non-structural carbohydrates fermenting bacteria derived 66 % of their N from peptides or amino acids and 34 % of their N from ammonia; so specific N incorporation mechanisms could allow the surveillance and growth of certain microbial populations under low ammonia-N concentration.

Recorded ruminal VFA concentration was similar to those described in other trials employing high-concentrate diets with rapidly degradable carbohydrates (Ludden and Cecava, 1995; Devant et al., 2000); and it was only penalized by protein restriction in finishing animals, in accordance with other authors working with animals of similar age (Ceconi et al., 2015). Protein reduction also decreased propionate concentration in the growing period (Reynal and Broderick, 2005), possibly due to the considerable quantities of propionate derived from the degraded protein (Van Bruchen et al., 1985).

4.2 Ruminal microbial community composition

Rumen microbiota represents a complex network where bacteria, archaea, protozoa and anaerobic fungi work in harmony contributing to the health and productivity of ruminants, as described by Kumar et al. (2015). Factors that influence the composition of gut microbiota, as well as other microbial communities, can include stochastic processes, such as dispersal, genetic diversification and ecological drift (Pereira and Berry, 2017). However, deterministic interactions between species, individuals and the environment also create defined niches and thus might influence community composition. We hypothesized that rumen microbiome changes, not only as ruminant animal ages, but under different dietary conditions and that these microbial shifts may be responsible for resilience to protein reduction. For this reason, we characterized ruminal bacterial and archaeal populations. Protozoa abundance in animals fed high-concentrate diets is proved to be minimal due to the reduction of rumen pH caused by this type of diets (Mackie et al., 1978); likewise, fungal population is known to be scarce in diets rich in starch and soluble carbohydrates (Grenet et al., 1989), so neither protozoa nor fungi were quantified in this study.

Bacteroidetes and Firmicutes were detected as the dominant phyla in rumen; Actinobacteria was the third phylum in order of importance and Proteobacteria and Fibrobacteres were scarcely found. According to existing studies of ruminal microbiota shifts when transitioning to a high-concentrate diet (Fernando et al., 2010; Petri et al., 2013; Tapio et al., 2017), Bacteroidetes abundance (aforementioned studies, 25 – 40 %) does normally rise with increasing amount of concentrate in diet (mainly due to a rise in its main component *Prevotella*). Moreover, Bacteroidetes titers have been proved to be higher when (i) the liquid fraction of ruminal content is analyzed, (ii) when the bead-beating time is short or (iii) when the QIAamp DNA Stool Mini Kit is used as the DNA extraction method (Henderson et al., 2013), being all those three conditions met in the present study. Actinobacteria role in ruminal

fermentation is not very well known (Šuľák et al., 2012) and, in our study, its abundance was higher than that previously observed in animals in high-concentrate diet conditions (aforementioned studies, 0.5 – 1.5 %). Proteobacteria abundance was low in comparison with the aforementioned studies (13 – 20 %) and a similar decrease has been previously related to feed restriction in bulls (McCabe et al., 2015). Fibrobacteres minimal abundance was in accordance with the cited literature in animals with such low fiber ingestion. High-concentrate diets are also known to reduce archaeal abundance (Belanche et al., 2012; Tapio et al., 2017), so its minor presence in the current study was already expected.

Microbial biodiversity levels in terms of richness, Shannon index or Simpson index were low because they do normally experiment a decrease when animals are fed a high-concentrate diet (Belanche et al., 2012; Petri et al., 2013; Tapio et al., 2017). The mentioned authors reported higher biodiversity levels than ours (Richness: 138 – 148 OTUs/animal; Shannon index: 3.2 – 4.6; Simpson index: 0.8 – 0.9) possibly because their studies used animals previously fed with high-forage diets and, consequently, the initial microbial biodiversity in rumen could have been more elevated than that registered in our animals. Moreover, excluding Petri et al. (2013) trial, diets employed in the other studies did not reach the 10/90 forage-to-concentrate ratio.

In the transit from growing to finishing period, microbial biodiversity levels tended to evolve towards a more heterogeneous microbiota, which partially agrees with Jami et al. (2013) and Dill-McFarland et al. (2019) who reported an age-dependent increase in bacterial diversity, as well as in within-age-group similarity. More specifically, CTR animals presented lower alpha diversity values than the rest of animals and periods; leading us to think that, at this early period, CP level was limiting and promoted an increase in ruminal microbial biodiversity levels in order to overcome such substrate limitation (Langenheder and Prosser, 2008). Shabat et al. (2016) have outlined a possible relationship between performance and alpha diversity

and it seems that more efficient animals present lower diversity and higher dominance of specific species. In this sense, the increased microbial biodiversity caused by the restriction in CP availability would have a detrimental effect on the efficiency of utilization of that nutrient by LP animals, which could ultimately be the reason of their reduced growth rate at the beginning of the fattening period.

Bulls' age did affect ruminal microbiota at phylum level: we found descending titers of Bacteroidetes and increasing titers of Firmicutes, while archaeal abundance was not altered by age in accordance with Liu et al. (2017a).

Rumen maturation did alter rumen microbiota also at genus level. *Prevotella* and *Bifidobacterium* abundance decreased while some Clostridiales (*Roseburia, Agathobacter, Ruminococcus)* and *Olsenella* increased. *Prevotella* genus can fill a variety of niches thanks to its diverse metabolic capabilities (it is mainly known for their starch-degrading and proteolytic capacity but they play a role in fiber degradation), being hard to interpret its ecological role in rumen (Stevenson and Weimer, 2007); its age-dependent decrease has already been reported elsewhere (Liu et al., 2017a). *Bifidobacterium* genus is normally abundant in the gastrointestinal tract of young mammals because milk glycans enhance its proliferation (Pacheco et al., 2015); we hypothesize that, after weaning, *Bifidobacterium* population started to progressively decrease its abundance in bulls' ruminal ecosystem, still being detectable at low abundance in GRO but not in FIN. *Roseburia* and *Agathobacter* genera are members of Clostridium Cluster XIV and they produce butyrate as a fermentation end product (Rosero et al., 2016); to our knowledge, their age-dependent variation in rumen has not been reported yet. *Ruminococcus* genus, which also increased its abundance as animals aged in the study of Liu et al. (2017a), includes cellulolytic and non-cellulolytic species (La Reau et al., 2016). *Olsenella* is a markedly peptidolytic and lactic acid producing

genus that is usually found in the gastrointestinal tract of both ruminants and non-ruminants (Kraatz et al., 2011); its age-shift is not mentioned in any of the consulted studies.

In the present study and as animals matured, microbial co-occurrence pattern evolved to a tighter and rich network but only in LP-fed individuals, while in CTR-fed ones microbial network remained stable. The observed evolution in LP-fed animals agrees with similar challenge in dairy cows: available literature reveals that the extent of co-occurrence among microbial domains in young ruminants is naïve but, with rumen maturation, there is a greater interdependency across microbial domains (Kumar et al., 2015).

Current data analyzing how the dominant ruminal microbiota species or their relationships respond to a reduced protein intake is not conclusive (Wang et al., 2017; He et al., 2018). However, in goats fed mixed or forage diets, the richness of fiber-, protein- and fat-degrading bacteria increased with their specific substrate content in the ration (Liu et al., 2017b). In a similar approach, Belanche et al. (2012) described that protein restriction could alter microbial populations involved in carbohydrate degradation, having cellulolytic microbiota more sensitivity to the challenge.

Animals fed LP diet presented higher levels of microbial biodiversity than CTR animals during the growing period, which to some extent agrees with data reported by Peng et al. (2017) who found increased bacterial diversity in pig's hindgut compartment when CP content was moderately reduced.

At phylum level, CP restriction reduced Firmicutes abundance, confirming previous values described by Luo et al. (2015) in the cecum and Fan et al. (2017) in the ileum of pigs.

At genus level, the CP optimization led to a lower abundance of the lactate producer *Sharpea* (Morita et al., 2008), which has not been seen by any of the aforementioned authors. In contrast, *Bifidobacterium* levels remained higher when dietary protein was reduced. Considering that Peng et al. (2017) observed just the opposite in their study, we suspect that

the rise in *Bifidobacterium* titers might be due to the difference in EE content of the two dietary treatments (72 g EE/kg DM in LP concentrate and 51 g EE/kg DM in CTR concentrate), as this genus has been proved to be actively involved in lipid metabolism in rumen (Gorissen et al., 2010).

Considering that i) the proportion of shared sequences was extremely high, ii) unique OTUs gathered scarce sequences and iii) there were no major differences in alpha biodiversity levels between treatments, we expected that microbial network architecture under different dietary conditions would have been similar. However, LP-fed animals had a more complex ruminal microbial community in terms of microbial co-occurrence patterns than CTR-fed animals. The fact that microbial associative patterns are diet specific has already been observed in dairy cows fed diets with different forage-to-concentrate ratios (Kumar et al., 2015; Tapio et al., 2017). In the same line, an increasing degree of functional interdependence among genera with a progressive decrease in nutritional status has been described when comparing gut microbiome of healthy and malnourished children (Ghosh et al., 2014), so describing a similar phenomenon as that observed in the present study.

Functional redundancy, or the co-existence of functionally-similar organisms, is often considered to be an important feature of the gut ecosystem that contributes to robustness and resilience (Moya and Ferrer, 2016). However, some key metabolic activities may be restricted to one or few species, called “keystone” species or taxa. A keystone species has a large impact on the rest of the community and, in most occasions, has a disproportionally low abundance relative to its impact on the ecosystem (Mills et al., 1993). The fact that the vast majority of correlating genera were low-abundant supports the earlier assumption that minor species may hold key functions in rumen, for example, in the fermentation of proteins, mucins and toxic plant metabolites (Prins and Stewart, 1997; Tapio et al., 2017).

In our study we observed a general lack of agreement between the abundance of bacterial and archaeal genera and rumen fermentation parameters across diets. This suggests that functional diversity may occur even with similar taxonomical distribution (Belanche et al., 2019), relying on ruminal microbial community redundancy; i.e., the overlapping distribution of physiological capabilities across multiple microbial taxa (Weimer, 2015). For long, it has been known that the peripheral pathways of polymer cleavage are more diverse than the ensuing monomers processing pathways; so it seems that for every microbial function there are several candidates, no one identical to each other (Prins and Stewart, 1997).

5. Conclusions

Reducing CP content from 140 g/kg to 120 g/kg (on a DM basis) did not have major impacts on either DM intake or animal performance. Crude protein limitation did not penalize DM or OM apparent digestibility but it did reduce CP apparent digestibility and N waste. Statistical differences in ammonia-N due to dietary CP content could not be found. Low protein-fed bulls showed more diverse and complex ruminal microbiota with greater functional interdependency among genera; thus indicating that ruminal microbiota may be playing a crucial role in cattle resilience to protein restriction.

Acknowledgements

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Supplementary information

Supplementary Table 1 Correlation between ruminal bacterial and archaeal taxa in growing cattle.

*Obtained in intensively reared Holstein bulls (160 d of age and 225 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 120 g/kg crude protein on a dry matter basis) plus barley straw. Only Spearman correlations with r > 0.60 and P < 0.05 are shown (n = 19). Green and red values indicate positive and negative correlations, respectively. * means that genera had statistically different ruminal abundance (P < 0.05) between CTR- and LP-fed animals.*

Phylum	Genera	CORRELATIONS											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Actinobacteria	*Arthrobacter*						-0.727		0.816		-0.816		-0.692
	*Bifidobacterium**											0.709	
	Corynebacterium	0.672											
	Dietzia	0.793											
	Leifsonia					0.711			0.837		-0.837		
	Nesterenkonia										-0.940		
	Prauserella								0.693				
	Sanguibacter								0.822		-0.822		
Bacteroidetes	*Bacteroides**	-0.638		-0.723			-0.767				-0.833		
	Chryseobacterium								0.693				
	Prevotella							-0.782		0.818	0.700	-0.721	
Fibrobacteres	*Fibrobacter*							0.854		-0.896			

Supplementary Table 1 Continued.

Phylum	Genera	CORRELATIONS[1]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Firmicutes	*Agathobacter*						-0.733		0.717		-0.750		
	Butyrivibrio						-0.792		0.911		-0.690		-0.885
	Clostridium		0.686				-0.929		0.828	-0.719			-0.828
	Desemzia							0.664		-0.710			
	Dialister							0.663		-0.644			0.700
	Eubacterium							0.661		-0.685		0.770	
	Facklamia	0.652											
	Phascolarctobacterium						-0.883		0.953		-0.831		-0.822
	Planomicrobium						-0.730		0.707				
	Roseburia						-0.800	0.745	0.733	-0.770	-0.733	0.721	
	Ruminococcus						-0.917	0.939	0.933	-0.927			-0.933
	Sharpea *									-0.685			
	Staphylococcus						-0.681		0.792		-0.800		
	Streptococcus					0.731	-0.738						-0.738
	*Weissella**	0.759									-0.732		
Proteobacteria	*Acinetobacter*						-0.863		0.932		-0.794		-0.794
	Alysiella		0.667										
	Brevundimonas						-0.671		0.820				-0.783
	Pseudomonas								0.817		-0.908	-0.674	
	Psychrobacter								0.678				
	Stenotrophomonas												-0.725

Supplementary Table 2 Correlation between ruminal bacterial and archaeal taxa in finishing cattle.

*Obtained in intensively reared Holstein bulls (280 d of age and 444 kg of body weight). Animals were fed two concentrates: control (CTR: 140 g/kg crude protein on a dry matter basis) or low protein (LP: 12 g/kg crude protein on a dry matter basis) plus barley straw. Only Spearman correlations with r > 0.60 and P < 0.05 are shown (n = 19). Green and red values indicate positive and negative correlations, respectively. * means that genera had statistically different ruminal abundance (P < 0.05) between CTR- and LP-fed animals.*

Phylum	Genera	CORRELATIONS											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Actinobacteria	*Bifidobacterium**							-0.748		0.632			
	Georgenia	0.757											
	Kocuria									0.658			
	Microbacterium									-0.722			
	Rothia				0.820								
	Serinicoccus									-0.662			
Bacteroidetes	*Bacteroides**			0.707		-0.683							
	Prevotella									0.794			
Euryarchaeota	*Methanobrevibacter*			0.675			0.736					0.782	
Firmicutes	*Agathobacter*								0.867		-0.750		
	Bacillus							-0.833		0.768		-0.638	
	Dialister												
	Eubacterium		0.723								-0.733		
	Globicatellla			0.733									
	Jeotgalicoccus									-0.665			
	*Mitsuokella**								-0.683				
	Planococcus		-0.729										
	Planomicrobium	0.737											

Supplementary Table 2 Continued.

Phylum	Genera	CORRELATIONS[1]											
		pH		Ammonia-N		VFA		Acetate		Propionate		Butyrate	
		CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP	CTR	LP
Firmicutes	*Pseudobutyrivibrio*									-0.693	-0.733		0.767
	Roseburia	-0.644									-0.733		
	Ruminococcus									-0.758			
	Selenomonas							-0.636					
Proteobacteria	*Alysiella*		0.698		0.935		-0.811						
	Bibersteinia										-0.694		
	Halomonas											0.642	
	Paracoccus	0.739		0.694		-0.850							
	Pseudaminobacter											0.655	
	Pseudomonas								-0.766				
Spirochaetes	*Treponema*							0.679		-0.673			

CHAPTER III

Nutrient utilization efficiency, ruminal fermentation and microbial community in Holstein bulls fed concentrate-based diets with different forage source

Abstract

Ruminal acidosis can penalize cattle performance and modify ruminal microbiota composition; in that sense, providing quality forage may be a useful tool to cope with such dysfunction. This assay aimed to control animals' performance and assess ruminal microbiota shifts and interactions when fattening Holstein bulls are fed corn-based concentrate and different quality forages. Thirty animals (from 119 to 332 d of age and from 164 to 511 kg body weight [BW]) were fed corn-based concentrate and were allotted to three experimental treatments or forage sources: barley straw, considered as control (*Hordeum vulgare*, CTR), oats haylage (*Avena sativa*, OATS) or vetch haylage (*Vicia sativa*, VETCH). Both concentrate and forage were supplied *ad libitum* in a free choice system and animals had free access to drinking water. Bulls' BW and concentrate intake were automatically recorded on a daily basis. Feces and ruminal fluid were sampled twice, during the growing period (158 d of age and 220 kg BW) and during the finishing period (280 d of age and 434 kg BW), for digestibility, ruminal fermentation and microbial population characterization. No differences in concentrate intake or BW could be detected between CTR and OATS-fed animals; however, VETCH-fed bulls had lower concentrate intake ($P < 0.047$) and slaughter BW ($P = 0.034$) than CTR. The use of oats haylage as forage source did not affect nutrient apparent digestibility rates but vetch haylage did penalize dry matter ($P = 0.048$) and crude protein ($P < 0.001$) digestibility in finishing animals. Differences in neither ruminal volatile fatty acids concentration nor pH were detected, but acetate-to-propionate ratio increased with the incorporation of vetch haylage in diet ($P < 0.041$). Ammonia-nitrogen concentration in ruminal fluid was low (20.63 ± 2.55 mg/L) but it significantly improved when oats ($P = 0.001$, only in finishing) and vetch ($P = 0.001$) haylage were provided. Core microbial community gathered 75 % of analyzed sequences; however, ruminal microbial community

was different between CTR and OATS ($P < 0.001$) or VETCH ($P < 0.001$) bulls. Shannon and Simpson diversity indexes were improved by quality forage feeding, mainly during the growing period ($P < 0.081$ in OATS and $P < 0.004$ in VETCH). Microbial network analysis revealed that the use of oats or vetch haylage promoted an increase in the overall network complexity, basically in terms of node degree and betweenness centrality. Some lactate-degrader genera were found to be central nodes in the microbial network when quality forage was provided, highlighting their probable implication in ruminal acidosis prevention.

1. Introduction

Climate conditions in the Mediterranean basin do not sustain a significant yield of quality forage; therefore, beef cattle are usually reared under intensive feeding systems characterized by the use of cereal products: cereal-based concentrate is provided as the main dietary component and cereal straw as the forage source (Preston, 1974). The high-nutrient availability of these concentrate-based diets enables calves to maximize intake, growth and feed efficiency (Ørskov, 1998). However, massive starch ingestion enhances ruminal microbial fermentation and volatile fatty acids (VFA) production, under a situation in which saliva buffering capacity is reduced by the decreased chewing and rumination activity induced by the low-roughage intake. Additionally, if the absorptive capacity of the ruminal wall is impaired by abnormal ruminal papillae or rumenitis (Zhao et al., 2018), the animals' ability to maintain a stable ruminal pH is disrupted (Nagaraja and Lechtenberg, 2007), leading to a situation of ruminal acidosis, under either acute or sub-acute (SARA) forms. Animals affected by ruminal acidosis may develop anorexia, dehydration, intermittent diarrhea and laminitis, and also present unexplained abscesses (Kleen et al., 2003). Under SARA, rumen dysfunction is neither intense nor long enough to cause obvious clinical signs, but intake and performance are reduced (Nagaraja and Lechtenberg, 2007).

The use of antimicrobials (i.e., ionophores as feed additives) does alleviate pH-depression symptoms; however, when in-feed antibiotics were banned (EU Regulation 1831/2003), ruminal acidosis incidence suddenly increased and that caused a need for identifying new methods to prevent rumen dysfunction. To date, a wide range of tools for controlling ruminal acidosis have been developed, both in dairy (Enemark, 2008; Krause and Oetzel, 2006) and beef (González et al., 2012) cattle. Appropriate diet balancing and feeding management is recurrently used (Calsamiglia et al., 2012; Kleen et al., 2003) and, specifically, the amount and quality of forage provided has awakened great interest because forage reduces ruminal fermentation intensity, stimulates salivation and reduces pH depression, and thus the risk of developing ruminal acidosis (Mialon et al., 2008; Yang and Beauchemin, 2009).

Over the last decades, previous research has focused on the evolution of single microbial populations directly involved in ruminal acidosis, such as lactate-producers (*Streptococcus bovis, Selenomonas ruminantium ssp. ruminantium, Lactobacilli*) and lactate-degraders (*Mehasphaera elsdenii, Selenomonas ruminantium ssp. lactilytica*) (Balcells et al., 2012). However, the high complexity of ruminal ecosystem suggests that interaction patterns between microbial populations should explain rumen function and its robustness against disturbances (Costa-Roura et al., 2020); therefore, it is necessary to study microbial shifts and correlations as a whole to reveal, from a holistic point of view, rumen's adaptive capacity to acidosis condition. So far, SARA has been observed to modify the ruminal microbial community, probably due to the increased fermentable substrate availability that favors the growth of amylolytic and other starch-degrading populations (Mao et al., 2013). In this scenario, both reduction in ruminal microbiota richness and diversity and increase in Firmicutes-to-Bacteroidetes ratio have been described (Plaizier et al., 2017).

This assay aimed to evaluate animals' performance and assess changes and interactions that occurred in ruminal microbiota when fattening Holstein bulls fed concentrate-based diets

received different forage sources. To authors' best knowledge, the applicability of such approach to young ruminants fed *ad libitum* in a free choice system has yet to be explored.

2. Materials and methods

2.1 Animals, diets and housing

This experiment was conducted at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* (Ivars d'Urgell, Spain, 41°41'50"N, 0°58'53"E) between January and August 2017. All procedures were carried out under Project License CEEA 01-07/16 and approved by the in-house Ethics Committee for Animal Experiments at the University of Lleida. Care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purpose.

Thirty Holstein bulls (mean ± standard error: 119 ± 1 d of age), raised under the same conditions from two weeks of age, were group-housed in three outdoor paved and covered pens equipped with two feedbunks each: an individual one for concentrate and a common one for forage. Animals were fed corn-based concentrate and, according to their body weight (BW, 164 ± 3 kg), they were allotted to three experimental treatments or forage sources: barley straw, considered as control (*Hordeum vulgare*, CTR) because it is the most commonly used forage in the diet of intensively reared fattening cattle at the north-east region of Spain, oats haylage (*Avena sativa*, OATS) or vetch haylage (*Vicia sativa*, VETCH) (concentrate and forage composition is detailed in Table 9). In each experimental treatment, both concentrate and forage were supplied *ad libitum* in a free choice system and animals had free access to drinking water.

Table 9 Ingredients and chemical composition of concentrate and forages.

ADF: acid detergent fiber; CP: crude protein; DDGS: distillers dried grains and solubles; DM: dry matter; EE: ether extract; FM: fresh matter; NDF: neutral detergent fiber; OM: organic matter; PDIN and PDIE: protein digestible in the small intestine allowed by protein and energy; UFC: forage unit for meat production.

Item	Concentrate	Barley straw	Oats haylage	Vetch haylage
Ingredient composition, g/kg FM				
Corn	431.2			
Bran	204.5			
Barley	99.5			
Corn DDGS	65.0			
Wet ear of corn haylage	69.8			
Palm kernel meal	30.0			
Palm kernel oil	23.6			
Calcium carbonate	26.9			
Sugarcane molasses	18.4			
Soybean hunks	15.6			
Sodium bicarbonate	7.8			
White salt	3.5			
Urea	2.5			
Vitamin/mineral premix	2.0			
Chemical composition, g/kg DM				
DM, g/kg FM	856.2	857.1	489.2	628.5
OM	934.8	842.8	925.8	897.9
CP	140.7	71.9	100.4	164.4
EE	51.4	14.2	26.5	18.7
NDF	202.5	754.5	517.2	442.1
ADF	70.7	437.7	285.0	281.8
Nutrient composition				
UFC, UFC/kg DM	1.00	0.36	0.55	0.73
PDIN, g/kg DM	89.75	40.83	61.70	93.65
PDIE, g/kg DM	89.90	52.61	57.41	58.02
PDIN/UFC	90.29	113.42	112.06	127.63
PDIE/UFC	90.44	146.14	104.27	79.07

2.2 Measurements and sampling

The experimental phase included the whole fattening period. The first 150 d of trial were considered as growing period, beginning when animals were 119 ± 1 d of age and 164 ± 3 kg of BW and ending when they were 269 ± 1 d of age and 423 ± 8 kg of BW. After that, the finishing period began and continued until animals reached commercial slaughter weight (332 ± 2 d of age and 511 ± 8 kg of BW).

Daily animal BW and concentrate intake were automatically controlled by a feed station, which was equipped with a feedbunk and an individual tunnel-type feeder both provided with a scale. When a calf entered the feeder, it was identified, its BW was registered and concentrate intake was obtained by difference between initial and final feedbunk weight.

Feces and ruminal fluid samples were taken on days 37, 38, 42 and 43 of the trial, corresponding to growing period (GRO) (158 ± 1 d of age and 220 ± 4 kg of BW), and on days 160, 161, 162 and 163 of the trial, corresponding to finishing period (FIN) (280 ± 1 d of age and 434 ± 7 kg of BW) for digestibility, ruminal fermentation and microbial population characterization.

Fecal excretion and forage intake were calculated based on concentrate intake and adapting the two indigestible markers system (Owens and Hanson, 1992), using chromium oxide (Cr_2O_3) as external marker and acid insoluble ash (AIA) as internal marker. Chromium oxide was mixed with concentrate (150 mg/kg in GRO and 90 mg/kg in FIN) and administered during 15 d. Fecal samples (approximately 50 g, one sample per animal and day, obtained in the morning on the last 4 d of Cr_2O_3 administration) were collected using rectal stimulation and stored at -20 ºC until further marker determination (both Cr_2O_3 and AIA) and proximate chemical analysis. After thawing, fecal samples from each animal were pooled and mixed to produce one grab sample per collection period. During sampling days, concentrate and forage samples were also collected and stored at 5 ºC until marker and proximate analysis.

Ruminal fluid samples (one sample per animal and collection period [GRO and FIN], obtained in the morning) were collected using an oral stomach tube connected to a vacuum pump. Each sample was obtained through two sequential collections: firstly, ruminal fluid (approximately 200 mL) was collected and discarded to avoid sample contamination with saliva that could get into the tube during its introduction through the animal's mouth and esophagus. After that, ruminal fluid (approximately 200 mL) was re-extracted, strained through a cheese-cloth and its pH recorded (Testo 205, Testo AG, Germany). Then, 15 mL of ruminal fluid were immediately frozen on dry ice and stored at -80 ºC for subsequent DNA extraction and molecular analysis. The remaining ruminal fluid was sampled for ammonia-nitrogen (N) (2 mL over 0.8 mL of 0.5 N HCl) and VFA concentration (4 mL over 1 mL solution of 0.4 M ortho-phosphoric acid and 0.02 M 4-methylvaleric acid as internal standard, in distilled water). Samples were immediately frozen on dry ice and stored at -20 ºC until analysis.

2.3 Chemical analysis

Feed and feces dry matter (DM) (index nº 934.01), ash (index nº 942.05), ether extract (EE) (index nº2003.05) and N (index nº 990.03) contents were determined according to the AOAC methods (AOAC, 2006).

Neutral detergent fiber and acid detergent fiber analyses in feed were carried out following the sequential procedure of Van Soest et al. (1991), with the Ankom200/220 fiber analyzer (Ankom Technology, USA). Neutral detergent fiber was assayed with a heat stable amylase.

Chromium as external marker was analyzed as follows. Feed and feces samples (0.5 g) were calcined (550 ºC, 2 h) and digested with 3 mL HCl (1:1) in a sand bath (60 ºC, until dry). Residue was then dissolved in 3 mL HCl (1:1), filtered and washed with 50 mL of hot

distilled water. Chromium concentration was quantified by inductively coupled plasma mass spectrometry (7700x, Agilent Tecnologies, USA).

Acid insoluble ash was analyzed according to a standard procedure (BOE, 1995) based on the method of Shrivastava and Talapatra (1962). Briefly, residues of ash content determination were introduced in an Erlenmeyer flask and then hydrolyzed with 75 mL of 3 N HCl and boiled for 15 min. Samples were then filtered through ash-free filter paper (cat nº 1004 150, Whatman) and then the residues were washed with 50 mL of hot distilled water. Filters with residues were dried (103 ºC, 2 h) and then ashed (550 ºC, 3 h) in a tared crucible. Both crucible and its content were left in a desiccator to settle at room temperature and weighed to calculate the AIA content.

Ammonia-N concentration was determined by the Chaney and Marbach (1962) method after sample centrifugation (13800 g, 30 min).

Volatile fatty acid concentration and molar VFA profile were determined by gas chromatography according to the technique proposed by Jouany (1982), using a capillary column (GS-BR-SWAX 30m x 0.25 mm D.I. x 0.25 µm, Bruker, USA) and a flame ionization detector with helium as the carrier gas at 1 mL/min. The sample was injected in split mode (1 µL, 1:50). The oven temperature program increased from 110 ºC to 158 ºC at 6 ºC/min. The injector and detector temperatures were 220 ºC and 230 ºC, respectively.

2.4 Extraction and sequencing of DNA

Extraction of DNA was carried out on freeze-dried ruminal fluid (the initial amount of sample was 60 mg) through physical disruption (1 min) using a bead beater (Mini-bead beater 1, BioSpec Products, USA) and subsequent DNA purification with the QIAamp DNA Stool Mini Kit (ID: 51504; QIAGEN N.V., Germany), with the modifications of greater temperature (95 ºC) to improve cell lysis and greater elution time (3 min). Amplification of

DNA was carried out by using primers 341F and 805R, which target the V3 and V4 regions of the bacterial and archaeal 16S rRNA. Sequencing was conducted on an Illumina MiSeq 2x300 platform. Sequencing of 16S rRNA genes was performed by Era7 Bioinformatics (Spain).

2.5 Bioinformatics

Sample reads were assembled by using FLASh software (Magoč and Salzberg, 2011) and quality filtered using FastQC software (Babraham Bioinformatics, Babraham Institute, U.K.). Operational Taxonomic Units (OTUs) were picked using BLAST software (National Center for Biotechnology Information [NCBI], USA) with a 98 % similarity threshold. Taxonomy assignment of OTUs was performed by comparing sequences to *Taxonomy* database by NCBI (USA), according to the lowest common ancestor method.

2.6 Calculations and statistical analysis

Fecal excretion (*FcE*) was calculated using chromium concentration in both concentrate and feces, as follows (Eq. 1):

$$(Eq.1)\ Fecal\ excretion\ = \frac{[Cr]c * CI}{[Cr]fc}$$

Where *[Cr]c* and *[Cr]fc* are chromium concentrations in concentrate and feces, respectively, and *CI* is concentrate intake.

Forage intake (*FrI)* was calculated using the estimated fecal excretion (Eq. 1) and the AIA concentration in both feed and feces, as follows (Eq. 2):

$$(Eq.2)\ Forage\ intake = \frac{[AIA]fc * FcE - [AIA]c * CI}{[AIA]fr}$$

Where *[AIA]fc*, *[AIA]c* and *[AIA]fr* are AIA concentrations in feces, concentrate and forage, respectively.

Nutrient total tract apparent digestibility was calculated using registered concentrate intake, estimated forage intake (Eq. 2), estimated fecal excretion (Eq. 1) and nutrient concentration in both feed and feces, as follows (Eq. 3):

$$(Eq.3)\ Apparent\ digestibility\ (\%) = \frac{([N]c * CI + [N]fr * FrI) - ([N]fc * FcE)}{([N]c * CI + [N]fr * FrI)}$$

Where *[N]c, [N]fr, [N]fc* are nutrient concentration in concentrate, forage and feces, respectively.

Performance, intake, apparent digestibility, ruminal fermentation parameters and microbial biodiversity data were analyzed with a mixed model; including treatment (CTR vs OATS vs VETCH), period (GRO vs FIN) and their interaction as fixed effects and animal as random effect, to account for repeated measurements (R Core Team, 2018; lme4 package). Contrasts between CTR and either OATS or VETCH data were performed by the Tukey's method (R Core Team, 2018; emmeans package). Individual samples out of three standard deviations of the mean were discarded and not included to the statistical analysis. Significant effects were declared at $P < 0.05$ and tendency to difference at P between 0.05 and 0.10.

Sequence data were normalized and alpha biodiversity indexes were calculated (R Core Team, 2018; Vegan package) to measure the variability of species within a sample.

To circumvent the compositional bias problem (Calle, 2019; Gloor et al., 2017; Tsilimigras and Fodor, 2016), we applied the Aitchison's centered log ratio (clr) transformation to carry the data to a Euclidean space, after replacing zeros by adding 1 to each value. To measure differences in microbiome composition between samples, beta diversity was approached through performing a partial least squares-discriminant analysis (PLS-DA) based on clr Euclidean distance (R Core Team, 2018; mixOmics package). To test whether differences in microbiota composition between treatments were statistically significant, a permutational multivariate analysis of variance (Adonis) was conducted, based on the clr Euclidean distance and calculating statistical significance after 10000 random permutations (R Core Team, 2018;

Vegan package). To decipher which genera abundance were responsible for the differences between treatments, an ANOVA-like differential expression (ALDEx) analysis was conducted over those genera present at least at 50 % of the individuals (R Core Team, 2018; Aldex2 package) (Fernandes et al., 2013). Finally, to describe the interactions within rumen microbial community, we performed a network analysis through Sparse Correlations for Compositional data (SparCC) technique (R Core Team, 2018; SpiecEasi package) (Friedman and Alm, 2012) over those genera present at least at 50 % of the individuals. Microbial networks were graphically represented (R Core Team, 2018; igraph package) and their complexity was described in terms of number of nodes (genera), number of edges (significant positive or negative correlations), node degree (number of connections that any node establishes with other nodes) and betweenness (measure of centrality in a graph based on shortest paths).

3. Results

3.1 Animal's performance through the whole experimental period

Evolution of animal performance is showed in Figure 9: data starts when animals had a BW of 164 ± 3 kg and finishes when they reached commercial slaughter weight (511 ± 8 kg). OATS feeding altered neither performance (Figure 9A) nor concentrate intake (Figure 9B) in relation to CTR. Contrarily, VETCH feeding decreased bulls average daily gain (ADG) at the beginning (Figure 9A; $P < 0.001$) and at the end (Figure 9A; $P < 0.020$) of the fattening period; therefore, they had lower BW than CTR at slaughter (Figure 9A; $P = 0.033$). Moreover, after the first month of trial, VETCH-fed bulls exhibited lower cumulated concentrate intake than CTR (Figure 9B).

3.2 Intake, apparent digestibility and ruminal fermentation parameters

Data on DM intake, both concentrate and forage, are presented in Table 10. OATS and CTR bulls showed similar concentrate and forage DM intake in both sampling periods; however, VETCH animals tended to consume less concentrate than CTR during GRO and forage intake remained unaffected.

Apparent digestibility coefficients for DM, organic matter (OM) and crude protein (CP) are shown in Table 10. OATS animals digested the same proportion of nutrients than CTR ones. Likewise, VETCH bulls exhibited the same apparent digestibility rates than CTR in GRO but, in FIN, DM and CP digestibility were reduced.

Ruminal fermentation parameters are detailed in Table 10. No differences in ruminal pH between OATS and CTR bulls were detected; however, VETCH animals tended to have higher ruminal pH than CTR in FIN. Ammonia-N data were quite variable (C.V. = 95 %) but statistical differences comparing to CTR were still detectable: ammonia-N concentration was higher in VETCH animals during the whole fattening period whereas OATS bulls presented higher ammonia-N concentration only during FIN. Volatile fatty acids concentration was unaffected by diet, however, main VFA proportion did vary among treatments. Straw substitution for haylage as forage source generally increased acetate and butyrate proportions and decreased propionate proportion. Acetate-to-propionate ratio was higher in either OATS or VETCH-fed bulls than in CTR; however, differences did reach statistical significance only when comparing VETCH and CTR in both sampling periods.

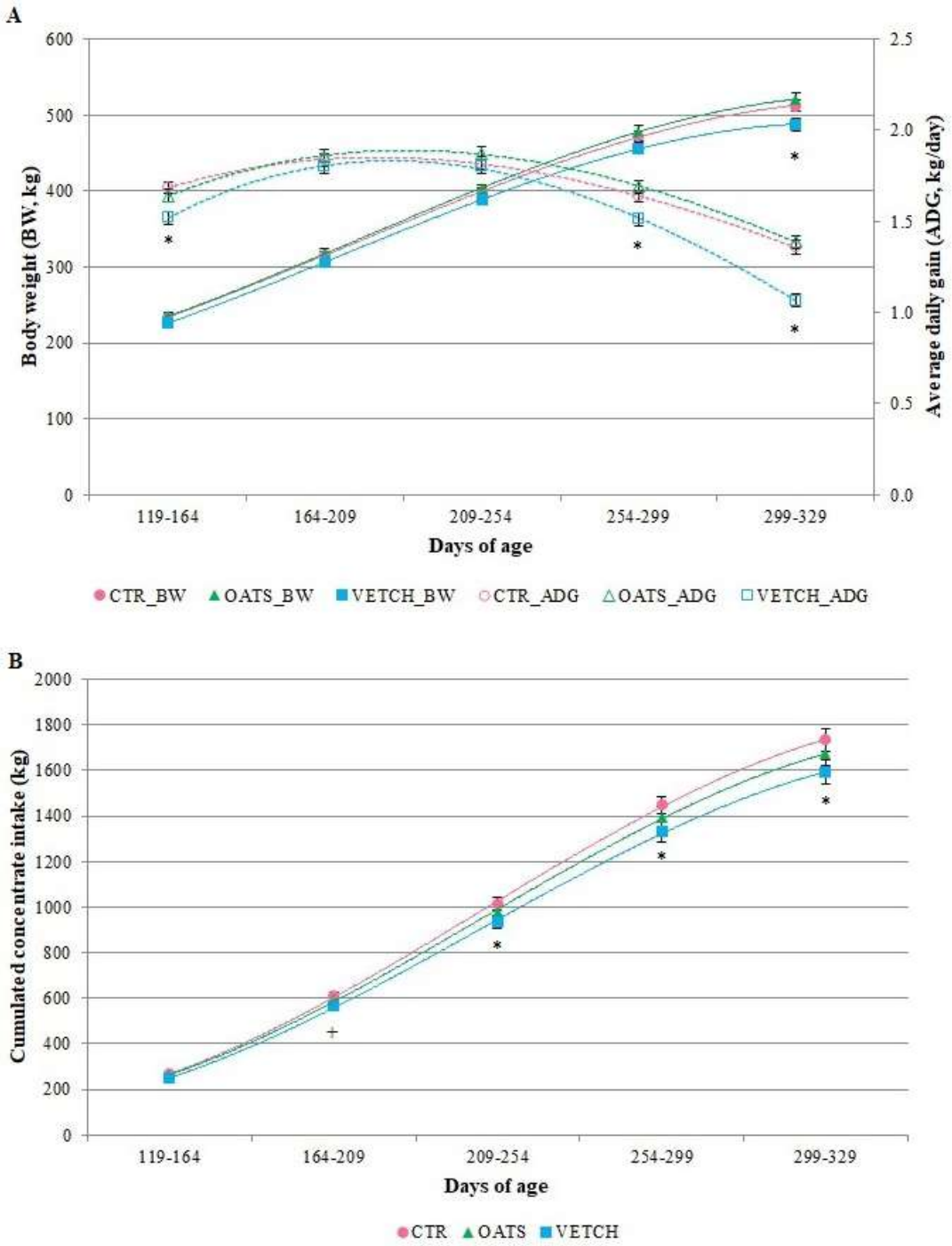

Figure 9 Performance and concentrate intake evolution.

*(A) Body weight and average daily gain and (B) cumulated concentrate intake obtained in intensively reared Holstein bulls (from 119 to 332 d of age). Animals were fed corn-based concentrate and either barley straw (CTR), oats haylage (OATS) or vetch haylage (VETCH) as forage. No statistical differences were found between CTR and OATS-fed animals. * means that statistical difference ($P < 0.05$) and + means that statistical tendency ($0.05 < P < 0.10$) were found between CTR and VETCH-fed animals.*

Table 10 Dry matter intake, nutrient apparent digestibility and ruminal fermentation parameters.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 158 d of age and 220 kg of body weight) and finishing (FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and either barley straw (CTR), oats haylage (OATS) or vetch haylage (VETCH) as forage. Standard error of the mean (SEM) and significance of contrasts between CTR and either OATS or VETCH in both periods are shown. A/P: acetate-to-propionate; CP: crude protein; DM: dry matter; N: nitrogen; OM: organic matter; VFA: volatile fatty acids.

Item	GRO			FIN			SEM	Contrast P-values with CTR			
								GRO		FIN	
	CTR	OATS	VETCH	CTR	OATS	VETCH		OATS	VETCH	OATS	VETCH
n	10	10	10	10	10	10					
Intake											
DM, kg/d	6.46	5.46	5.33	9.45	10.02	9.07	0.397	0.420	0.296	0.889	0.982
Concentrate DM	5.71	5.16	4.76	8.08	7.85	7.21	0.253	0.617	0.086	0.983	0.151
Forage DM	0.76	0.29	0.58	1.36	1.75	1.85	0.242	0.711	0.994	0.857	0.686
Apparent digestibility coefficients											
DM	0.737	0.712	0.749	0.744	0.742	0.690	0.1311	0.696	0.983	0.999	0.048
OM	0.746	0.720	0.763	0.753	0.754	0.708	0.1353	0.685	0.943	0.999	0.172
CP	0.717	0.692	0.677	0.725	0.704	0.633	0.1257	0.685	0.170	0.804	<0.001

Table 10 Continued.

Item	GRO			FIN			SEM	Contrast P-values with CTR			
								GRO		FIN	
	CTR	OATS	VETCH	CTR	OATS	VETCH		OATS	VETCH	OATS	VETCH
n	10	10	10	10	10	10					
Ruminal fermentation parameters											
pH	7.29	7.27	7.13	6.45	6.77	6.83	0.091	0.999	0.825	0.141	0.056
Ammonia-N, mg/L	4.15	9.41	29.86	9.05	32.78	32.58	3.947	0.917	<0.001	0.001	0.001
VFA, mmol/L	66.96	54.99	68.63	91.75	68.58	74.63	6.361	0.767	0.999	0.122	0.412
VFA, mol/100 mol											
Acetate	46.30	50.73	52.09	46.25	49.51	53.09	1.122	0.037	0.003	0.223	0.001
Propionate	43.59	36.51	35.49	41.57	34.05	30.43	1.558	0.027	0.007	0.015	<0.001
Butyrate	6.42	8.01	7.61	7.97	9.97	8.63	0.407	0.055	0.258	0.010	0.824
Ratio A/P	1.07	1.42	1.48	1.14	1.50	1.70	0.107	0.129	0.041	0.102	0.004

3.3 Ruminal microbial community

3.3.1 Data set features

Sequencing procedure yielded an average of 21020 ± 2113 sequences per sample, resulting in 1261229 overall sequences from the study. A total of 816 OTUs were obtained at the 98 % sequence similarity cut-off levels with 116 ± 5 as the mean number of OTUs per sample. Good's coverage value was 99.69 ± 0.03 %, suggesting that more than 99 % of bacterial and archaeal phylotypes were identified. The unclassified rate of OTUs at genus level was 0.67 ± 0.07 %. Shared OTUs by all individuals in each treatment and sampling period were deemed to be core bacterial/archaeal communities. Core community gathered 75.14 ± 1.74 % of analyzed sequences and was composed of 6 OTUs: *Prevotella ruminicola*, unclassified *Prevotella* (both representing more than 80 % of shared sequences), unclassified *Roseburia*, *Sharpea azabuensis*, *Ruminococcus flavefaciens* and unclassified *Methanobrevibacter*.

3.3.2 Microbial community biodiversity

Alpha biodiversity was assessed by Shannon and Simpson indexes and microbial richness (values presented in Table 11). OATS animals had or tended to have higher microbial biodiversity, in terms of Shannon and Simpson indexes, in GRO but not in FIN; although no differences in microbial OTUs richness were detected. In a similar way, Shannon and Simpson index values were higher in VETCH than CTR bulls in GRO but only tended to be in FIN, and differences in microbial OTUs richness were only observed in GRO.

Proteobacteria-to-Firmicutes-plus-Bacteroidetes (P/F+B) ratio is also shown in Table 11 and it was only affected by treatment when comparing VETCH-fed animals and CTR during FIN.

Table 11 Ruminal microbial alpha biodiversity.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 158 d of age and 220 kg of body weight) and finishing (FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and either barley straw (CTR), oats haylage (OATS) or vetch haylage (VETCH) as forage. Standard error of the mean (SEM) and significance of contrasts between CTR and either OATS or VETCH in both periods are shown. P/F+B: Proteobacteria-to-Firmicutes-plus-Bacteroidetes.

Item	**GRO**			**FIN**			**SEM**	**Contrast P-values with CTR**			
								GRO		**FIN**	
	CTR	**OATS**	**VETCH**	**CTR**	**OATS**	**VETCH**		**OATS**	**VETCH**	**OATS**	**VETCH**
n	10	10	10	10	10	10					
Shannon index	1.03	1.52	1.71	1.86	2.26	2.34	0.125	0.081	0.004	0.224	0.091
Simpson index	0.34	0.54	0.63	0.62	0.75	0.78	0.043	0.020	<0.001	0.308	0.100
Richness	97.3	77.0	133.1	108.2	114.2	118.60	7.06	0.338	0.009	0.991	0.902
Ratio P/F+B ($x10^{-3}$)	1.95	1.46	7.57	3.27	4.86	2.67	5.48	0.999	0.972	0.999	0.034

Beta biodiversity is graphically represented in Figure 10. It can be appreciated that samples are clearly clustered by diet, comparing both OATS and VETCH individuals to CTR (Figure 10A and Figure 10B, respectively). Adonis test results confirmed that foreseen differences in ruminal microbiota composition were significant, either when comparing OATS vs CTR ($P < 0.001$) and VETCH vs CTR ($P < 0.001$). ALDEx analysis identified which genera were responsible for differences between dietary treatments and its results are presented in Figure 11 and Figure 12. With respect to OATS vs CTR comparison during GRO (Figure 11A), OATS bulls had significantly higher abundance of *Clostridium*, *Weissella* and *Agathobacter* genera, and significantly lower abundance of *Aerococcus* and *Staphylococcus* genera than CTR. During FIN (Figure 11B), OATS animals had significantly higher presence of *Lactobacillus*, *Bacillus*, *Staphylococcus* and *Alisonella* genera, and significantly lower presence of *Corynebacterium*, *Peptoclostridium* and *Dietzia* genera than CTR. In the case of VETCH vs CTR comparison during GRO (Figure 12A), VETCH animals had significantly more abundant *Fibrobacter* and *Treponema* genera, and significantly scarcer *Prevotella*, *Selenomonas*, *Bifidobacterium*, *Dietzia*, *Candidatus_Phytoplasma*, *Corynebacterium*, *Lactobacillus* and *Aerococcus* genera than CTR. During FIN (Figure 12B), VETCH individuals had significantly higher presence of *Butyrivibrio* genus, and significantly lower presence of *Corynebacterium*, *Dietzia* and *Dialister* genera than CTR.

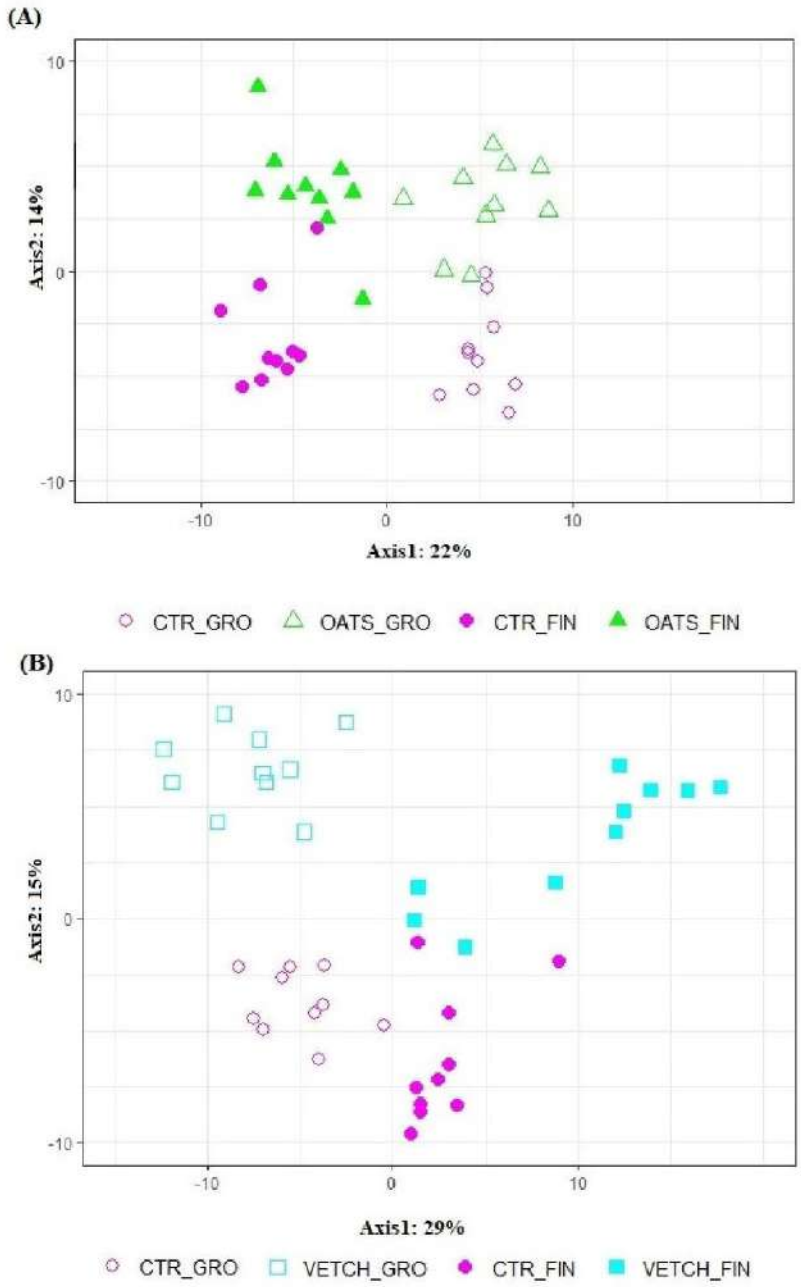

Figure 10 Graphical representation of partial least squares-discriminant analysis (PLS-DA) on bacterial and archaeal OTUs in ruminal fluid.

Obtained in intensively reared Holstein bulls in two periods: growing (GRO: 158 d of age and 220 kg of body weight) and finishing (FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and either barley straw (CTR), oats haylage (OATS) or vetch haylage (VETCH) as forage. Plots show the spatial distribution of CTR in comparison with (A) OATS or (B) VETCH treatments, in both periods. Each point represents a different sample and a greater distance between two points infers a higher dissimilarity between them.

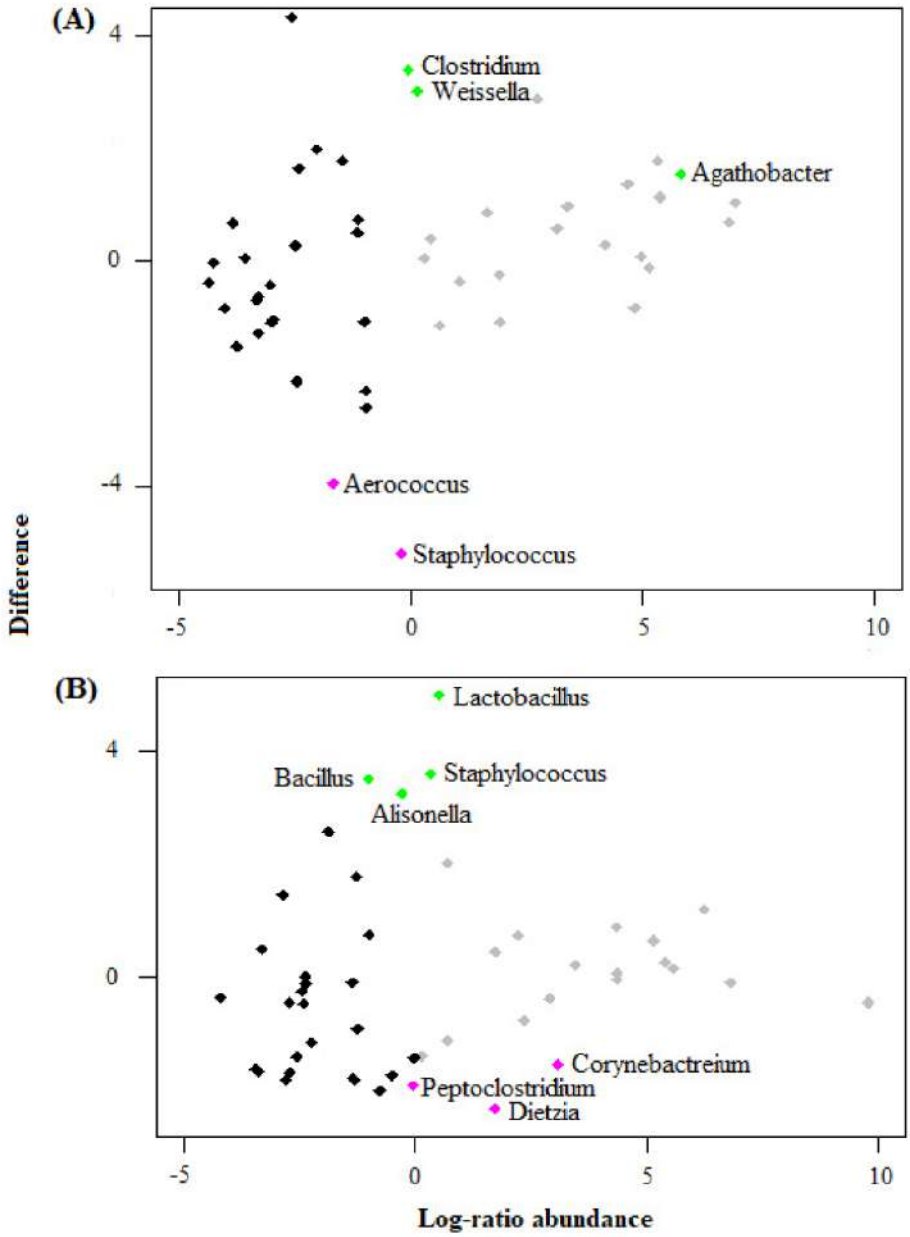

Figure 11 Graphical representation of bacterial and archaeal genera in ruminal fluid. *Obtained in intensively reared Holstein bulls in two periods: growing (A, GRO: 158 d of age and 220 kg of body weight) and finishing (B, FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and either barley straw (CTR) or oats haylage (OATS) as forage. Plots show the relationship between genera abundance and their difference in abundance between treatments. Each point represents a different genus: black dots represent rare genera that are not significantly different between treatments, grey dots represent abundant genera that are not significantly different between treatments, magenta dots represent genera that are significantly more abundant in CTR animals and green dots represent genera that are significantly more abundant in OATS animals.*

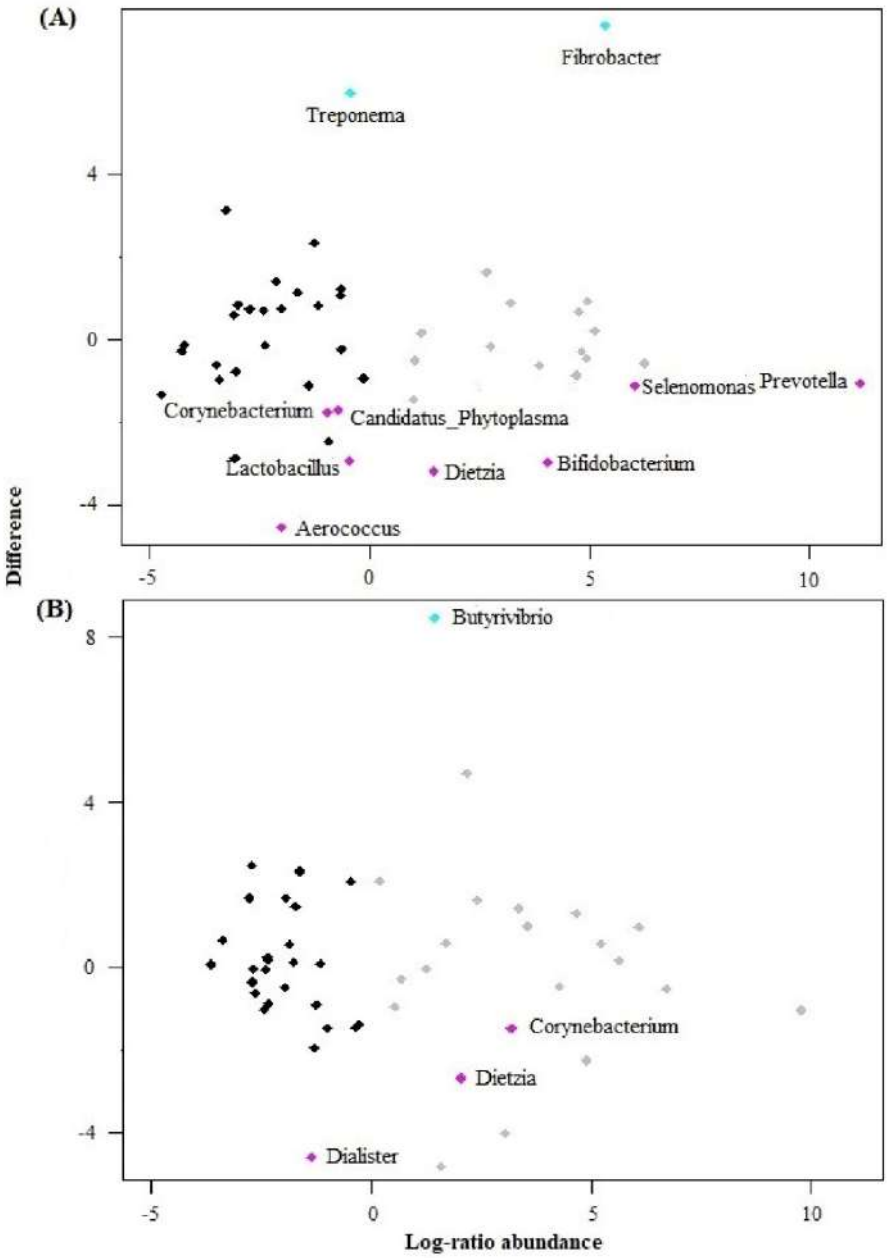

Figure 12 Graphical representation of bacterial and archaeal genera in ruminal fluid. *Obtained in intensively reared Holstein bulls in two periods: growing (A, GRO: 158 d of age and 220 kg of body weight) and finishing (B, FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and either barley straw (CTR) or vetch haylage (VETCH) as forage. Plots show the relationship between genera abundance and their difference in abundance between treatments. Each point represents a different genus: black dots represent rare genera that are not significantly different between treatments, grey dots represent abundant genera that are not significantly different between treatments, magenta dots represent genera that are significantly more abundant in CTR animals and cyan dots represent genera that are significantly more abundant in VETCH animals.*

3.3.3 Microbial network

Microbial networks were built to test bacterial and archaeal genera interactions (Figure 13, Figure 14, Figure 15). Degree of interaction was studied through the number of genera (nodes) that established significant interactions (edges) with other genera, as well as node degree, i.e., number of interactions established per node. When comparing OATS and CTR animals during GRO, OATS bulls had similar number of nodes taking part in the microbial network as CTR (24 in OATS vs 23 in CTR) but higher number of edges (44 in OATS vs 27 in CTR) and average node degree (3.67 in OATS vs 2.35 in CTR). During FIN, microbial network complexity in OATS and CTR dietary treatments became similar, in terms of number of nodes (19 in OATS vs 28 in CTR), number of edges (24 in OATS vs 33 in CTR) and average node degree (2.53 in OATS vs 2.36 in CTR).

When comparing VETCH and CTR bulls during GRO, VETCH animals had higher number of nodes (31 in VETCH vs 23 in CTR), edges (47 in VETCH vs 27 in CTR) and average node degree (3.03 in VETCH vs 2.35 in CTR). In FIN, microbial network structure changed: though VETCH individuals had similar number of nodes building their network as CTR (30 in VETCH vs 28 in CTR), they noticeably increased the number of edges (71 in VETCH vs 33 in CTR) and node degree (4.73 in VETCH vs 2.36 in CTR).

Moreover, we investigated microbial genera that act as main information gateways in networks in terms of betweenness centrality, i.e., extent to which one node lies on paths that connect other nodes. OATS bulls' networks presented higher betweenness centrality than CTR, in both GRO (15.71 in OATS vs 3.13 in CTR) and FIN (9.68 in OATS vs 7.79 in CTR). Likewise, VETCH animals presented more connected networks than CTR, either in GRO (38.94 in VETCH vs 3.13 in CTR) or FIN (29.33 in VETCH vs 7.79 in CTR).

Finally, we identified those genera responsible for networks' betweenness centrality, i.e., the most central and connected genera in the network. Beginning with CTR

bulls, nodes with the highest betweenness centrality were *Ruminococcus*, *Fibrobacter* and *Sharpea* in GRO and *Bifidobacterium*, *Pseudobutyrivibrio* and *Dialister* in FIN. Regarding OATS animals, the most central nodes were *Clostridium*, *Treponema* and *Dialister* in GRO and *Fibrobacter*, *Megasphaera* and *Alisonella* in FIN. In the case of VETCH individuals, the most connected nodes were *Eubacterium*, *Roseburia* and *Bifidobacterium* in GRO and *Selenomonas*, *Methanobrevibacter* and *Olsenella* in FIN.

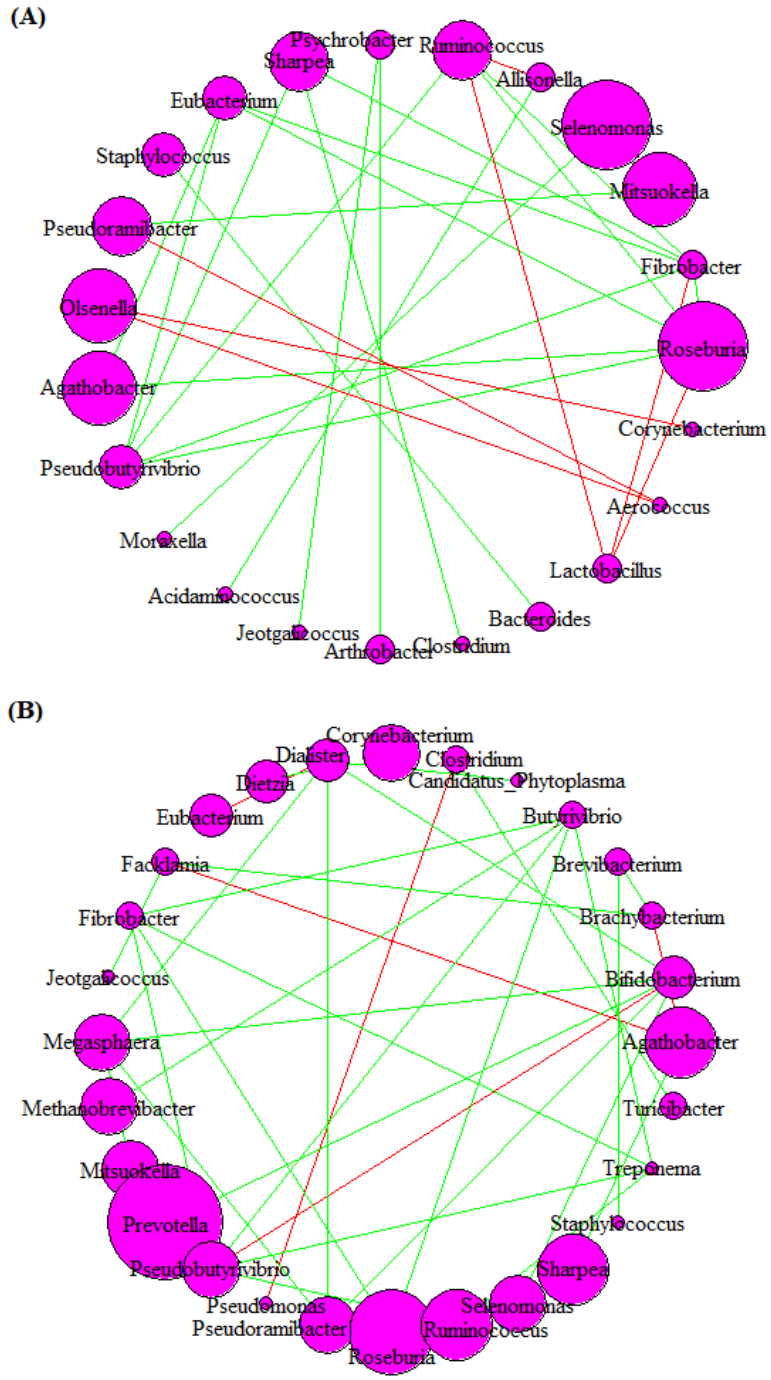

Figure 13 Microbial networks in the rumen of fattening cattle.

Obtained in the rumen of intensively reared Holstein bulls in two periods: growing (A, GRO: 158 d of age and 220 kg of body weight) and finishing (B, FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and barley straw as forage (CTR). Networks were generated based on those genera establishing significant correlations ($r > 0.60$ and $P < 0.05$). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genus abundance.

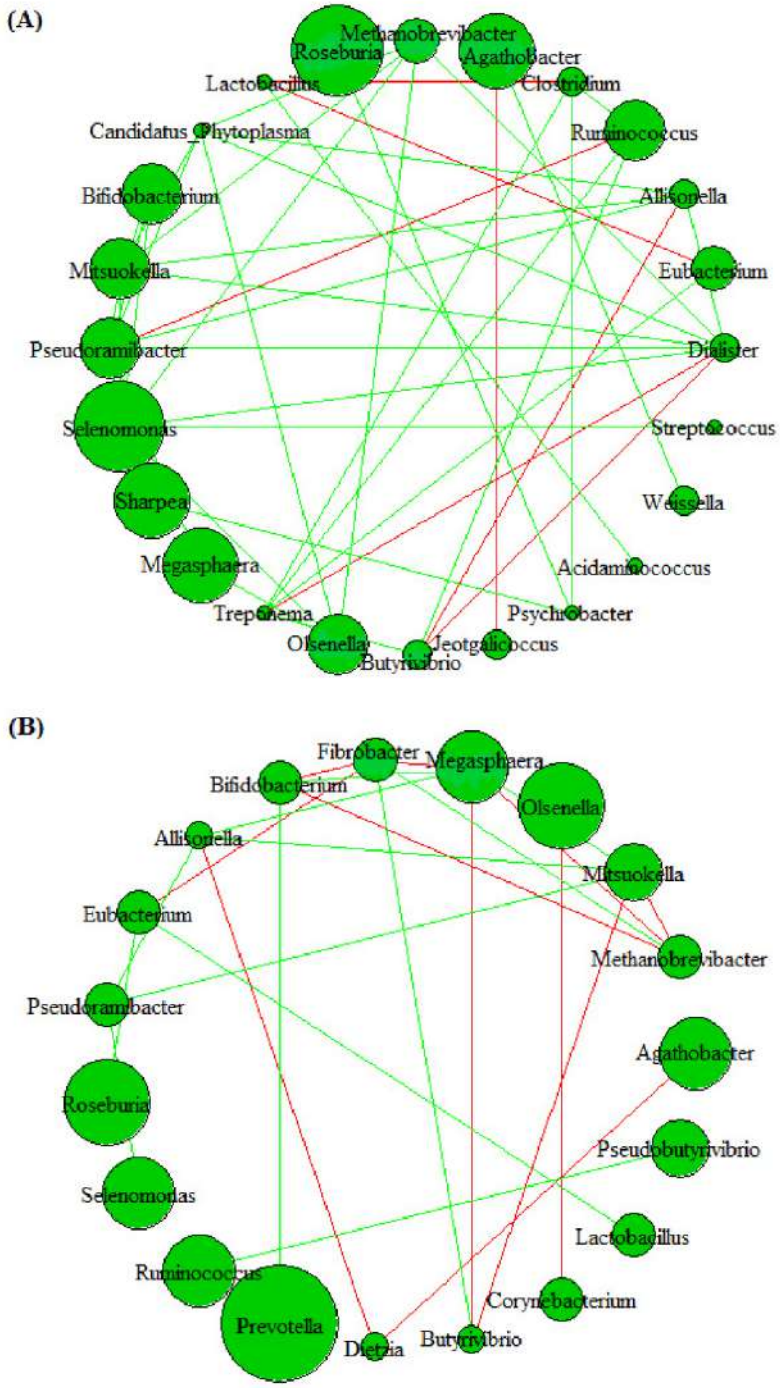

Figure 14 Microbial networks in the rumen of fattening cattle.

Obtained in the rumen of intensively reared Holstein bulls in two periods: growing (A, GRO: 158 d of age and 220 kg of body weight) and finishing (B, FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and oats haylage as forage (OATS). Networks were generated based on those genera establishing significant correlations ($r > 0.60$ and $P < 0.05$). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genus abundance.

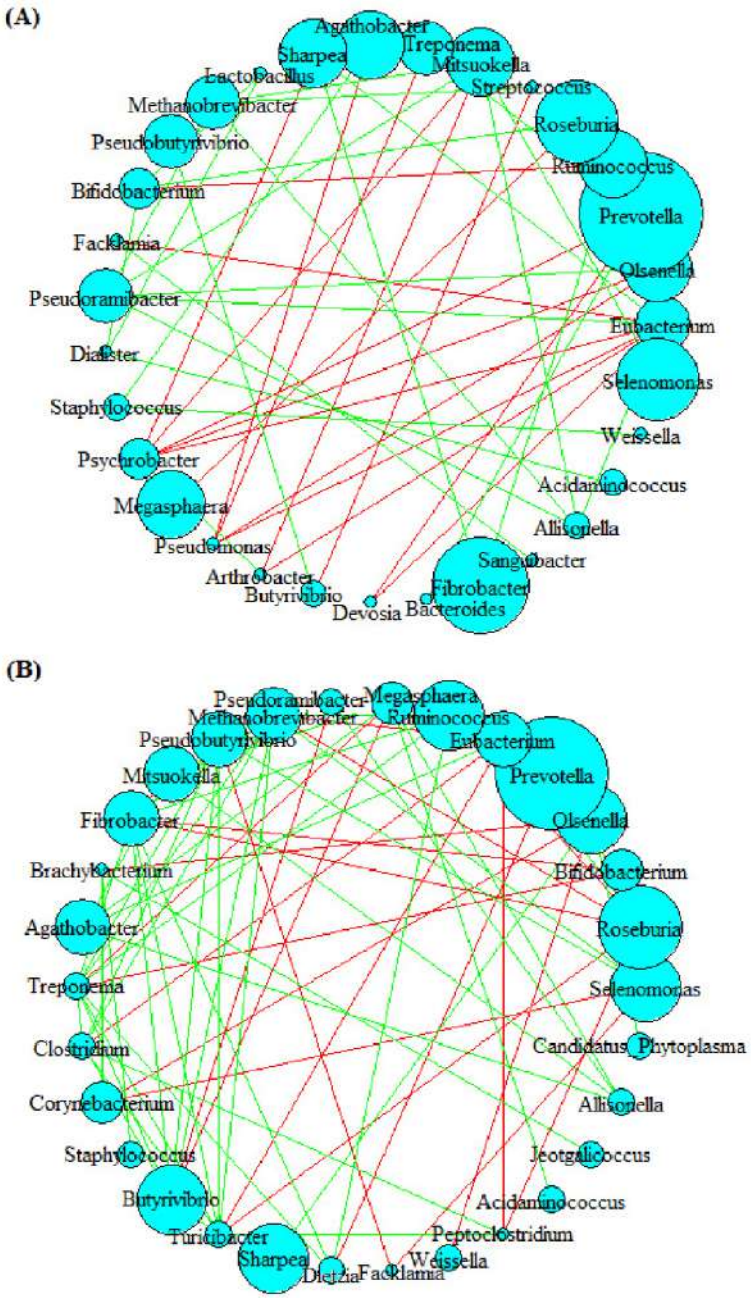

Figure 15 Microbial networks in the rumen of fattening cattle.

Bacterial and archaeal genera network in the rumen of intensively reared Holstein bulls in two periods: growing (A, GRO: 158 d of age and 220 kg of body weight) and finishing (B, FIN: 280 d of age and 434 kg of body weight). Animals were fed corn-based concentrate and vetch haylage as forage (VETCH). Networks were generated based on those genera establishing significant correlations ($r > 0.60$ and $P < 0.05$). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genus abundance.

4. Discussion

4.1 Performance, intake, digestibility and ruminal fermentation

Providing quality forage to concentrate-fed ruminants is known to be a useful strategy to avoid ruminal pH depression (Mialon et al., 2008; Yang and Beauchemin, 2009). Nevertheless, a question arises: may quality forage feeding compromise concentrate intake and animals' performance? No relevant discrepancies were stated between our reported ADG or DM intake and literature data, in intensively reared Holstein bulls consuming high-concentrate diets in a free choice system (Mora-Gil, 2019; Verdú et al., 2017). However, specific differences in relation to the forage source were evidenced: oats haylage inclusion did not compromise bulls' performance but vetch haylage utilization did depress ADG, at the beginning (119 – 164 d of age) and at the end (254 – 329 d of age) of the trial, and consequently bulls' slaughter weight was penalized. In that sense, VETCH-fed animals had the highest forage intake, probably because vetch haylage had the lowest fiber content, as fiber fraction is known to be negatively correlated with forage digestibility and voluntary intake (Jung and Allen, 1995). The incremented forage intake of VETCH-fed bulls caused a reduction in their cumulated concentrate intake that may ultimately explain the reported differences in ADG.

Apparent digestibility rates of DM, OM and CP were similar to those reported in previous studies (Ceconi et al., 2015; Mora-Gil, 2019) employing concentrate-based diets. In spite of the low roughage intake observed in the present study, forage source did alter total tract apparent digestibility; more specifically, vetch haylage inclusion did reduce DM and CP apparent digestibility in FIN. It is commonly accepted that ruminal CP degradability is higher in legumes than in grasses (Amrane and Michalet-Doreau, 1993; Bowen et al., 2008); however, the opposite has also been demonstrated in sheep consuming iso-nitrogenous diets

(Abbeddou et al., 2011). In any case, authors are unaware of data comparing CP digestibility from legumes and grasses in young ruminants fed concentrate-based diets.

Ammonia-N concentration recorded in the present study was below the threshold level defined to attain microbial requirements (Satter and Slyter, 1974). However, ruminal ammonia-N concentration reflects the equilibrium between N sources degradation and microbial uptake and, when concentrate-based diets are employed, ammonia-N assimilation may be increased (as a result of more readily available carbohydrates which intensifies microbial growth) and bacterial recycling decreased (due to the lower abundance of rumen protozoa) (Hristov et al., 2001). In this sense, observed ammonia-N concentration was similar to that reported by other studies using concentrate-based diets (Devant et al., 2001, 2000; Hristov et al., 2001; Ludden and Cecava, 1995; Rotger et al., 2006a). Ruminal ammonia-N concentration was variable but significantly higher in OATS and VETCH-fed animals than in CTR ones: haylage does normally have higher ammonia-N content than straw, due to protein breakdown that occur during haylage fermentation, with values ranging between 10 – 20 % ammonia-N of total N content in most grass and legume silages (Wilkins et al., 1971).

Concentrate intake, as a proportion of total intake, was considerable (up to 90 % in GRO and 80 % in FIN); nevertheless, registered ruminal pH was not far from neutrality. Intensive feeding systems place the rumen on the verge of the dysfunction but young cattle are able to adapt to specific challenges, and consequent acidosis is less severe than that experienced in adult cattle (Gozho et al., 2005). Given that young ruminants are unlikely to increment their VFA absorption capacity through the ruminal epithelium, they probably can cope with acidosis thanks to the high resilience and malleability of young rumen microbial ecosystem.

Registered ruminal pH values were similar to those reported by other authors working with high-concentrate diets (Devant et al., 2001, 2000; Hristov et al., 2001; Ludden and Cecava, 1995). Nevertheless, pH values need to be treated with caution due to their marked circadian

rhythm (Rotger et al., 2006b, 2006a): pH records are close to neutrality in the morning but they suddenly decrease at night, bellow SARA threshold levels (pH < 5.8 for 167 min: D. Villalba, personal communication, 2019).

Ruminal pH decreased as bulls aged but such phenomenon seemed to be alleviated by improving forage quality, even though only a statistical tendency between VETCH and CTR-fed animals could be found. In this sense, the substitution of high-lignocellulose straw for haylage would ameliorate ruminal fermentation conditions, enhancing the young ruminal environment to adapt to acidotic features.

Recorded ruminal VFA concentration did not differ from those described in other trials also employing high-concentrate diets (Devant et al., 2001, 2000; Hristov et al., 2001; Ludden and Cecava, 1995). In general terms, quality forage feeding did not affect DM intake, hence, OM fermentation and VFA production were similar between groups. The inclusion of a quality forage raised acetate-to-propionate ratio, phenomenon that has already been described when dietary forage-to-concentrate ratio is increased (Bayat et al., 2017; Lechartier and Peyraud, 2010).

4.2 Ruminal microbial community

In this study, high-throughput sequencing was applied to reveal the effect of forage source on composition, biodiversity and connectance of ruminal microbial community. Illumina sequencing procedure resulted in the identification of a huge number of bacteria and archaea, enabling a detailed description of ruminal microbial community. Protozoa abundance in animals fed high-concentrate diets is proved to be minimal due to the reduction of rumen pH caused by this type of diets (Mackie et al., 1978); likewise, fungal population is known to be scarce in diets rich in starch and soluble carbohydrates (Grenet et al., 1989), so neither protozoa nor fungi were quantified in the present study.

Regardless of dietary treatment, *Prevotella ruminicola* (54 % of bacterial sequences) and *Methanobrevibacter* (99 % of archaeal sequences) were the most abundant bacterial and archaeal OTUs in ruminal fluid, respectively. *Prevotella* is known to be one of the most abundant taxa of rumen bacteria; its role in the degradation and utilization of starch, plant cell wall polysaccharides and proteins ensures its presence in ruminants fed on a wide range of diets (Hobson and Stewart, 1997). For its part, *Methanobrevibacter* has been previously observed to dominate within rumen methanogenic archaeal species (Seedorf et al., 2015). The impact of dietary forage-to-concentrate ratio on methanogenic archaea community has awakened great interest (Hook et al., 2011, Rooke et al., 2014), as it is known that both quantity and quality of forage provided can impact on methane emissions from ruminants (De La Fuente et al., 2019). However, it is unclear whether these differences are due to any specific archaeal species, since a lack of correspondence between methane emissions and the abundance of most common rumen hydrogenotrophic methanogens has been reported (Tapio et al., 2017b). In the present study, no differences between dietary treatments were observed in *Methanobrevibacter* abundance; nevertheless, authors cannot discard a potential impact on methane emissions due to the alternative sources of forage provided.

Resistance, resilience and functional redundancy are properties exhibited by robust microbial communities (Moya and Ferrer, 2016). Microbial diversity tends to be correlated positively with ecosystem stability, which depends on the differential response of species to variable conditions, as well as the functional redundancy of species (McCann, 2000). Moreover, maintaining diversity requires the existence of variability in the ecosystem and populations able to exploit this variability (Chesson and Huntly, 1997). In the present study, microbial alpha biodiversity was low because it usually undergoes a reduction in concentrate-fed animals (Fernando et al., 2010). However, an effect of forage source could be seen, since OATS and VETCH-fed animals' microbial communities were more diverse than CTR,

especially in GRO. The positive effect of quality forage on microbial biodiversity and robustness over other types of roughage, like cereal straw or corn stover, has been already demonstrated elsewhere (Jin et al., 2016; Kong et al., 2010), likely because quality forage offers a wider range of nutrients that demand a higher complexity of microbes to make the best use of forage components.

Within the human gut, the positive correlation between a bloom of Proteobacteria and some metabolic disorders (i.e., obesity, insulin-resistance) has been proved, being Proteobacteria abundance proposed as a potential biomarker for dysbiosis and disease (Shin et al., 2015). The same applies in ruminants, establishing that P/F+B ratio should not exceed 0.19 in properly balanced ruminal microbial ecosystems (Auffret et al., 2017). Even though concentrate diets can increase Proteobacteria abundance (Petri et al., 2013), obtained P/F+B ratio in the present study was far from the proposed dysbiosis threshold.

Even though the proportion of shared sequences was high, unique OTUs were consistently different in each dietary treatment, with OATS and VETCH-fed bulls' ruminal microbial community significantly differing from CTR. Among those genera with higher presence in CTR-fed animals, we could identify (i) *Bifidobacterium* and *Selenomonas*, both known to be abundant in concentrate-fed animals' rumen and implicated in lactate metabolism (Hobson and Stewart, 1997); (ii) *Dietzia*, *Aerococcus* and *Candidatus_Phytoplasma* genera that, to authors knowledge, have not been reported in rumen yet, and (iii) *Dialister* genus, whose high abundance has been associated to hyposalivation in humans (Hayashi et al., 2014) and may also be compromising ruminal buffering capacity (Myer et al., 2015). In the case of OATS-fed animals, we detected a consistent presence of (i) lactate-producers *Weissella* (Fusco et al., 2015) and *Lactobacillus*; (ii) *Clostridium* and *Bacillus* genera, which can ferment a wide range of substrates participating in the degradation of plant tissue, starch and proteins (Hobson and Stewart, 1997; Sneath, 1986); (iii) butyrate-producer *Agathobacter*, likely

responsible for the high butyrate proportion observed in OATS-fed animals (Rosero et al., 2016) and (iv) histamine-producer *Alisonella*, commonly found in silage-fed cattle rumen and previously related to laminitis syndrome (Garner et al., 2004). Finally, all those genera characteristic of VETCH-fed animals were involved in plant cell wall degradation by either (i) degrading cellulose, like *Fibrobacter*, or (ii) utilizing as substrates those soluble carbohydrates released from fiber degradation, like *Treponema* and *Butyrivibrio* (Hobson and Stewart, 1997), indicating that vetch haylage consumption was promoting the establishment of certain microbial populations specialized in fiber degradation.

Highly connected communities are generally more robust to the removal of the most central nodes (Dunne et al., 2002). In the present study, microbial network analysis revealed that quality forage feeding promoted an increase in the overall network complexity, basically in terms of node degree and betweenness centrality. Given that Kumar et al. (2015) described the disappearance of some syntrophic interactions between microbial taxa when adult cows were changed to high-concentrate diets, and Belanche et al. (2019) observed an increase in microbial network complexity and positive symbiotic interactions through the adaptation to a pasture diet in sheep; it could be hypothesized that quality forage feeding has a positive effect on ruminal ecosystem robustness. In that sense, SARA and acidogenic diets are amongst the leading causes of death in intensively-reared beef cattle (between 30 – 42 % of the monthly mortality in US feedlots [González et al., 2012]); so, the improved ruminal fermentation robustness might counterbalance the slight reduction in animals' growth rate due to the decreased concentrate intake. Indeed, the substitution of cereal straw by higher quality forage can penalize concentrate intake but it can also constitute an opportunity to cut feedstuff costs, since the unstably higher concentrate price. Such economic benefits would be especially true in mixed farms producing their own cost-competitive quality forages.

Microbial associative patterns pointed out that each dietary treatment had its own set of correlating nodes, but some degree of functional redundancy was detected (Taxis et al., 2015): all microbial networks included (i) starch-degrading populations, i.e., *Roseburia* (Duncan et al., 2006, 2002), *Sharpea* (Morita et al., 2008), *Bifidobacterium* and *Selenomonas*; (ii) fiber-degrading populations, i.e., *Fibrobacter* (Montgomery et al., 1988), *Treponema* and *Eubacterium* (Hobson and Stewart, 1997); (iii) versatile populations with the ability to ferment both starch and fiber, i.e., *Clostridium* and *Ruminococcus* (Hobson and Stewart, 1997) and (iv) lactate-producing populations, i.e., *Sharpea*, *Bifidobacterium*, *Eubacterium*, *Selenomonas* and *Olsenella* (Kraatz et al., 2011). The most notorious difference between dietary treatments was the presence of lactate-degrading populations acting as central nodes in the networks of animals provided with quality forage sources, i.e., *Megasphaera* (Hobson and Stewart, 1997) in OATS bulls and *Selenomonas* in VETCH ones; suggesting that those populations may be playing a role in the alleviation of ruminal acidosis via quality forage feeding. Such observations support the idea that, despite the existence of a core microbiome across diets and hosts (Henderson et al., 2015), diet is an important regulating factor defining which taxa should act as node and dictate the rest of microbial interactions (Tapio et al., 2017a).

5. Conclusions

Providing oats haylage as forage source in concentrate-fed cattle penalized neither performance nor apparent nutrient digestibility; contrarily, feeding vetch haylage reduced concentrate intake, slaughter BW and nutrient apparent digestibility. Animals provided with quality forage sources presented both higher alpha biodiversity levels and more connected microbial networks, which supports the hypothesis that those bulls could host a more robust

ruminal ecosystem. Thus, quality forage feeding should take into account the trade-off between a lower animals' performance and a more robust ruminal microbiota.

Acknowledgments

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CHAPTER IV

Ruminal microbiota is associated with feed efficiency phenotype of fattening bulls fed high-concentrate diets

Abstract

Context: Improving feed efficiency in livestock production is of great importance to reduce feeding costs.

Aims: To examine the relationship between ruminal microbiota and variation in feed efficiency in beef cattle fed concentrate-based diets.

Methods: Residual feed intake of 389 fattening bulls, supplied with corn-based concentrate and forage *ad libitum*, was used to estimate animals' feed efficiency. Feces and ruminal fluid samples, from 48 bulls chosen at random, were collected to estimate their forage intake and to determine their apparent digestibility, ruminal fermentation and microbiota. Those animals with extreme values of feed efficiency (high-efficiency [HE, n = 12] and low-efficiency [LE, n = 13]) were subjected to further comparisons. Alpha biodiversity was calculated based on the normalized sequence data. Beta diversity was approached through performing a canonical correspondence analysis based on log transformed sequence data. Genera differential abundance was tested with an ANOVA-like differential expression analysis and genera interactions were determined applying the Sparse Correlations for Compositional data technique.

Key results: No differences in dry matter intake were found between the two categories of feed efficiency (P = 0.699); however, HE animals had higher apparent digestibility of dry matter (P = 0.002), organic matter (P = 0.003) and crude protein (P = 0.043). The concentration of volatile fatty acids was unaffected by feed efficiency (P = 0.676) but butyrate proportion increased with time in LE animals (P = 0.047). Ruminal microbiota was different between HE and LE animals (P = 0.022): both alpha biodiversity and genera network connectance increased with time in LE bulls (P = 0.005 for Shannon index and P = 0.020 for Simpson index); which suggests that LE animals hosted a more robust ruminal microbiota.

Certain genera usually related to high energy loss through methane production were found to establish more connections with other genera in LE animals' rumen than in HE ones. Microbiota function capability suggested that methane metabolism was decreased in HE finishing bulls.

Conclusions: Rumen microbiota was associated with feed efficiency phenotypes in fattening bulls fed concentrate-based diets.

Implications: The possible trade-off between feed efficiency and ruminal microbiota robustness should be taken into account for the optimization of cattle production, especially in systems with intrinsic characteristics that may constitute a disturbance to rumen microbial community.

1. Introduction

Improving feed efficiency (FE) in beef cattle production systems provides an opportunity to cut down on the cost of feeding livestock. In that sense, residual feed intake (RFI) can be used as an index of FE that is independent of variation in body weight (BW) and average daily gain (ADG) (Arthur et al., 2001; Arthur and Herd, 2008; Schenkel et al., 2004), and is the gold standard index to examine biological mechanisms associated with inter-animal differences in FE. Moreover, some studies have demonstrated the possibility of selection for low RFI as a strategy for greenhouse gas mitigation, as it has been correlated to lower methane emission and greater diet digestibility (Herd and Arthur, 2009). Limitations in conducting RFI trials (recording BW and feed intake for a long time) and searching for rumen microbial markers to identify efficient animals with low RFI have become a contemporary challenge.

Research in cattle has focused mostly on the microbial response to dietary changes and management practices, whereas trials for understanding the relationship between host FE phenotype and rumen microbiota are scarce and yet to be done (Myer et al., 2015). Previous

studies have shown that rumen microbes are responsible for energy supply through producing organic acids (Huntington, 1990), and most taxa associated with variation in FE have been related to cellulolytic, fermentative, and metabolic activities (Myer et al., 2015). Therefore, differences in the production rate of organic acids lead to variation in nutrient digestibility and fermentation that ultimately change animals' phenotypic efficiency (Herd and Arthur, 2009). This experiment aimed to understand the relationship between ruminal microbiota and variation in FE of beef cattle fed concentrate-based diets.

2. Materials and Methods

2.1 Animals, diets, and housing

Residual feed intake data from two feeding experiments comprising 389 fattening bulls were used to explore relationships between ruminal microbiota and FE. This dataset included 317 animals raised at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* (Ivars d'Urgell, Spain, 41°41'50"N, 0°58'53"E) and 72 animals from *CITA-La Garcipollera* Research Station (Jaca, Spain, 42°37'34"N 0°30'10"W). All procedures were carried out under Project License CEEA 01-07/16 and approved by the in-house Ethics Committee for Animal Experiments at the University of Lleida. Care and use of animals were in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Animals raised at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* were distributed in four batches: batches nº 1 – 3 included 231 Holstein bulls (63 – 83 animals per batch) and batch nº 4 included 86 Montbeliard bulls. Body weight and feed intake data were collected on a daily basis. Animals raised at *CITA-La Garcipollera* Research Station were distributed in three batches: batches nº 5 – 6 included 28 and 32 Parda de Montaña bulls,

respectively, and batch nº 7 included 12 Pirenaica bulls. For these animals, BW was measured weekly and feed intake data were collected on a daily basis.

Body weight and feed intake data were recorded throughout the entire fattening phase: the first 150 days were considered as the growing phase (121 d old [SD: 37 d] and 162 kg BW [SD: 49 kg]), followed by a finishing phase which lasted until animals reached slaughter weight (336 d old [SD: 31] and 501 kg BW [SD: 56 kg]).

Animals were fed concentrate and forage *ad libitum*, which were provided separately in two different bunkers, and they had free access to drinking water, following the conventional beef cattle feeding system in Spain. The concentrates used were very similar in composition and their main ingredients were raw corn, corn gluten feed, raw barley, corn dried distillers grains with solubles and raw chickpea; whereas forage used was mainly barley straw (349 animals), oats haylage (20 animals) and vetch haylage (20 animals). Feed chemical and nutritional composition is shown in Table 12.

2.2 Measurements and sampling

Intake of concentrates was recorded automatically at both research facilities using automatic feed stations, which were equipped with a feedbunk (provided with a scale) and an individual feeder. When a calf entered the feeder, it was identified and its concentrate intake was obtained by difference between initial and final feedbunk weight. Feed stations available at the research facilities of *Cooperativa d'Ivars d'Urgell, SCCP* were additionally equipped with a scale under the individual feeder by which the animals were automatically weighed at each visit; whereas, at *CITA-La Garcipollera* Research Station, BW data were recorded manually once a week.

Table 12 Feed chemical and nutritional composition.

Values presented are mean, with minimum and maximum in brackets. ADF: acid detergent fiber; CP: crude protein; DM: dry matter; EE: ether extract; NDF: neutral detergent fiber; OM: organic matter; PDIN and PDIE: protein digestible in the small intestine allowed by protein and energy; UFV: forage unit for meat production.

Parameters	Concentrate	Forage
Chemical composition (g/kg DM)		
DM (g/kg fresh weight)	871.0 (856.2 – 879)	658.3 (489.2 – 857.1)
OM	946.4 (939.9 – 950.9)	888.8 (842.8 – 925.8)
CP	129.7 (117.7 – 140.7)	112.2 (71.9 – 164.4)
EE	418.5(247.9 – 720)	19.8 (14.2 – 26.5)
NDF	165.7 (135.1 – 207.1)	571.3 (442.1 – 754.5)
ADF	59.1 (45.6 – 78.5)	334.8 (281.8 – 437.7)
Nutritional composition		
UFV (UFV/kg DM)	1.02 (0.97 – 1.03)	0.55 (0.36 – 0.73)
PDIN (g/kg DM)	91.8 (79.8 – 95.9)	65.4 (40.8 – 93.7)
PDIE (g/kg DM)	87.4 (80.3 – 94.9)	56.0 (52.6 – 58.0)

Feces and ruminal fluid samples from 48 bulls (selected at random within batches) were collected twice, at mid-growing period (GRO: 159 d old and 225 kg BW) and mid-finishing period (FIN: 266 d old and 434 kg BW), for forage intake estimation and digestibility, ruminal fermentation and microbial community characterization.

Fecal excretion and forage intake were calculated based on concentrate intake and adapting the two indigestible markers system (Owens and Hanson, 1992), using chromium oxide (Cr_2O_3) as external marker and acid insoluble ash as internal marker. Then, apparent digestibility of dry matter (DM), organic matter (OM) and crude protein (CP) were estimated. Detailed information about markers administration, feed and feces analytical determinations and apparent digestibility calculations are described in Costa-Roura et al. (2020).

Ruminal fluid was sampled in the morning using an oral stomach tube connected to a vacuum pump. Each sample was obtained through two sequential collections. First, ruminal fluid (approximately 200 mL) was collected and discarded to avoid sample contamination with

saliva that could get into the tube during its introduction through the animal's mouth and esophagus. After that, ruminal fluid (approximately 200 mL) was reextracted, strained through a cheesecloth and its pH recorded (Testo 205, Testo AG, Germany). Then, ruminal fluid was sampled for DNA extraction, ammonia-nitrogen (N) and volatile fatty acids (VFA) concentration, and immediately frozen on dry ice. Sample preservation conditions and analytical procedures for ammonia-N and VFA determination are detailed in Costa-Roura et al. (2020).

2.3 Extraction and sequencing of DNA

Extraction of DNA was carried out on freeze-dried ruminal fluid (the initial amount of sample was 60 mg) through physical disruption (1 min) using a bead beater (Mini-bead beater 1, BioSpec Products, USA) and subsequent DNA purification with the QIAamp DNA Stool Mini Kit (ID: 51504; QIAGEN N.V., Germany), with the modifications of greater temperature (95 ºC) and greater elution time (3 min) to ensure maximum DNA concentration in the final elute. Amplification of DNA was carried out by using primers 341F and 805R, which target the V3 and V4 regions of the bacterial and archaeal 16S rRNA. Sequencing was conducted on an Illumina MiSeq 2x300 platform by Era7 Bioinformatics (Spain).

Assembly and filtration of sample reads, as well as Operational Taxonomic Units (OTUs) preparation are detailed in Costa-Roura et al. (2020).

2.4 Estimation of residual feed intake and clustering

Weight data were fitted to a third-degree polynomials model in function of age (Eq. 4) that allows the estimation of the ADG of each animal at any age.

$$(\text{Eq. } 4)\ \text{Weight}_{i,age} = \sum_{j=0}^{j=3} \left(b_{BATCH,j} + A_{i,j}\right) \cdot \text{age}_j + e_{i,age}$$

Where $b_{BATCH,j}$, is the batch effect (fixed); $A_{i,j,}$ is the j random coefficient for the i animal effect; *age* is the age of the animal (days) and $e_{i,age}$ the residual term.

Thereafter, ADG_dev is obtained as the first derivative of Eq. 4 for each month using the monthly average age of each animal (Eq. 5). The individual ADG deviation (ADG_dev) will account for the difference of growth of the animal compared with the average of the batch at each age.

$$(\text{Eq. } 5)\ \text{ADG\_dev}_{i,age} = \sum_{j=1}^{j=3} j \cdot A_{i,j} \cdot \ age^{(j-1)}$$

A total of 86 records (3 %) out of three standard deviations of the mean were considered as outliers and excluded from the dataset.

Residual feed intake was modeled (Eq. 6) using the random regression coefficients approach proposed by Savietto et al. (2014). The model included batch, age (months), ADG_dev, metabolic weight (MW; monthly mean $BW^{0.64}$) and defined as follows.

(Eq. 6) $FI_{ij} = B_{0,animal\ i} + Batch \cdot age_j + (Batch + B_{1,animal\ i}) \cdot MW_{ij} + (Batch + B_{2,animal\ i}) \cdot ADG\_dev_{ij} + e_{ij}$

Where FI_{ij} is DM intake measured for animal i in month j and $B_{k,animal\ i}$ are the random coefficients for animal effect modelled using an unstructured matrix of variances between them.

The inclusion of batch effect in Eq. 3 and Eq. 6 assures that the FE calculated is not affected by diet differences.

Based on the individual coefficients of ADG ($B_{1,animal\ i}$) and MW ($B_{2,animal\ i}$), animals were segregated into four categories of FE, as follows.

i) Animals with both positive coefficients of ADG and MW belonged to “low-efficiency in ADG and low-efficiency in MW” category.

ii) Animals with positive coefficient of ADG but negative coefficient of MW belonged to "low-efficiency in ADG and high-efficiency in MW" category.

iii) Animals with negative coefficient of ADG but positive coefficient of MW belonged to "high-efficiency in ADG and low-efficiency in MW" category.

iv) Animals with both negative coefficients of ADG and MW belonged to "high-efficiency in ADG and high-efficiency in MW" category.

For the purpose of the present study, the two extreme categories (i and iv) were considered as high-efficiency (HE, Positive RFI) and low-efficiency (LE, Negative RFI) animals, respectively. This clustering (HE vs LE) was subjected to bioinformatic analyses of apparent digestibility, ruminal fermentation and microbiota data as explained below.

2.5 Bioinformatics

Sequence data were normalized and alpha biodiversity indices were calculated to measure the variability of OTUs within a sample (R Core Team, 2020; Vegan package).

To measure differences in microbiota composition between samples, beta diversity was approached through performing a canonical correspondence analysis (CCA), based on log transformed OTUs data (zeros were replaced by adding 1 to each value), and including FE (HE vs LE), Period (GRO vs FIN) and both ADG and MW coefficients as explanatory variables (R Core Team, 2020; Vegan package).

To circumvent the compositional bias problem (Calle 2019; Gloor et al., 2017; Tsilimigras and Fodor, 2016), we applied the Aitchison's centered log ratio (clr) transformation to carry the data to a Euclidean space, after replacing zeros by adding 1 to each value. To test the significance of the following effects: FE (HE vs LE), period (GRO vs FIN) and both ADG and MW coefficients on microbiota composition, a permutational multivariate analysis of variance (Adonis) was conducted based on the clr Euclidean distance and calculating

statistical significance after 10000 random permutations (R Core Team, 2020; Vegan package). To decipher which genera abundance were responsible for the differences between groups, an ANOVA-like differential expression (ALDEx) analysis was conducted over those genera present at least at 50 % of the individuals (R Core Team, 2020; Aldex2 package) (Fernandes et al., 2013). Finally, to describe the interactions within rumen microbial community, we performed a network analysis through Sparse Correlations for Compositional data (SparCC) technique (R Core Team, 2020; SpiecEasi package) (Friedman and Alm, 2012) over those genera present at least at 50 % of the individuals. Microbial networks were graphically represented (R Core Team, 2020; igraph package) and their complexity was described in terms of number of nodes (genera), number of edges (significant positive or negative correlations), node degree (number of connections that any node establishes with other nodes) and betweenness (measure of centrality in a graph based on shortest paths).

Microbiota functional content was assessed using a topic model approach (R Core Team, 2020; themetagenomics package) that consists on (i) capturing groups of co-occurring taxa termed “topics”, (ii) uncovering within-topic functional potential, and (iii) linking these topics and their functional content to specific sample features (e.g., FE phenotypes) (Woloszynek et al., 2019).

2.6 Statistical analyses

The models of RFI were solved using MIXED procedure of SAS statistical software (SAS v9.4, Cary, USA). Intake, apparent digestibility, ruminal fermentation parameters and microbial alpha-biodiversity data were analyzed with a mixed model; including FE (HE vs LE), period (GRO vs FIN) and their interaction as fixed effects and animal as random effect, to account for repeated measurements (R Core Team, 2020; lme4 package). Differences between least square means were assessed using Tukey multiple comparison test (R Core

Team, 2020; emmeans package). Individual samples out of three standard deviations of the mean were discarded and not included to the statistical analysis. Results were reported as least square means and standard error of mean. Significant effects were declared at $P < 0.05$ and tendency to difference at P between 0.05 and 0.10.

3. Results

The four defined FE categories based on random regression coefficients included between 85 and 108 animals each; the two extreme categories corresponding to HE and LE animals had statistically different means for ADG and MW coefficients (Supplementary Table 3). The set of 48 bulls which were sampled for apparent digestibility, ruminal fermentation and microbiota characterization were equally distributed within the four categories.

The FE by period interactions were not significant for any of the response variables measured in this study, thus, only the main effects means are presented and discussed.

3.1 Intake, apparent digestibility and ruminal fermentation parameters

Data on DM intake (Table 13) indicate that bulls' concentrate and forage intake were similar between FE categories (HE vs LE). However, animals classified as HE had greater apparent digestibility coefficients of DM, OM and CP than their LE counterparts.

Data on ruminal fermentation parameters (Table 13) showed no differences in ruminal pH between HE and LE bulls. Ammonia-N concentration was low and variable among animals, therefore, no statistical differences between FE categories were found. Although total VFA concentration remained unaffected by FE, numerical differences were found in molar proportions of the main VFA: HE animals had lower proportion of acetate and higher proportion of propionate than LE ones. Butyrate proportion increased with time in the case of LE animals (7.46 % vs 9.30 % for GRO and FIN periods respectively; $P = 0.047$) whereas it

remained equal for HE animals (7.95 % vs 8.42 % for GRO and FIN respectively; P = 0.908). Contrarily, branched chain VFA proportion (isobutyrate and isovalerate) incremented with time only in the case of HE bulls (1.55 % vs 2.31 % for GRO and FIN respectively; P = 0.006).

3.2 Microbial data set features

Sequencing procedure yielded an average of (mean ± SEM) 19862 ± 2215 sequences per sample, resulting in 973259 sequences of the whole study. A total of 787 OTUs were obtained at the 98 % sequence similarity cut-off levels with 114 ± 5 as the mean number of OTUs per sample. Good's coverage value was 99.69 ± 0.03 %, suggesting that more than 99 % of bacterial and archaeal phylotypes were identified. The unclassified rate of OTUs at genus level was 0.75 ± 0.09 %. Shared OTUs by all individuals in each FE category and period were deemed to be core bacterial/archaeal communities. Core community gathered 69.90 ± 2.94 % of analyzed sequences and was composed of 5 OTUs: *Prevotella ruminicola*, unclassified *Prevotella* (both representing more than 84 % of shared sequences), unclassified *Roseburia*, *Sharpea azabuensis* and unclassified *Methanobrevibacter*.

3.3 Microbial community biodiversity

Alpha biodiversity (Table 14) was found to be similar between bulls differing in their FE; however, Shannon and Simpson indexes values incremented with time only in LE animals (Shannon index 1.51 vs 2.13, P = 0.005; Simpson index 0.53 vs 0.70, P = 0.020, for GRO and FIN, respectively).

Table 13 Dry matter intake, nutrient apparent digestibility and ruminal fermentation parameters.

Obtained in intensively reared bulls in two periods: growing (GRO: 159 d old and 225 kg body weight) and finishing (FIN: 266 d old and 434 kg body weight). Residual feed intake was modelled to classify animals into two categories of feed efficiency: high-efficiency (HE, n = 12) and low-efficiency (LE, n = 13). Standard error of the mean (SEM) and significance of Feed Efficiency and Period effects are shown. No Feed Efficiency by Period interaction did reach statistical significance (P > 0.05) and were not included in the table. A/P: acetate-to-propionate; CP: crude protein; DM: dry matter; N: nitrogen; OM: organic matter; VFA: volatile fatty acids. Mean values within a row with unlike superscript letters differ (P < 0.05).

Parameters	Feed Efficiency		Period		SEM	P-values	
	HE	LE	GRO	FIN		Feed Efficiency	Period
Intake							
DM (kg/d)	7.52	7.64	5.88^{b}	9.28^{a}	0.235	0.699	<0.001
Concentrate DM	6.40	6.59	5.28^{b}	7.72^{a}	0.191	0.479	<0.001
Forage DM	1.09	0.93	0.60^{b}	1.42^{a}	0.137	0.397	<0.001
Apparent digestibility coefficients							
DM	0.752^{a}	0.708^{b}	0.729	0.731	0.0096	0.002	0.875
OM	0.762^{a}	0.719^{b}	0.738	0.743	0.0100	0.003	0.760
CP	0.715^{a}	0.685^{b}	0.696	0.704	0.0102	0.043	0.579

Table 13 Continued.

Parameters	Feed Efficiency		Period		SEM	P-values	
	HE	LE	GRO	FIN		Feed Efficiency	Period
Ruminal fermentation parameters							
pH	6.93	6.97	7.16[a]	6.75[b]	0.080	0.710	<0.001
Ammonia-N (mg/L)	12.36	19.27	12.03	19.59	3.618	0.181	0.095
VFA (mmol/L)	70.87	74.41	68.22	77.05	6.102	0.676	0.183
VFA (mol/100 mol)							
Acetate	49.63	51.42	49.32	51.73	1.532	0.403	0.093
Propionate	37.70	35.88	38.89[a]	34.69[b]	1.867	0.487	0.015
Butyrate	8.18	8.38	7.70[b]	8.86[a]	0.425	0.737	0.025
Branched chain VFA	1.93	1.99	1.66[b]	2.27[a]	0.128	0.724	<0.001
Ratio A/P	1.42	1.58	1.30[b]	1.69[a]	0.148	0.431	0.017

Table 14 Ruminal microbial alpha biodiversity.

Obtained in intensively reared bulls in two periods: growing (GRO: 159 d old and 225 kg body weight) and finishing (FIN: 266 d old and 434 kg body weight). Residual feed intake was modelled to classify animals into two categories of feed efficiency: high-efficiency (HE, n = 12) and low-efficiency (LE, n = 13). Standard error of the mean (SEM) and significance of Feed Efficiency and Period effects are shown. No Feed Efficiency by Period interaction did reach statistical significance (P > 0.05) and were not included in the table. Mean values within a row with unlike superscript letters differ (P < 0.05).

Parameters	Feed Efficiency		Period		SEM	P-values	
	HE	LE	GRO	FIN		Feed Efficiency	Period
Shannon Index	1.76	1.82	1.53[b]	2.05[a]	0.149	0.760	<.001
Simpson Index	0.61	0.61	0.54[b]	0.68[a]	0.050	0.942	0.002
Richness	101.10	107.69	96.87[b]	111.92[a]	5.824	0.408	0.072

Beta biodiversity is graphically represented in Figure 16, as well as the effects of explanatory variables included in the model: FE, period, and both MW and ADG coefficients in the RFI model. Samples are clearly clustered by period and FE, being the effects of MW and ADG coefficients less graphically evident. Adonis test results confirmed the foreseen differences in ruminal microbiota composition when comparing sampling period (GRO vs FIN, $P < 0.001$), FE categories (HE vs LE, $P = 0.022$) and MW coefficient values ($P = 0.021$), but not in the case of ADG coefficient values ($P = 0.276$). Statistical differences in genera abundance between FE categories (HE vs LE) could not be detected by ALDEx analysis, regardless of sampling period (Supplementary Figure 1).

3.4 Microbial network

Microbial networks were built to test bacteria and archaea genera interactions (Figure 17). Degree of interaction was studied through the number of genera (nodes) that established significant interactions (edges) with other genera, as well as the number of interactions established per node (node degree). During the growing period, HE bulls had similar number of nodes taking part in the microbial network as LE (26 in HE vs 24 in LE) but higher number of edges (57 in HE vs 28 in LE) and average node degree (4.38 in HE vs 2.33 in LE). During the finishing period, microbial network architecture changed: HE bulls continued to have more correlating nodes than LE (30 in HE vs 21 in LE) but LE animals drastically incremented their number of edges (53 in HE vs 59 in LE) and node degree (3.53 in HE vs 5.62 in LE).

Moreover, we investigated microbial genera that act as main information gateways in networks in terms of betweenness centrality, i.e., the extent to which one node lies on paths that connect other nodes. Networks of HE animals presented higher betweenness centrality

than those of LE in growing (3.88 in HE vs 1.04 in LE), but not in finishing period (2.37 in HE vs 2.52 in LE).

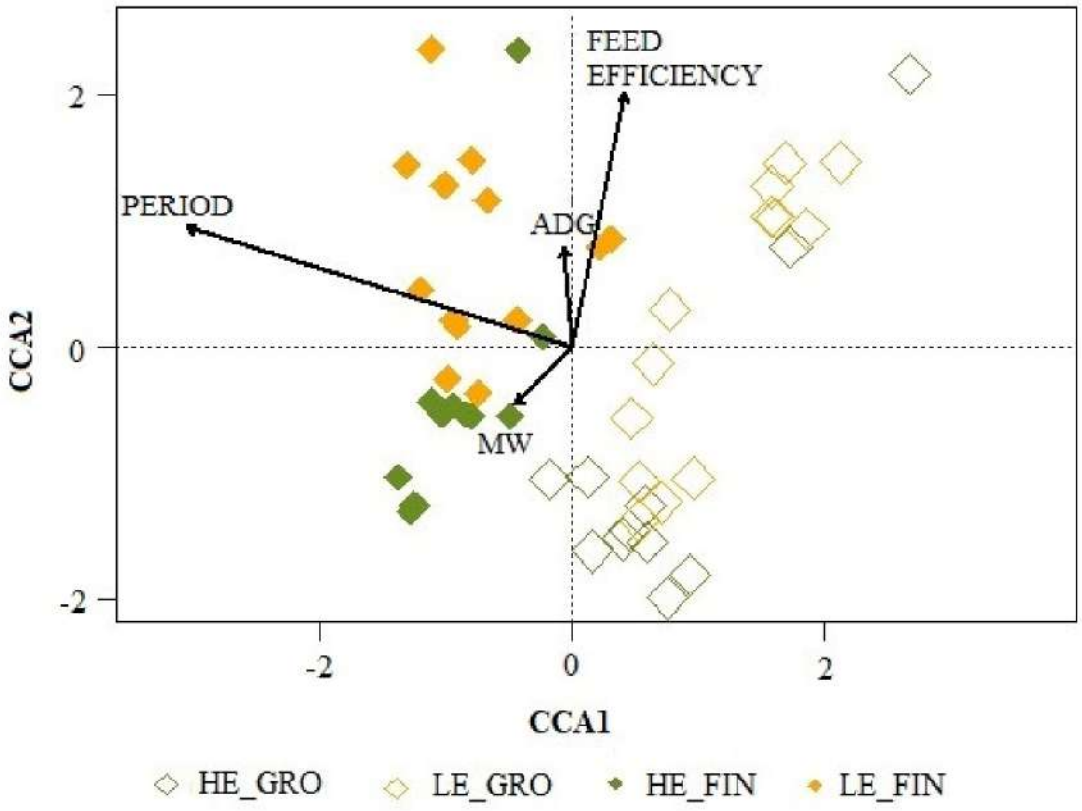

Figure 16 Graphical representation of canonical correspondence analysis (CCA) on bacterial and archaeal OTUs in ruminal fluid.

Obtained in intensively reared bulls in two periods: growing (GRO: 159 d old and 225 kg body weight) and finishing (FIN: 266 d old and 434 kg body weight). Residual feed intake was modelled to classify animals into two categories of feed efficiency: high-efficiency (HE) and low-efficiency (LE). The analysis included feed efficiency, period, and both average daily gain (ADG) and metabolic weight (MW) coefficients as explanatory variables.

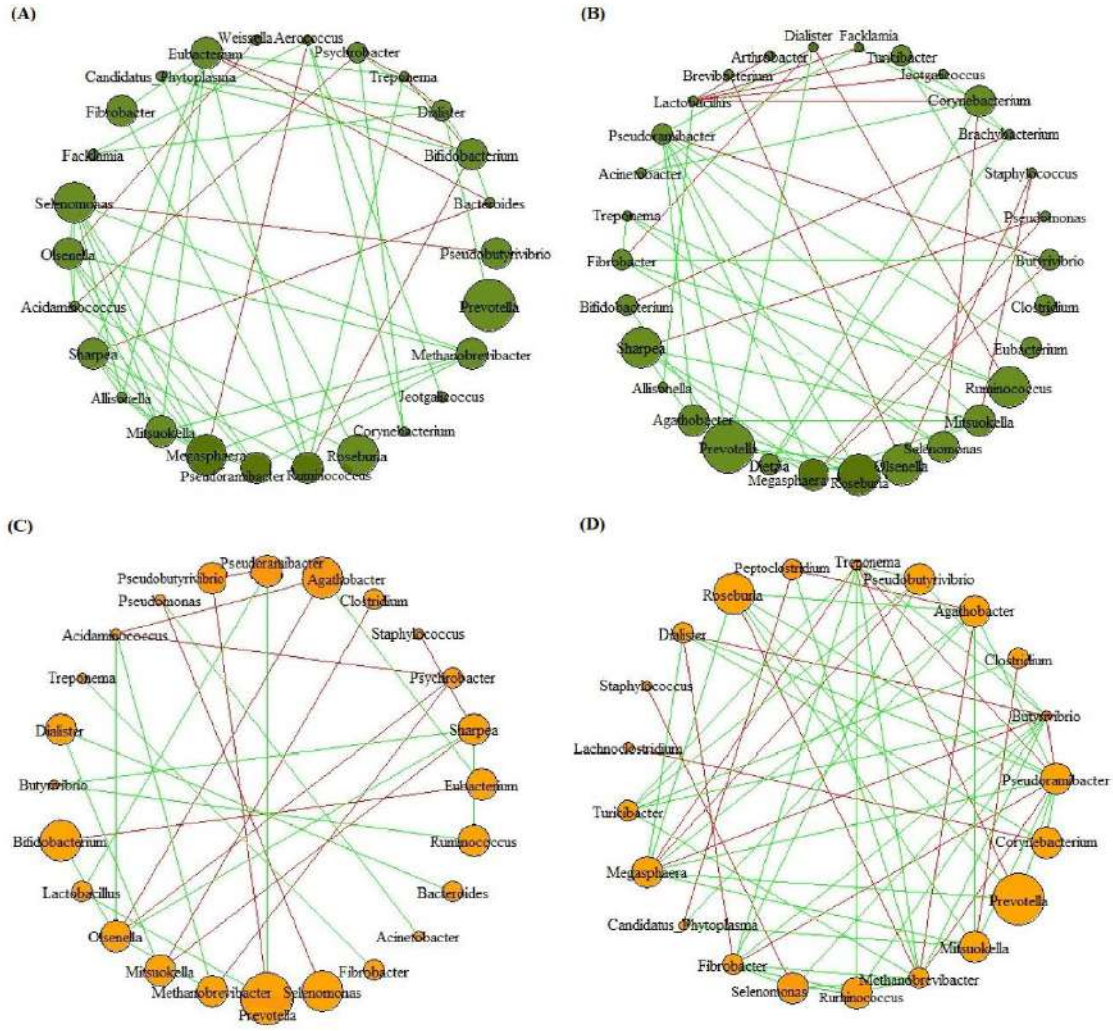

Figure 17 Microbial networks in the rumen of fattening cattle.

Obtained in intensively reared fattening bulls (A – C, GRO: 159 d old and 225 kg body weight; B – D, FIN: 266 d old and 434 kg body weight). Residual feed intake was modelled to classify animals into two categories of feed efficiency: high-efficiency (A – B, HE) and low-efficiency (C – D, LE). Networks were generated based on those genera establishing significant correlations ($r > 0.60$ and $P < 0.05$). Green and red edges indicate positive and negative correlations, respectively. Node size is proportional to genus abundance in ruminal fluid.

3.5 Microbial functional capability

After predicting ruminal microbiota functional content, two pathways were found to be differentially expressed depending on animals' FE phenotype. In the growing period, ABC transporters pathway (ATP-dependent transport of molecules across cell membrane) was more active in HE animals than in LE; and, in the finishing period, methane metabolism was downregulated in HE individuals' rumen when compared to LE.

4. Discussion

4.1 Residual feed intake and mechanisms underlying the variability of feed efficiency

Variations in RFI occur due to potential physiological mechanisms such as digestion, fermentation and metabolism (Herd and Arthur, 2009). Our findings show that HE animals apparently digested more feed, in terms of DM, OM and CP, compared to LE ones. These results are in accordance with previous studies in which more efficient animals showed higher nutrient digestibility and less nutrient loss through waste and methane emission (Nkrumah et al., 2006; Richardson et al., 1996). Negesse et al. (2017) also observed improved apparent digestibility coefficients of DM, OM and CP in HE heifers: these animals excreted a smaller proportion of N through feces and their N biological value (N balance/digestible N) was higher compared to less efficient heifers, suggesting that CP digestion and metabolism may be enhanced in HE animals. In comparison, de Assis Lage et al. (2019) did not find differences in digestibility coefficients of such nutrients but they reported a tendency of HE heifers to better digest ether extract fraction.

Volatile fatty acids are products of rumen microbial fermentation of carbohydrates, constituting the main energy source for ruminants (Bergman, 1990). Although differences between HE and LE bulls did not reach significance for any ruminal fermentation parameter, numerical values indicate that LE animals had a fermentation pattern oriented towards the

production of higher molar proportion of acetic and less propionic acids than HE animals, with the consequent effect on acetate-to-propionate ratio. These observed differences in rumen fermentation pattern may be playing a role in bulls' FE phenotype, since metabolic hydrogen produced in the first step of acetic acid pathway is later taken up by methanogens, increasing energy loss through gas emissions (Ungerfeld, 2020).

During the growing period, molar proportions of the major VFA were similar to those observed by Yuste et al. (2020), in beef heifers fed a similar *ad libitum* concentrate plus straw diet. On the other hand, during the finishing period, the higher amount of total VFA, concomitant with significantly lower ruminal pH, were rooted in the increased DM intake and, consequently, in the higher extent of fermentation process. However, propionic acid showed a different trend and it was higher in younger animals. Hernandez-Urdaneta et al. (1976) reported that forage-to-concentrate ratio affects the molar proportions of VFA, which for high-concentrate diets varies in direction of decreased acetic and increased propionic acid; therefore, in our experiment, the lower forage-to-concentrate ratio during the growing (11%) respect to the finishing period (18 %) can explain the observed decreased proportion of propionic acid with time.

4.2 Residual feed intake and ruminal microbial community

In the present study, *Illumina* sequencing technology was used to analyze bacterial and archaeal composition, biodiversity, connectance and functional capability within the rumen of intensively reared bulls differing in their FE.

A negative correlation between ruminal microbial alpha biodiversity and FE has been previously described in milking cows (Shabat et al., 2016); suggesting that efficient microbiotas are less complex but more specialized in providing higher concentrations of relevant output metabolites that can be used to meet host's energy requirements. In a similar

manner, our results showed that microbial alpha diversity values significantly increased with time only in LE bulls. Microbial diversity is also positively correlated with community stability and robustness, as both differential response to variable conditions and functional redundancy of species are enhanced (McCann, 2000; Moya and Ferrer, 2016). Thus, it seems reasonable to hypothesize that microbiota alpha biodiversity has positive and negative coexisting effects on ecosystem robustness and feed utilization efficiency, respectively. The fact that LE bulls considerably increased their genera networks connectance with time, while HE bulls kept it constant or even diminished it, supports the hypothesis that LE animals' ruminal microbiota could be more robust and have an enhanced ability to cope with possible disturbances (Dunne et al., 2002).

Even though beta diversity representation showed clear clustering of bulls' microbial community, no statistical differences in main genera abundance could be found between FE categories. Considering that some studies had success in reporting a relationship between certain microbial taxa and animal's FE (Delgado et al., 2019; McCann et al., 2014; Myer et al., 2015; Perea et al., 2017), authors consider that the following factors could hinder detection of such relationship: (i) there can be substantial animal-to-animal variation in the rumen microbial community, thus requiring a greater number of animals to observe a significant association between microbial taxa and FE (Brulc et al., 2009; Weimer et al., 2010), and (ii) the lack of differences observed between FE categories at the main genera level may indicate that the important variation in microbial communities lies at a finer resolution (i.e., at species level or low-abundance genera).

Kittelmann et al. (2014) described the existence of three ruminal microbial communities linked with different methane yields in sheep, ruminotype H was characterized by the highest methane emissions and harbored higher abundance of species belonging to *Ruminococcus*, other Ruminococcaceae, Lachnospiraceae, Catabacteriaceae, *Coprococcus*, other

Clostridiales, *Prevotella*, other Bacteroidales, and Alphaproteobacteria. In a recent study in sheep, Ghanbari Maman et al. (2020) also identified certain genes from Lachnospiraceae, *Ruminococcus*, *Butyrivibrio* and *Selenomonas* taxa that can have significant effects on methane production pathway. In accordance with these studies, our co-abundance analysis showed that certain genera previously related with high methane emission (i.e., *Methanobrevibacter, Roseburia, Agathobacter, Butyrivibrio, Pseudobutyrivibrio, Ruminococcus, Selenomonas*) either were more central or evolved to be more central in LE animal's networks during the transition from growing to finishing periods (Supplementary Table 4; Supplementary Table 5), which could at least partially cause their lower FE.

Recent studies have highlighted a possible relationship between microbial metabolic functions and animal's FE, but the nature of such relationship is still unclear. Li et al. (2016) observed that HE cattle had more active metabolism of nucleotides, as well as of various energy-generating molecules (i.e., propanoate, glyoxylate and dicarboxylate, starch and sucrose), hypothesizing that such increased metabolic activity could enhance feed digestion and provide the host with more nutrients. Li et al. (2016) and Elolimy et al. (2020) also reported that rumen microbiota of the most efficient cattle was more active in cell proliferation and survivability, inducing cellular growth and increasing tolerance to viral infection; likewise, our results showed enhanced cell membrane transport functions in HE growing animals. Finally, the observed decrease of methane metabolism activity in HE finishing bulls (Shabat et al., 2016) supports the previous idea that high and low methane emitters can have similar abundance of ruminal methanogens but differential expression and transcription of methanogenesis pathway genes (Shi et al., 2014).

5. Conclusions

The exploration of the relationship between rumen microbial community and host FE revealed increased nutrient digestibility in HE animals. Alpha biodiversity and genera network connectance increased with time in LE bulls, highlighting a possible trade-off between FE and ruminal microbiota robustness. Moreover, certain genera previously related with high methane emission were more central in LE animals' genera networks. Our results provide evidence that the rumen microbiota could be one of the biological factors associated with variation in cattle FE.

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Supplementary information

Supplementary Table 3 Feed efficiency classification

Animals were classified into four categories of feed efficiency according to their random regression coefficients for average daily gain (ADG) and metabolic weight (MW) obtained in the residual feed intake model. Mean values within a column with unlike superscript letters differ (P < 0.05). [*] *Feces and ruminal fluid samples were obtained and animals were subjected to further apparent digestibility, ruminal fermentation and microbiota characterisation.* [+] *One animal in the category iv was excluded from the trial due to a respiratory illness.*

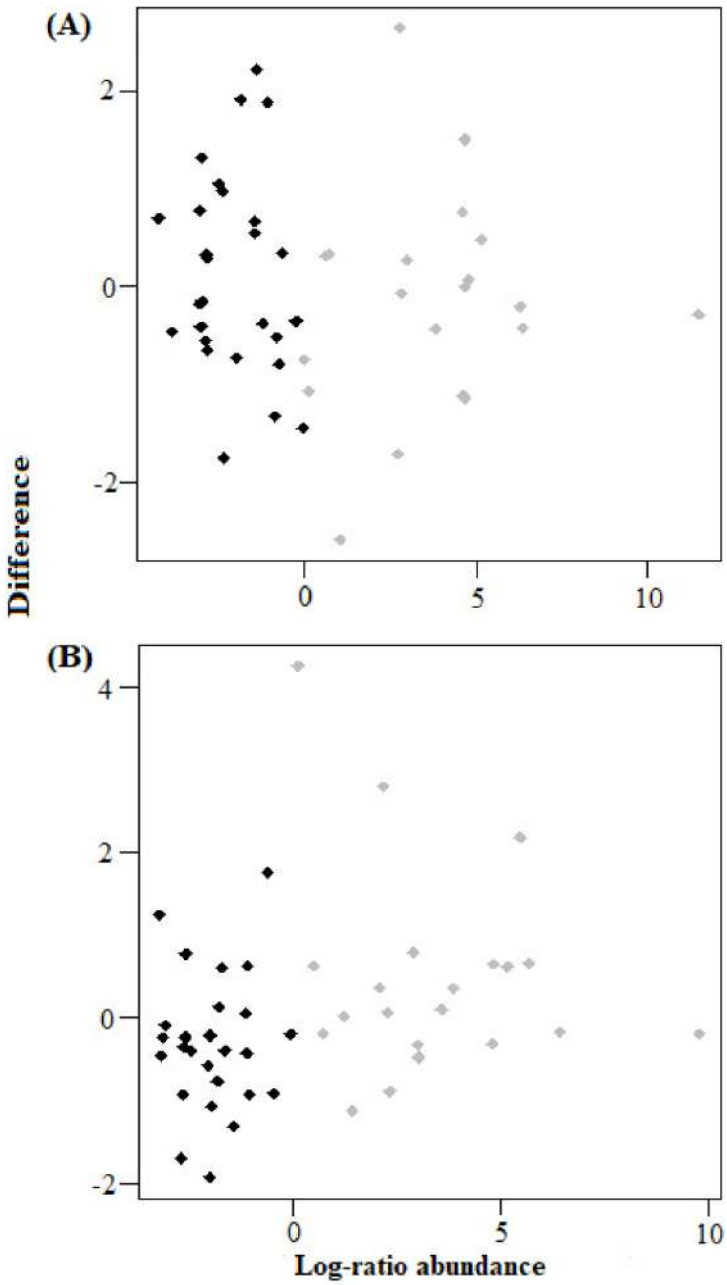
Feed efficiency category	Description	Animals	Sampled animals[*]	Random regression coefficients mean (standard error)	
				ADG	MW
i	Low-efficiency in ADG and low-efficiency in MW	100	13	+0.22 (0.026)[a]	+0.012 (0.001)[a]
ii	Low-efficiency in ADG and high-efficiency in MW	108	12	+0.33 (0.027)[b]	-0.015 (0.001)[b]
iii	High-efficiency in ADG and low-efficiency in MW	85	11	-0.25 (0.024)[c]	+0.12 (0.001)[a]
iv	High-efficiency in ADG and high-efficiency in MW	94	12[+]	-0.26 (0.025)[c]	-0.014 (0.001)[b]

Supplementary Table 4 Node degree values obtained in genera networks.

Nodes	Growing period		Finishing period	
	High-efficiency	Low-efficiency	High-efficiency	Low-efficiency
Acidaminococcus	5	4	0	0
Acinetobacter	0	1	2	0
Aerococcus	4	0	0	0
Agathobacter	0	3	6	7
Allisonella	5	0	1	0
Arthrobacter	0	0	1	0
Bacteroides	3	1	0	0
Bifidobacterium	6	2	2	0
Brachybacterium	0	0	4	0
Brevibacterium	0	0	1	0
Butyrivibrio	0	2	3	10
Candidatus_Phytoplasma	3	0	0	3
Clostridium	0	1	1	2
Corynebacterium	2	0	5	3
Dialister	6	2	4	5
Dietzia	0	0	2	0
Eubacterium	8	2	1	0
Facklamia	2	0	4	0
Fibrobacter	2	1	5	8
Jeotgalicoccus	1	0	2	0
Lachnoclostridium	0	0	0	1
Lactobacillus	0	2	6	0
Megasphaera	9	0	7	9
Methanobrevibacter	5	3	0	10
Mitsuokella	8	3	6	6
Olsenella	7	6	3	0
Peptoclostridium	0	0	0	3
Prevotella	1	3	6	4
Pseudobutyrivibrio	1	2	0	5
Pseudomonas	0	2	2	0
Pseudoramibacter	5	3	8	11
Psychrobacter	4	3	0	0
Roseburia	2	0	6	6
Ruminococcus	5	2	3	5
Selenomonas	8	1	3	5
Sharpea	6	5	5	0
Staphylococcus	0	1	2	1
Treponema	5	1	3	9
Turicibacter	0	0	2	5
Weissella	1	0	0	0

Supplementary Table 5 Betweenness centrality values obtained in genera networks.

Nodes	Growing period		Finishing period	
	High-efficiency	Low-efficiency	High-efficiency	Low-efficiency
Acidaminococcus	6.3	3.0		
Acinetobacter		0.0	0.0	
Aerococcus	0.0			
Agathobacter		4.0	1.0	7.5
Allisonella	0.0		0.0	
Arthrobacter			0.0	
Bacteroides	0.0	0.0		
Bifidobacterium	2.3	2.0	4.0	
Brachybacterium			6.0	
Brevibacterium			0.0	
Butyrivibrio		0.0	0.0	5.2
Candidatus_Phytoplasma	3.0			0.5
Clostridium		0.0	0.0	1.0
Corynebacterium	0.0		9.0	0.0
Dialister	18.5	0.0	0.0	0.8
Dietzia			1.0	
Eubacterium	16.6	3.0	0.0	
Facklamia	7.0		4.0	
Fibrobacter	0.0	0.0	3.0	8.5
Jeotgalicoccus	0.0		0.0	
Lachnoclostridium				0.0
Lactobacillus		0.0	8.0	
Megasphaera	12.6		5.0	8.9
Methanobrevibacter	0.0	0.0		0.0
Mitsuokella	7.3	0.0	0.0	0.0
Olsenella	3.3	6.0	0.0	
Peptoclostridium				0.0
Prevotella	0.0	0.0	11.0	0.0
Pseudobutyrivibrio	0.0	0.0		0.9
Pseudomonas		0.0	0.0	
Pseudoramibacter	0.0	0.0	11.0	10.9
Psychrobacter	0.0	0.0		
Roseburia	0.0		6.0	2.5
Ruminococcus	0.0	0.0	0.0	0.0
Selenomonas	5.1	0.0	0.0	2.0
Sharpea	5.8	7.0	2.0	
Staphylococcus		0.0	0.0	0.0
Treponema	13.3	0.0	0.0	4.2
Turicibacter			0.0	0.0
Weissella	0.0			



Supplementary Figure 1 Graphical representation of ANOVA-like differential expression (ALDEx) analysis on bacterial and archaeal OTUs in ruminal fluid.

Obtained in intensively reared bulls in two periods: growing (A: 159 d old and 225 kg body weight) and finishing (B: 266 d old and 434 kg body weight). Residual feed intake was modeled to classify animals into two categories of feed efficiency: high-efficiency (HE) and low-efficiency (LE). Plots show the relationship between genera abundance and their difference in abundance between feed efficiency categories. Each point represents a different genus: black dots represent rare genera that are not significantly different between feed efficiency categories and grey dots represent abundant genera that are not significantly different between feed efficiency categories

GENERAL DISCUSSION

According to the latest FAO projections, meat demand will increase by a further 80 % by 2030 and by over 200 % by 2050. Such rapid increase in production and trade is expected to be rooted in the growth of intensive production systems, with relatively little contribution from smallholder producers (FAO, 2018). Intensive livestock farming is highly efficient and can provide a regular supply of clean, affordable and nutritious food but it also entails an environmental burden. Intensive beef cattle production is not an exception to that and it has a large potential of acidification, eutrophication, energy consumption and water depletion (Bragaglio et al., 2018; Huerta et al., 2016; Ogino et al., 2016). In that context, diverse strategies related with nutritional management have been thoroughly reviewed as a means to increase systems' profitability while mitigating its environmental impact, e.g., forage-to-concentrate ratio (Capper, 2012; Molossi et al., 2020), nitrogen (N) use efficiency (Angelidis et al., 2019; Salami et al., 2020), antimicrobials (Capper and Hayes, 2012), diet ingredients (Kinley et al., 2020; Lagrange et al., 2020), etc. However, fewer studies have focused on the effects of such strategies on ruminal microbiota composition and, specially, on the interactions established among microbes. The research undertaken in this thesis provides additional insight into the feasibility of three strategies to redesign intensive beef cattle production: (i) dietary protein restriction, (ii) increasing dietary forage-to-concentrate ratio, and (iii) improving animal feed efficiency; with a particular eye on their effects on rumen community state and functioning.

1. Animal performance and ruminal fermentation

1.1 Dry matter intake and body weight gain

In spite of the dietary strategy used, no relevant discrepancies were found between our average daily gain (ADG) or dry matter intake (DMI) and literature data (Mora-Gil, 2019;

Verdú et al., 2017); however, specific differences between dietary treatments could be detected.

As described in chapter II (Table 6, Figure 5), dietary protein restriction penalized animals ADG (11 %) only during the first month of the trial and no effects on DMI or slaughter body weight were detected. Other authors have obtained similar results (Schiavon et al., 2010; Segers et al., 2014), concluding that the lack of differences in performance could stem from the compensatory growth undergone by animals fed low-protein diets in the middle fattening period, and that feeding restricted levels of dietary protein could be a favorable strategy to increase cattle production efficiency at a commercial-farm level. Moreover, dietary protein restriction decreased nitrogen waste by 29 % in the growing period and by 10 % in the finishing period (Table 6); in that sense, previous studies have reported similar reductions in nitrogen waste in response to moderate limitations of protein intake (He et al., 2018; Zhang et al., 2017), highlighting the viability of dietary protein restriction as a means to alleviate beef cattle environmental footprint.

In chapter III, low-quality barley straw (control) was replaced by either oats or vetch haylage in order to increase bulls' voluntary forage intake and dietary forage-to-concentrate ratio. The obtained results proved our initial hypothesis and a moderate increase in forage-to-concentrate ratio could be observed during the finishing period (14/86 vs 18/82 vs 22/78 in bulls receiving barley straw, oats and vetch haylage, respectively). In relation to DMI, no statistical differences could be found between dietary treatments; however, a moderate decrease (about 17 %) could be observed due to haylage feeding during the growing period (Table 10). Animals consuming oats haylage later incremented their DMI and ended up ingesting the same amount of feed as control bulls in the finishing period; thus, no differences in performance could be stated between those two dietary treatments (Figure 9). Contrarily, vetch haylage inclusion also depressed DMI in the finishing period (5 %), leading to a

reduced ADG at the beginning (10 %) and at the end (22 %) of the trial, and to a penalization of slaughter body weight (5 %) (Figure 9). The observed decreased DMI and performance in vetch-fed animals may be explained because these bulls reduced concentrate intake throughout the whole fattening period; the increase in forage intake may depress concentrate intake as vetch haylage had the lowest fiber content, and fiber content is negatively correlated with forage digestibility and voluntary intake (Jung and Allen, 1995). Moreover, improving forage quality seemed to alleviate the drop in ruminal pH that was observed as bulls aged (Table 10); in that sense, the substitution of high-lignocellulose straw for haylage would ameliorate ruminal fermentation conditions. Nevertheless, in no dietary treatment the mean pH values recorded were below the defined 5.9 threshold for diagnosing subacute ruminal acidosis in ruminal fluid samples obtained via stomach tubing (Duffield et al., 2004), therefore, animals' performance was not expected to be impaired by such metabolic disorder.

Feed efficiency is a measure to determine the ability of beef cattle to turn feed nutrients into meat: animals with high-efficiency are able to gain more weight, per kg of DMI, than their low-efficiency counterparts. As stated in chapter IV (Table 13), although differences in DMI between feed efficiency groups did not reach statistical significance, high-efficiency bulls had higher apparent digestibility coefficients for dry matter, organic matter and crude protein (Nkrumah et al., 2006; Richardson et al., 1996). Another possible explanation of the observed differences in bulls' feed efficiency phenotype could be that low-efficiency animals had a fermentation pattern oriented towards the production of higher molar proportion of acetic and less propionic acids than their counterparts (Table 13), being known that metabolic hydrogen produced in the first step of acetic acid pathway is later taken up by methanogens, increasing energy loss through gas emissions (Ungerfeld, 2020), and that tissue utilization of propionate is more efficient than acetate one (McDonald et al., 2010).

1.2 Rumen contents sampling and representativeness of the results

Ruminal fermentation and microbiota have been widely studied because of the importance of this digestion compartment in ruminants' nutrition and, thus, in feed conversion to animal products. The main pitfall of analyzing ruminal fermentation and its microbiota is that no direct access to the rumen compartment does exist, so, a wide variety of sampling techniques have been developed in order to obtain a representative sample of rumen contents. For long, rumen sampling through a cannula has been considered the gold standard technique; however, it is an invasive method that alters rumen anaerobic environment and requires skilled surgery, animal care and, in most countries, formal governmental permission, which increases its cost and limits the number of experimental animals (Tapio et al., 2016). Rumen cannulation may also compromise animals' performance, which can be a major drawback in those trials performed under commercial conditions. Alternatively, rumenocentesis (Nordlund and Garrett, 1994) has been widely performed but, despite being less invasive than rumen cannulation, it also involves surgical preparation of the centesis site, physical discomfort for the animal and risk of localized abscesses or peritonitis (Duffield et al., 2004). With the purpose of avoiding surgical procedures, stomach tubing has been developed as a non-invasive, cheap procedure that can be used to sample a large group of animals (Geishauser, 1993); however, it is still unpleasant for the animal and certain concerns exist about saliva contamination and sample representativeness, inconsistent position of the tube in the rumen and lack of recovery of the solid fraction (Paz et al., 2016). Alternatively, diverse authors have proposed the suitability of using buccal swabs to determine the composition of ruminal microbiota, as ruminants regurgitate large amounts of rumen contents to their oral cavity in order to chew the partially digested feed material (Kittelmann et al., 2015); indeed, Tapio et al. (2016) assessed the similarity between ruminal microbiota composition sampled by stomach tubing and buccal swabs and observed that bacteria, archaea and anaerobic fungi

populations present in buccal swabs samples closely matched those present in stomach tube samples (Pearson correlation coefficients: 0.98, 0.96 and 0.99, respectively), while the correlation between ciliate protozoa was lower (0.65).

Different authors have tried to identify the most optimal technique to study ruminal fermentation parameters, obtaining a wide range of results. Ramos-Morales et al. (2014) and Lodge-Ivey et al. (2009) concluded that sampling through ruminal cannula or stomach tubing give similar results regarding to fermentation parameters. Conversely, Duffield et al. (2004) and Terré et al. (2013) reported that rumen pH was between 0.1 and 0.4 units higher when samples were obtained via stomach tubing instead of ruminal cannula. In that sense, the results presented in this thesis support the suitability of stomach tubing as a non-aggressive sampling procedure to characterize ruminal fermentation, as the observed pH values, volatile fatty acids and ammonia-N concentrations (Table 7, Table 10, Table 13) were in the range obtained by other authors working with animals fed similar diets (Table 15).

When comparison between rumen cannulation and stomach tubing was extended to ruminal microbiota, both Henderson et al. (2013) and Paz et al. (2016) concluded that the two techniques give an equally valid qualitative representation of microbial community structure: the samples obtained by both methods could not be distinguished by multivariate analysis graphical representation and only the relative abundance of certain microbial groups would differ depending on the method used. In this regard, the present results relative to ruminal microbiota composition are in agreement with those previously reported by other authors working with cannulated animals. On the one hand, microbial alpha diversity was low (Table 8, Table 11, Table 14), as it usually experiments a decrease when ruminant animals are fed high-concentrate diets (Fliegerova et al., 2021; Kim et al., 2018; Plaizier et al., 2017; Zhang et al., 2019). On the other hand, the bacterial genera identified as core microbiota in the experiments included in this thesis (i.e., *Prevotella*, *Roseburia*, *Selenomonas*, *Sharpea* and

Ruminococcus) have already been described to be present or to increment when animals are fed a high-concentrate diet (Callaway et al., 2010; Fliegerova et al., 2021; Plaizier et al., 2017; Zhang et al., 2019).

1.3 Ruminal pH and ammonia-nitrogen availability

Though concentrate proportion of total intake was high (between 80 – 90 %), registered ruminal pH was not far from neutrality. In theory, animals receiving such amount of concentrate would have a massive volatile fatty acids production that would induce a critically low ruminal pH, leading to a subacute or acute ruminal acidosis condition in almost the whole herd; however, young cattle seems to be able to adapt to specific challenges, and consequent acidosis is less severe than that experienced in adult cattle (Gozho et al., 2005). In fact, previous pH data reported by other authors also working with cattle fed high-concentrate diets (Table 15) are similar than the results obtained in the present thesis (Table 7, Table 10, Table 11, Table 13). In any case, pH values need to be treated with caution due to their marked circadian rhythm (Rotger et al., 2006a, 2006b): pH records are close to neutrality in the morning but they suddenly decrease at night, therefore, some periods when rumen environment was bellow SARA threshold levels could not be dismissed (pH < 5.8 for 167 min: D. Villalba, personal communication, 2018).

Ammonia is the main N source for microbes and, in the seventies, the threshold level to fulfil microbial N requirements was fixed at a value of 50 mg/mL (Satter and Slyter, 1974). Since then, that definition has been refined and, nowadays, ammonia-N needs are adjusted in function of fermentable organic matter availability (Song and Kennelly, 1990), presence or absence of preformed protein (Broudiscou and Jouany, 1995) or microbes function (Balcells et al., 1993). Moreover, it has for long been recognized that the necessary ammonia-N concentration to fulfill microbial requirements is lower when high-concentrate diets are

employed as ammonia-N assimilation may be increased (as a result of more readily available carbohydrates which intensifies microbial growth) and bacterial recycling decreased (due to the lower abundance of rumen protozoa) (Hristov et al., 2001). In that sense, ammonia-N concentrations presented in this thesis (Table 7, Table 10, Table 13) are in agreement with those reported in the existing literature (Table 15) and, considering that the protein content in our diets was lower than that used in the consulted works, it is logical that our ammonia-N values are placed at the bottom range.

2. Issues derived from statistical analysis of microbial abundance data

The rapid development of high throughput sequencing techniques has provided an opportunity to better understand the structure of microbial community in the rumen; in that sense, amplicon sequencing targeting the 16S rRNA in bacteria and archaea constitutes the most cost-effective and facile tool to provide valuable phylogenetic information for the comparison of microbial community in large number of environmental samples, such as ruminal contents (McGovern et al., 2018). The 16S rRNA gene contains both highly conserved areas and hypervariable sites, denoted as V1 – V9; the conserved regions can be targeted with PCR primers while the hypervariable regions are specific to each microbial species and make possible to distinguish the different microbes. Briefly, amplicon sequencing approach involves the following steps (Dong et al., 2017): (i) extraction of DNA from a set of samples, (ii) amplification of the target region by PCR, (iii) barcoding of the amplicons of each sample, and (iv) high throughput, “multiplexed” sequencing of the combined amplicons from all samples. The resulting nucleotide sequences, named as “reads”, are then subjected to bioinformatic analysis that consists on: (v) processing and quality control filtering of the reads, (vi) OTUs binning, (vii) taxonomy assignment of each OTU, (viii) construction of the abundance table, and (ix) statistical analysis.

Table 15 References on ruminal pH values, volatile fatty acids and ammonia-nitrogen concentrations in cattle fed high-concentrate diets.

****** *Only concentrate composition was provided. CP: crude protein; DM: dry matter; N: nitrogen; ND: no data provided; NDF: neutral detergent fiber.*

Authors	**Sampling procedure**	**Ruminal pH (daily mean)**	**Ammonia-N (mg/L)**	**Volatile fatty acids (mmol/L)**	**Dietary CP (g/kg DM)**	**Dietary NDF (g/kg DM)**
Ludden and Cecava, 1995	Ruminal cannulation	6.1 – 6.3	21.2 – 42.3	71.0 – 95.5	124	No data
Devant et al., 2000	Ruminal cannulation	6.2 – 6.4	11.7 – 53.9	88.2 – 95.6	138 – 173*	202 – 258*
Devant et al., 2001	Ruminal cannulation	6.2 – 6.4	5.9 – 13.8	98.1 – 111.6	135 – 139*	142 – 159*
Hristov et al., 2001	Ruminal cannulation	5.9 – 6.2	17.4 – 35.1	118 – 133	139	229
Beauchemin and McGinn, 2005	Stomach tubing	5.69 – 6.22	ND	106.1 – 115.4	134 – 151	127 - 204
Rotger et al., 2006a	Ruminal cannulation	6.5 – 6.6 Daily max: 7.4 – 7.8 Daily min: 5.7 – 6.1	22 - 55	109.9 – 122.5	130 – 145	181 – 256

Table 15 Continued.

Authors	**Sampling procedure**	**Ruminal pH (daily mean)**	**Ammonia-N (mg/L)**	**Volatile fatty acids (mmol/L)**	**Dietary CP (g/kg DM)**	**Dietary NDF (g/kg DM)**
Rotger et al., 2006b	Ruminal cannulation	6.0 Daily max: 7.1 Daily min: 5.4	105.5	158.8	153	219
Verdú et al., 2015	Rumenocentesis	5.9 – 6.2	ND	115.9 – 134.3	144 – 163*	204 – 282*
D. Villalba, Personal communication, 2018	Ruminal boluses	6.05 Daily max: 7.97 Daily min: 4.73	ND	ND	112 – 114	170 - 207
Present thesis, chapter II	Stomach tubing	6.45 – 7.29	0.58 – 7.06	45.51 – 47.79	111 – 133	267 – 285
Present thesis, chapter III	Stomach tubing	6.45 – 7.29	4.15- 32.78	54.99 – 91.75	133 – 146	219 – 260
Present thesis, chapter IV	Stomach tubing	6.75 – 7.16	12.03 – 19.56	68.22 – 77.05	127 – 128	207 - 229

The abundance tables obtained in amplicon sequencing studies are large matrices with the different samples in rows and all the detected OTUs in columns; each entry of the matrix represents the number of reads corresponding to each OTU in each sample. Such abundance tables present some relevant characteristics (Calle, 2019):

(i) They contain large proportions of zeros, which requires the application of zero-dealing strategies.

(ii) The total number of reads per sample is highly variable along samples, which requires the application of normalization procedures.

(iii) The total number of counts per sample is constrained by the maximum number of sequence reads of the DNA sequencer, which causes strong dependencies between the abundances of different taxa: an increase in abundance of one taxon requires the decrease of the observed number of counts of some others.

In abundance tables, the value of each entry is not informative by itself and the relevant information is contained in the ratios between the components; therefore, microbiota abundance data convey relative information of the components of a whole and are considered to be “compositional data” (Gloor et al., 2017).

One key condition that should be fulfilled for a proper analysis of compositional data is subcompositional coherence, implying that the results obtained when a subset of components is analyzed should not contradict those obtained when analyzing the whole composition (Aitchison, 2005). In the context of microbial abundance studies, this condition is important because it is common to work with subcompositions, obtained after filtering out the most low-abundant OTUs. If the compositional nature of microbial abundance data is ignored, four main issues may arise (Rivera Pinto, 2018):

(i) Spurious correlations: as a consequence of the total count constraint that is implicit in microbial abundance data, negative correlations between components (OTUs) may arise, regardless of the underlying relationship between them.

(ii) Increase of type I error: ignoring the compositional nature of data may lead to an increase of false-positive findings in differential abundance tests.

(iii) Subcompositional incoherence of correlations: when working on subcompositions, not only size differences but also changes in the sign of associations can be found with respect to the correlations obtained in the whole composition.

(iv) Subcompositional incoherence of distances: commonly used distance metrics do not fulfill the condition of subcompositional dominance, by which the distance between two points in a multi-dimensional space should always be larger than their distance when projected in a lower dimensional space.

Statistical analysis of microbial abundance data usually starts with a normalization step followed by the calculation of alpha diversity in each sample and beta diversity across samples. Typically, beta diversity is assessed using dissimilarity measures, based on the calculation of a distance matrix, and then is graphically represented in ordination plots that take high-dimensional data and represents them in a viable number of dimensions. Finally, approaches as differential abundance testing and network inference can be performed in order to better determine microbiota state and functioning, enabling a deeper comparison of microbial communities between samples. Because of the fact that many researchers are unaware of the compositional nature of microbial abundance data, statistical analyses are still performed using standard methods; however, alternative methods that account for data compositionality are available and their use must be encouraged (Table 16).

Table 16 Standard and composition-friendly methods for the statistical analysis of microbial abundance data.

Adapted from Calle (2019), Gloor et al. (2017), Kalivodová et al. (2015) and Weiss et al. (2017).

Statistical analysis	Standard methods	Composition-friendly methods
Normalization	Rarefaction Scaling	Zero imputation plus log-ratio transformation
Distance	Bray-Curtis Unique fraction metric (UniFrac)	Aitchison
Ordination	Principal coordinates analysis (PCoA) Non-metric multi-dimensional scaling (NMDS)	Principal component analysis (PCA) Partial least squares-discriminant analysis (PLS-DA)
Multivariate comparison	Permutational multivariate analysis of variance (PERMANOVA) Analysis of similarity (ANOSIM)	Permutational multivariate analysis of variance (PERMANOVA) Analysis of similarity (ANOSIM)
Differential abundance testing	Linear discriminant analysis effect size (LEfSe) Differential expression analysis for sequence count data (DESeq2)	ANOVA-like differential expression analysis (ALDEx2) Analysis of composition of microbiomes (ANCOM)
Network inference	Pearson Spearman	Sparse correlations for compositional data (SparCC) Sparse inverse covariance estimation for ecological association and statistical inference (SpiecEasi)

3. Economic insights on the strategies to improve ruminal microbiota robustness

The composition of rumen microbial community is very diverse and highly variable, depending on diet, host and age (Henderson et al., 2015; Jami et al., 2013), making difficult the definition of a normal or healthy microbiota. Alternatively, robustness has been put

forward as a surrogate marker of a healthy microbial community (Dogra et al., 2020; Fassarella et al., 2020).

As noted in chapter I, a microbial community is considered to be robust if it exhibits temporal stability, resistance, resilience and functional redundancy. In other words, when external stress factors do occur, e.g., dietary changes (Plaizier et al., 2020), digestive disorders (Azad et al., 2019), metabolic diseases (Ogunade et al., 2019) or drugs administration (Ji et al., 2018), robust microbiotas will be able to resist to such disturbances, to return to their initial state if they have been perturbed or to evolve to an alternative state with the same functional capabilities as the initial one. The main biologic characteristics attributed to robust microbial communities are increased microbial alpha diversity and network complexity, parameters that can be measured to predict microbiota robustness to face disturbances. To date, there are a few evidences supporting such link between microbiota robustness and host health. In human studies, a lower microbiota alpha diversity has been associated to a bloom of the opportunistic pathogen *Enterobacter cloacae* after antibiotic administration (Raymond et al., 2016); similarly, microbiota network complexity has been positively associated with Chron disease remission after ileocolonic resection (Mondot et al., 2016).

The results presented in this thesis showed that the three strategies tested to redesign beef cattle production can affect ruminal microbiota robustness. First of all, dietary protein restriction increased both alpha diversity (mainly in growing animals, Table 8) and network complexity of rumen microbial community (Figure 8). Similarly, increasing dietary forage-to-concentrate ratio also raised microbial alpha diversity (Table 11) and network complexity (Figure 13, Figure 14, Figure 15). Finally, although the effects of animal feed efficiency on their ruminal microbiota robustness were less evident, numerical differences indicated that both alpha diversity (Table 14) and network complexity (Figure 17) incremented with time in the rumen of less efficient animals, phenomena that could not be detected in more efficient

animals. Therefore, the present results would suggest that dietary protein restriction and forage-to-concentrate ratio increase can enhance ruminal microbiota robustness, while improving animal feed efficiency can have the opposite effect.

The question that remains unanswered is whether enhancing ruminal microbiota robustness with the purpose of building a healthier rumen with an increased ability to cope with potential disturbances is economically viable at a commercial farm level. To try to estimate how much does it cost to build a robust rumen, we have calculated the gross margin of beef cattle production, based on intake and slaughter weight data presented in chapter II and chapter III and feedstuffs and animal products price during the last 20 years available on EUROSTAT database (Eurostat, 2021). The results presented in chapter IV have not been included in these gross margin estimations because decreased feed utilization efficiency will always penalize the economic results of beef cattle production.

In Figure 18A, feed cost, gross margin per animal and soy-to-corn price ratio, from years 2000 to 2019, are presented. Only when soybean meal is available at a low price, there are no differences in production gross margin between feeding the animals a standard diet or a low protein diet; however, when soybean meal price is high, dietary protein restriction would increase the profitability of beef cattle production. These preliminary estimations suggest that enhancing ruminal microbiota robustness through reducing protein intake would not penalize the economic results of beef cattle production. Alternatively, Figure 18B shows the feed cost and gross margin per animal, from 2000 to 2019, when animals are fed different forage sources: it can be seen that providing grasses as forage source is the most profitable dietary option while the use of legume forages penalizes the economic results; such estimations indicate that grasses should be favored over legumes as forage source because the formers leaded to the most optimal equilibrium between animal performance and rumen microbial robustness.

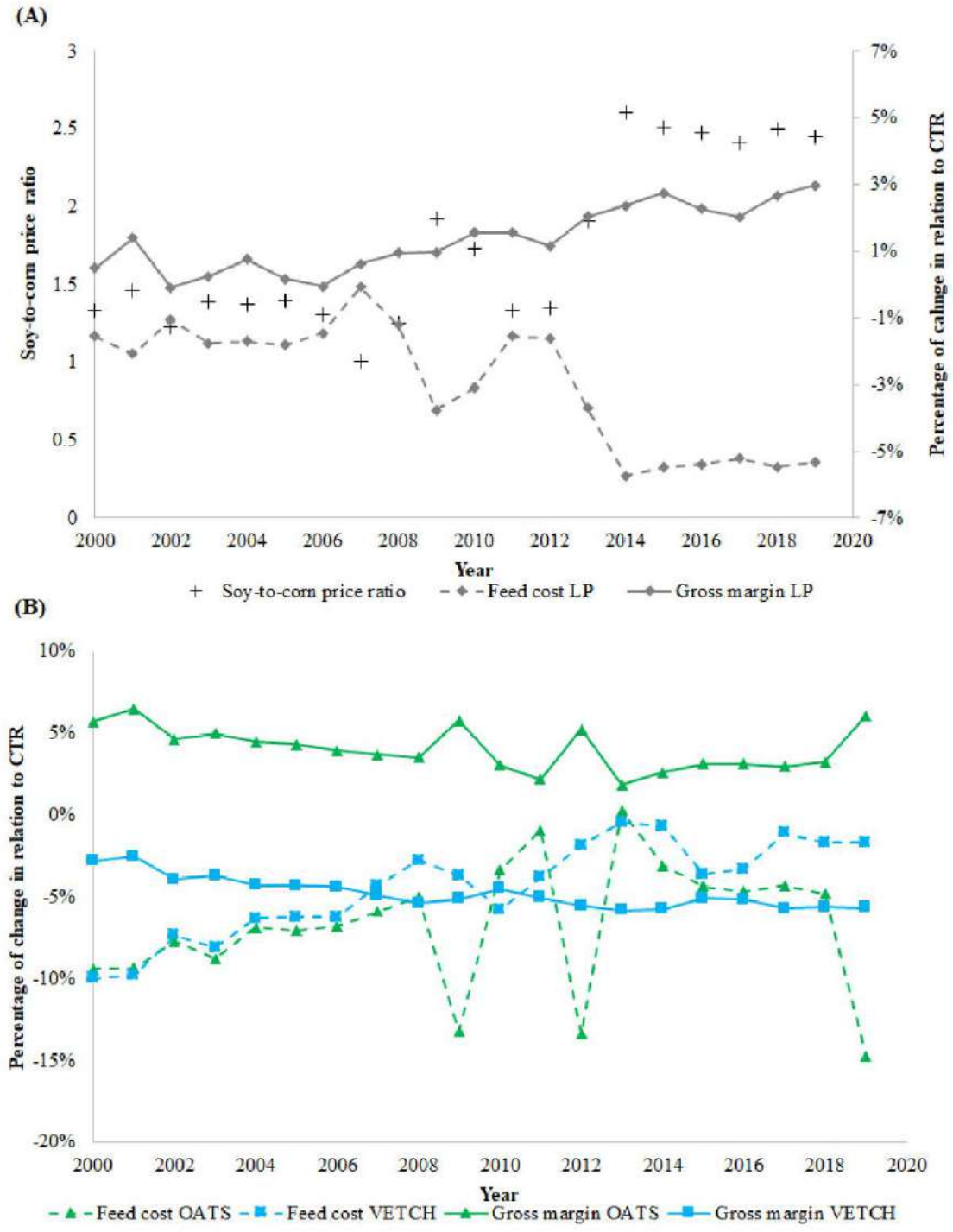


Figure 18 Feed cost and gross margin of beef cattle production in different scenarios of feed price and diet composition.

Dietary strategies tested were (A) protein intake restriction (CTR, standard diet vs LP, low protein diet) and (B) increasing forage-to-concentrate ratio via improving the quality of forage source (CTR, cereal straw vs OATS, grass forage vs VETCH, legume forage).

Despite the fact that providing legumes as forage source resulted in a tighter gross margin in relation to the control diet, the improved ruminal robustness acquired due to such dietary strategy could bring better results at a commercial farm level. Indeed, cattle fed high-concentrate diets can be in a subclinical state of acidosis, as reflected in the hyperkeratosis of the ruminal mucosa and signs of rumenitis detected at slaughterhouse (58 % and 30 % of the inspected rumens, respectively, according to Magrin et al. [2021]). Considering that digestive disorders are responsible of 30 – 42 % of beef cattle monthly mortality rates in North America (González et al., 2012), further studies are needed to accurately determine the real link between ruminal microbiota robustness, animal health and economic results at a commercial farm level. Long term experimental trials that apply a disturbance to animals with either robust or fragile microbial communities in rumen, following up the posterior shifts in microbiota composition and function, would be of great interest to elucidate the practical benefits of building a more robust rumen.

4. Personal thoughts about future research on rumen microbial community

The number of papers studying rumen microbial community has increased in the recent years (Figure 19). Most of these studies focus on the shifts that occur on alpha diversity and populations abundance due to the experimental treatments tested; however, few studies report microbial network analysis (Figure 19), leaving the interactions between ruminal microbes fully unexplored.

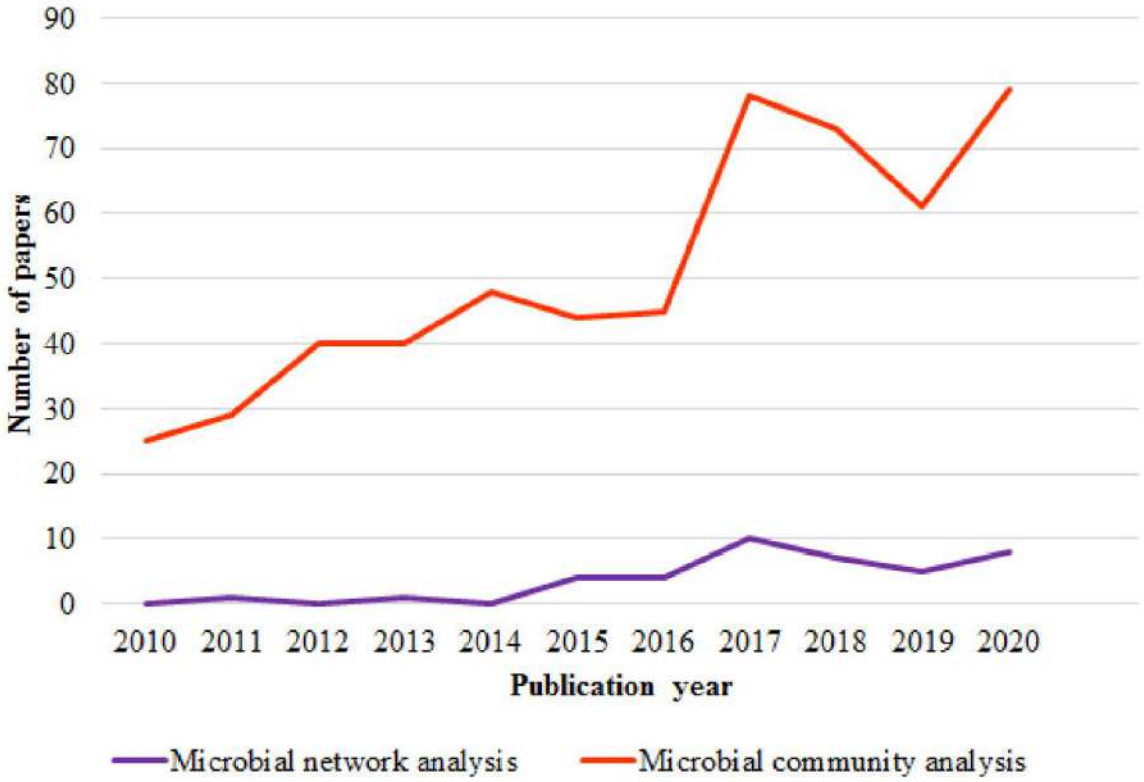


Figure 19 Number of papers reporting microbial community analysis and microbial network analysis in rumen.

Data are based on the Web of Science database, time span 2010-2020, keywords "microbial network analysis rumen" and "microbial community analysis rumen".

Excluding microbial associations from the routine analysis of rumen microbial community may constitute a considerable loss of information. It is known that, when a disturbance occurs, biotic interactions are the first to be affected and thus can alter the community functioning even before the species disappear (Valiente-Banuet et al., 2015); in other words, certain alterations in microbial community state and function may not be detectable with alpha diversity determinations and differential abundance testing, so analysis at a finer resolution, such as microbial co-abundance patterns, are highly needed.

Microbial network analysis is also useful to identify potential keystone taxa, defined as highly connected taxa that exert a considerable influence on microbiome structure and functioning

irrespective of their abundance across space and time. Keystone taxa may selectively modulate other microbial groups by secreting metabolites, antibiotics or toxins with the purpose of gaining direct benefits, e.g., replacing indigenous microflora, obtaining competitive advantage in the community or promoting further own growth (Banerjee et al., 2018). A common strategy to identify keystone taxa is to determine the most associated nodes in a microbial network and, more specifically, the combination of high node degree, high closeness centrality and low betweenness centrality has been proposed as an accurate indicator of keystone taxa (Berry and Widder, 2014). Therefore, the identification of keystone taxa could be a good option to better describe rumen microbial community and to determine in detail its potential shifts in response to a disturbance.

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CONCLUSIONS

Conclusions based on performance, intake and ruminal fermentation results

(1) Moderate restriction in dietary protein supply, increasing forage quality and forage-to-concentrate ratio and improving feed efficiency were all proved to be feasible options to redesign intensive beef cattle production, alleviating its environmental footprint while maintaining or improving its profitability.

(2) Reducing dietary protein content did not have major impacts on either dry matter intake or animal performance. Protein limitation, compared to commercial levels, did not penalize ammonia-nitrogen concentration in ruminal fluid but it significantly reduced nitrogen waste.

(3) Replacing barley straw by oats haylage as forage source in concentrate-fed cattle did not modify dry matter intake, performance or nutrient apparent digestibility; however, vetch haylage feeding reduced concentrate intake, slaughter body weight and nutrient apparent digestibility.

(4) The exploration of the relationship between ruminal fermentation and cattle feed efficiency revealed increased nutrient apparent digestibility and a fermentation pattern oriented towards the production of propionate in high-efficiency animals.

Conclusions based on ruminal microbiota determinations

(5) Dietary protein restriction, increasing forage-to-concentrate ratio and improving feed efficiency were all proved to significantly modify the composition and functioning of ruminal microbiota, evidencing that microbial community in rumen could play a crucial role in animals' adaptation to different dietary strategies, as well as explain, to some extent, the observed variations in animals' feed efficiency.

(6) Ruminal microbiota robustness is defined as the community's ability to cope with disturbances; it depends on its resistance, resilience and functional redundancy and it can be evaluated via alpha diversity metrics and network complexity inference.

(7) Both reducing dietary protein and providing high-quality haylage as forage source did increase ruminal microbiota alpha diversity and network complexity, suggesting that these dietary strategies can enhance rumen microbial community robustness.

(8) Alpha diversity and genera network complexity increased with time in low-efficiency bulls, highlighting a possible trade-off between feed efficency and ruminal microbiota robustness.

(9) Although alpha diversity is commonly reported, network complexity information is still missing in most of ruminal microbiota literature, thus, further studies providing a full vision of ruminal microbiota would be of great interest to better understand community robustness and to unravel its possible link with animal health and systems profitability.