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“Effect of olive oil and hydrogenated vegetable oil
supplementation on rumen bacterial composition in dairy
cows”

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DEDICATION

To my dear family and friends.

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CHAPTER 1

1. General Introduction

Rumen is one of the most diverse ecosystems in nature because it harbors a complex microbial community such as different types of bacteria, archaea, fungi, and ciliated protozoa (Sirohi *et al.*, 2012a). These microorganisms play an important role in animal health and productivity, they also participate in degrading plant fibers into short-chain fatty acids that later are absorbed to meet the requirement for essential processes such as growth, thermoregulation, and immunity (Wang *et al.*, 2016). Moreover, ruminal microbial community of ruminants is dynamic and could be affected by diet, host, physiological status, and environment (Pitta *et al.*, 2014; Henderson *et al.*, 2015).

Fibrolytic bacteria are particularly important for ruminants, because these animals lack of enzymes that degrade fiber, which are essential to degrade the forage-based diets (Snelling and Wallace, 2017). *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Butyrivibrio fibrisolvens* are the most studied and characterized bacteria in rumen due to the fact they have been *in vitro* cultured (Krause *et al.*, 1999; Koike and Kobayashi, 2009; Sirohi *et al.*, 2012b). Additionally, these bacteria play a fundamental role in the biohydrogenation of dietary unsaturated fatty acids (Potu *et al.*, 2011; Vargas-Bello-Pérez *et al.*, 2016), but at the same time they are susceptible to this type of fatty acids (Lourenço *et al.*, 2010).

Several studies have performed lipid supplementation to modify the milk fatty acid profile, using different lipid sources such as extruded soybeans (Khanal *et al.*, 2005; supplementation for 42 days), extruded linseeds (Lerch *et al.* 2015; supplementation for 35 days), fish oil (Vargas-Bello-Perez *et al.* 2015a; supplementation for 21 days), soybean oil and hydrogenated vegetable oil (Vargas-Bello-Perez *et al.* 2015b; supplementation for 21 days) and calcium salts of palm and fish oil in combination with soybean products (Allred *et al.* 2006; supplementation for 42 days). However, to our knowledge, no study has been published reporting relative long-term (more than 42 days) effects of dietary supplementation with unrefined olive oil (OO; widely available in central Chile) and hydrogenated vegetable oil (HVO; a saturated fatty acid source) on the composition of rumen microbiota.

In particular, palm oil have been widely employed in animal nutrition as energy source (Tomkins and Drackley, 2010) and to compare their effect with other lipids (Vargas-Bello-Pérez *et al.*, 2015a; b; Adeyemi *et al.*, 2016) or to evaluate their influence in rumen fermentation and microbial communities (Abubakr *et al.*, 2014; Lunsin, 2018). On the other hand, olive oil and derivatives have been used in dairy cattle (Meo Zilio *et al.*, 2015; Vargas-Bello-Pérez *et al.*, 2018) and ewes (Vargas-bello-pérez *et al.*, 2013; Gallardo *et al.*, 2014; Pallara *et al.*, 2014; Mannelli *et al.*, 2018), in order to study the effect of these additives in milk production and quality, and improve nutritional value cheese and meat.

Currently, ruminal microbial studies utilize molecular techniques to improve the knowledge in composition of rumen communities and their dynamics. However, the lack of information related to the length of exposure to lipid supplements limits the use of vegetable oils as permanent ingredients in diets of dairy cows. Therefore, the aim of this thesis is to contrast and investigate the effect of two different lipid sources, saturated (palm oil; HVO) and unsaturated (olive oil; OO) on rumen bacterial composition, in a relative long-term supplementation and through Next Generation Sequencing approach.

The **hypothesis** of this research is that supplementation with sources of unsaturated fatty acids will decrease diversity of rumen microbiota, and that effect on bacteria community would depend on the length of exposure to lipids.

The **general objective** is to characterize the effect of lipid supplementation on the composition of rumen bacterial community. **Specifically**, this study pursuit to determine the effect of lipid supplementation with olive oil (an unsaturated FA source) and palm oil (a saturated FA source) on rumen bacterial composition and the possible differences between the dietary treatments. Also, to analyze the changes on rumen bacterial communities throughout the relative long-term exposure to lipid sources.

2. Literature Review

2.1 Rumen microbiome

The group of microorganisms that inhabit the gut of multicellular organisms is often termed as microbiome (Jami *et al.*, 2014), and the DNA extracted from a microbiome sample is called metagenome (Ross *et al.*, 2012). Due to the important role of the rumen microbiome, it is referred as the “second” genome of the ruminant (AlZahal *et al.*, 2017).

The rumen microbiome is a very complex and diverse community formed by bacteria, fungi, protozoa, archaea and viruses (Martinez-Fernandez *et al.*, 2019). Among different rumen microorganisms, ruminal bacteria are the most diverse and accounts for 10^{10} – 10^{11} cells/mL of rumen content, followed by ciliate protozoa (10^4 – 10^6 cells/ mL), archaea (10^7 – 10^9 cells/mL), fungi (10^3 – 10^6 zoospores/mL), and phages (10^9 – 10^{10} cells/mL) (Bainbridge *et al.*, 2016a; AlZahal *et al.*, 2017). Until 1998, approximately more than 200 bacterial species were isolated from the rumen through *in vitro* culture techniques (Pinloche *et al.*, 2013). However, with the development of culture-independent approaches such as the use of 16S rRNA gene analyses it has been demonstrated that the rumen bacterial community is much more diverse than it was previously reported (Kong *et al.*, 2010). In dairy cattle, the rumen microbiome plays a key role in milk production, well-being and health of the animals (Biscarini *et al.*, 2018).

Rumen microorganisms actively metabolize polysaccharides, proteins, and lipids into different products such as volatile fatty acids (VFA's), microbial proteins, and vitamins that are used by the animal (St-Pierre *et al.*, 2015). In the first step, Protozoa, Fungi, and Bacteria carry out the hydrolysis of polymers, such as cellulose and other complex carbohydrates, proteins and lipids to low molecular weight compounds (Wirth *et al.*, 2018). Subsequently, fermentative bacteria convert these metabolites to short chain fatty acids (SCFAs) like acetate, propionate and butyrate, CO₂, H₂, alcohols and other compounds. Methanogenic Archaea produce methane in the final step of the microbial food-chain (Wirth *et al.*, 2018). Energy from VFA (acetate, propionate, and butyrate) and protein synthesized by rumen microbes can fulfill approximately 70% of the total energy requirements and 90% of amino acids that are supplied to the small intestine (Hou *et al.*, 2011; Li and Guan, 2017).

2.2 Ruminal bacteria

Ruminal bacteria make the greatest contribution to digestion and conversion of feeds into short-chain fatty acids and microbial proteins, both essential to bovines (Kim *et al.*, 2011). Bacteria inhabiting the rumen have been classified into five groups dependent on their environmental existence: a) free-living bacteria associated with rumen liquid phase; b) bacteria loosely associated with feed particles; c) bacteria firmly adhered to feed particles; d) bacteria associated with rumen epithelium; and e) bacteria attached to the surface of protozoa or fungal sporangia (Miron *et al.*, 2010).

Moreover, different studies have found that Firmicutes, Bacteroidetes and Proteobacteria are the most abundant bacterial phyla in rumen (Jewell *et al.*, 2015; Tapio *et al.*, 2017) of dairy cows (Kong *et al.*, 2010; Pitta *et al.*, 2010), sheep (Castro-Carrera *et al.* (2014) and goats (Wetzels *et al.*, 2015; Mao *et al.*, 2016). Biological function associated with bacteria from the phylum Bacteroidetes is polysaccharide degradation, meanwhile phylum Firmicutes have a lower ability for polysaccharide degradation and are more known for their production of butyric acid (Bainbridge *et al.*, 2016b).

Fibrobacter succinogenes, *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Butyrivibrio fibrisolvens* are the most studied and recognized fibrolytic bacterial species (Koike and Kobayashi, 2009; Sirohi *et al.*, 2012b; Ribeiro *et al.*, 2016). Plant cell walls are degraded by a combination action of bacteria, fungi and protozoa, with bacteria and fungi contributing approximately 80% of the degradative activity, and protozoa a 20% (Wang and Mcallister, 2002). *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes* are cellulose-degrading bacteria and are generally considered as the primary organisms responsible for degradation of plant cell walls in the rumen (Singh *et al.*, 2014). These bacteria produce cellulolytic enzymes such as endoglucanases, exoglucanases, β -glucosidases and hemicellulases. *Butyrivibrio fibrisolvens* and *Prevotella ruminicola* lack the ability to digest cellulose but degrade xylan and pectin, utilizing the degraded soluble sugars as substrates (Cai *et al.*, 2010). Three adaptive strategies have been identified in the ruminal ecosystem for degrading plant cell walls: a) production of the

full slate of enzymes required to cleave the numerous bonds within cell walls; b) attachment and colonization of feed particles; c) and synergetic interactions among ruminal species (Wang and Mcallister, 2002).

Rumen bacteria also play a fundamental role in dietary lipids metabolism in rumen (Potu *et al.*, 2011). This process identified two important microbial transformations of fats in the rumen: lipolysis and biohydrogenation. Lipolysis causes the release of free fatty acids from esterified plant lipids whereas the biohydrogenation reduces the number of double bonds (Buccioni *et al.*, 2012). Lipid metabolism consisting of sequential fatty acids isomerization and saturation performed by some bacteria to reduce the toxicity of unsaturated lipids for microbial growth (Toral *et al.*, 2018).

2.3 Effect of diet on Rumen Microbiome

Rumen microbial community structure is affected by the specie, breed, (Malmuthuge and Guan, 2017), age of animals age (Jami *et al.*, 2013; Rey *et al.*, 2014), parity (Pitta *et al.*, 2014), and lactation cycle stage (Zhu *et al.*, 2018). However, diet is the major determinant of ruminal microbial composition and changes in diet can lead to rapid and dramatic changes in gut microorganisms (Henderson *et al.*, 2015; Yáñez-Ruiz *et al.*, 2015, Tapio *et al.* 2017). The mechanisms that originate these alterations are not fully understood, especially when animals are fed with lipids.

The chemical composition of diets is the most important factor that determines the diversity and functionality of rumen (Derakhshani *et al.*, 2017). Most research has emphasized that differences in the amount and type of dietary components such as

complex carbohydrates and proteins are the main reason for changes in the microbiome (Petri *et al.*, 2013), therefore rumen nutritionists have developed and used several supplementary products to manipulate the rumen microbiome and harness benefits, such as improved productivity (AlZahal *et al.*, 2017).

2.4 Lipid supplementation in dairy cows

Energy is and will continue to be the major nutritional challenge to increase lactation productivity of dairy cows (Loften *et al.*, 2014). For high-producing dairy cows it is difficult to reach the required energy level in the diet without adding high-energy feed ingredients, such as fat. In dairy production, the diet composition is designed to improve efficiency and production of animals. One strategy is to increase energy content of the diet through lipid supplementation from different sources (McCann *et al.*, 2014). Enrichment of diets with plant oils or oilseeds have been used to increase diet energy density of lactating dairy cows to meet the high energy requirements during early and mid-lactation (Bayat *et al.*, 2017, Tapio *et al.*, 2017).

Naturally, consumption of lipids by herbivorous animals is low because most forages contain only limited amounts of fats. Lipids are mainly found in plant membranes that have a lipid bilayer structure, which are composed of 40–50% lipid and 50–60% protein. Lipids in plant membranes are predominantly composed by phospholipids, triglycerides (mainly found in fats and oils, storing energy), waxes that are found on the plant surface, and steroids (Elgersma, 2015). Moreover, fresh grass and maize silage and reported changes to the content of C18:0, C18:1, unsaturated FA (especially C18:3), and saturated fatty acids (especially C16:0) (Liu *et al.*, 2016).

Also, linoleic acid (LA; C18:2, cis-9, cis-12) and α -linolenic acid (ALA; C18:3, cis-9, cis-12, cis-15) are the most abundant fatty acids in forage and most concentrates (Enjalbert *et al.*, 2017). Consequently, when fat is added to the diet of dairy cows, it is fed normally at no more than 5% to 7% of the total diet DM (Szumacher-Strabel *et al.*, 2002), and should be less than 50 g/kg of DM, because digestive problems can occur if the total fat content of the diet is more than 100 g/kg of DM (Garnsworthy, 1997).

Polyunsaturated fatty acids (PUFA) have been related to the inhibition of biohydrogenation and reduction of fiber digestibility due to toxic effects on ruminal bacteria (Lourenço *et al.*, 2010), especially by the interaction of lipid supplements with cellulolytic bacteria (Cremonesi *et al.*, 2018). However, certain bacteria are able to protect themselves against PUFA toxicity through hydrolysis and hydrogenation of dietary lipids (Maia *et al.*, 2007; Nam and Garnsworthy, 2007), and PUFA may be massively converted into stearic acid (C18:0). In conclusion, the main role of biohydrogenation in the rumen is to decrease the abundance of unsaturated FA in the rumen in order to minimize the inhibitory effects on microbial growth (Ventto *et al.*, 2017)

Due to the lipid biohydrogenation in the rumen, most unsaturated fatty acids are rapidly converted to saturated molecules. Thus, this metabolic process limits the content of unsaturated fatty acids in milk and dairy products (Benchaa *et al.*, 2012). The use of different polyunsaturated lipid source in the diet of dairy ruminant has been extensively adopted with the objective to modify the milk fatty acid composition

toward a more desirable profile for human health (Cremonesi *et al.*, 2018), for example increasing concentrations of PUFA and conjugated linoleic acids (CLA) which have related to beneficial effects (Patra and Yu, 2013). Altering the fatty acid composition of milk offers an opportunity to lower consumption of SFA without losing the benefits of other nutrients and bioactive lipids present in milk (Bayat *et al.*, 2017).

Unsaturated fatty acids (UFA) that avoided biohydrogenation then are absorbed through the intestinal epithelium. In the enterocytes the fatty acids are re-esterified to triacylglycerols and arranged mainly in chylomicrons. Through the lymphatic system they reach the bloodstream where, being directed to peripheral tissues such as the mammary gland, they increase the UFA in milk fat (Welter *et al.*, 2016).

Lipid supplementation could also result in negative production effects, decreasing the feed intake and milk fat content. The reduction in milk fat content with little or no change in milk protein and lactose is recognized as milk fat depression (MFD)(Shingfield *et al.*, 2010). Milk fat depression in dairy cows is linked to a biohydrogenation shift toward the *trans*-10 pathway due to a decreased capacity of the microbiota to isomerize the unsaturated acid via the *trans*-11 pathway, triggering a necessary adaptation of the microbiota to shift toward the *trans*-10 biohydrogenation to avoid the toxic effects of UFA (Enjalbert *et al.*, 2017). C18:1 *trans*-10, which originates in the rumen from the hydrogenation of C18:2 *trans*-10, *cis*-12 that is formed in the biohydrogenation of C18:2, n-6 is the only intermediate shown unequivocally to inhibit milk fat synthesis in lactating cows (Ventto *et al.*, 2017).

Palm oil is a rich source in palmitic acid (C16:0) which is the most common saturated fatty acid found in plants, animals, and several microorganisms (Loften *et al.*, 2014). Inclusion of palmitic acid in diet of dairy cows increase milk fat yield and is used as an energy source for milk production, especially during periods of negative energy balance (Lourenço *et al.*, 2010).

The effect of supplementation with vegetal sources or marine lipids on rumen microbiome has also been studied, however to our knowledge; the possible effect on rumen microbiota when dairy cows are fed with crude olive oil has not been reported. To allow the use of this dietary intervention, new data should be obtained in *in vivo* trials with relative long-term supplementation studies because the resilience of the rumen microbiota or its adaptation to the degradation of plant compounds could alter effects over time.

2.5 Use of olive oil in ruminant diets

Olives are a major crop in Mediterranean weather such as Central Chile and usually olive oil plantations co-exist with dairy farms. Olive oil extraction is associated with production of large quantities of residues (unrefined olive oil) that require extra processing to convert them into virgin olive oil (Beltran-Ortega *et al.* 2016), which represent an economic loss for the company.

Feeding crude (unrefined) olive oil to dairy cows is not common and is rarely reported. Crude olive oil, however, represents a potentially valuable feed source for dairy cows that might enhance the fatty acid composition of milk and dairy products.

Meo Zilio *et al.*, (2015), used dried stoned olive pomace for dairy cows' supplementation and found that their addition in the diet did not cause detrimental effects in animal performance and thus these ingredients could represent an alternative supplement for dairy cattle. Gómez-Cortés *et al.*, (2008) used olive oil in diet of dairy ewes and observed that olive oil does not have detrimental effects on animal performance. Moreover, Vargas-Bello-Pérez *et al.*, (2013), evaluated the effect of a dietary supplementation of lampante olive oil on the fatty acid profiles of the milk and cheese of ewes. They found that lampante olive oil increases monounsaturated fatty acids and decreases saturated fatty acids in milk and cheese.

The effect of olive oil or related products has been studied in ruminant nutrition in order to establish their influence in animal performance and chemical composition of dairy products. However, the effect of this lipid supplement on rumen microbiome has not been studied and neither in relative long-time exposure (for more than 42 days).

On the other hand, palm oil has a balanced fatty acid composition with approximately 50% saturated and 50% unsaturated fatty acids, specifically palmitic and oleic acids as the major fatty acids, along with linoleic acid and only a trace amount of linolenic acid (Pande *et al.*, 2012), and also has emerged as one of the dominant vegetable oils in animal nutrition and is second only to soya bean oil in world production of vegetable oils (Palmquist, 2004).

Saturated long-chain fatty acid such as palmitic is generally considered better for ruminant animals, whereas unsaturated fatty acids and triglycerides are considered preferable for monogastric animals (Tomkins and Drackley, 2010), but the high inclusion of palm oil (in the same way as others vegetable oils) could result in rumen disorders, metabolic problems and reduced milk fat content (Jalaludin, 1997). Finally, palm oil has been used successfully to increase the energy content of diet of dairy cows, nevertheless due to their fatty acid profile its utilization could not improve the lipid composition of milk and dairy products.

2.6 Use of molecular techniques to study rumen microbiome

The study of the microbial life is a complicated task because up to 80% microbes that coexist in any environmental sample are non-culturable or not readily culturable, and represent what is called 'microbial dark matter' (Ortiz-Estrada *et al.*, 2019). In the rumen case, culture-based techniques accounted for only 10% to 20% of the bacterial species present in the rumen (AlZahal *et al.*, 2017).

The poor understanding of rumen microbiota is related to the difficulty of culturing the ruminal bacteria *in vitro*, mainly because of the challenging growth requirements and complex environment of rumen (Taxis *et al.*, 2015). Therefore, the lack of information about of the ruminal microbiome is one of the major knowledge gaps that hinder effective enhancement of rumen functions (Kim *et al.*, 2011).

Molecular techniques are transforming our understanding of the microbial world and have determine the larger diversity of microorganisms present in rumen even when the majority of microbes are still unknown (Nathani *et al.*, 2013). Methods based on DNA are attractive to investigate rumen community diversity and have been essential to characterize this complex community, allowing the scientists to determine changes and functions within these microbial groups (Ross *et al.*, 2013; Martinez-Fernandez *et al.*, 2019).

Different efforts have been made to obtain deeper understanding of the rumen microbiome composition, function of microorganisms and community symbiosis. Global Rumen Census, Hungate1000 project (both part of the Rumen Microbial Genomics network) and RuminOmics project are examples of the growing interest in the rumen ecosystem and have instigated research projects investigating the host link to the rumen microbiota, generating cutting-edge publications in the area of animal sciences.

The Global Rumen Census is a collaborative project that relies on the participation of researchers from 70 organizations in 35 countries, which aim is to survey the microbial communities in a large variety of rumen samples across a broad geographical area, covering a wide range of ruminant species, breeds, feeds and locations and using a standardized pipeline to generate a global 16S rRNA-based census of rumen microbial constituents (Henderson *et al.*, 2015; McAllister *et al.*, 2015; Wilkinson *et al.*, 2018). By the same token, the goal of the Hungate1000 project is to generate a reference set of rumen microbial genome sequences from

cultivated rumen bacteria and archaea, together with representative cultures of rumen anaerobic fungi and ciliate protozoa (Creevey *et al.*, 2014; Seshadri *et al.*, 2018). Currently, the “Hungate1000” catalog contains 410 genomes of rumen microorganisms, including different strains (Zehavi *et al.*, 2018). Finally, RuminOmics is a European Union-funded international project, which involves the analysis of rumen microbiomes and animal genotypes of 1000 dairy cows, in order to improve digestion efficiency and the environmental impacts of ruminant livestock production (Bath *et al.*, 2013; Tapio *et al.*, 2017b).

Recent advances in high-throughput sequencing and bioinformatic analyses have helped to reveal how the composition of the rumen microbiome varies significantly during the development of the ruminant host, and with changes in diet (Gruninger *et al.*, 2019). The application of these techniques has advanced our understanding of the major microbial populations and functional pathways that are used in relation to lower methane emissions, higher feed efficiencies and responses to different feeding regimes (Denman *et al.*, 2018).

The introduction of high-throughput sequencing techniques in rumen studies, utilizing the 16S gene as universal target, has led to many advances in rumen microbial communities characterization (Cremonesi *et al.*, 2018). In particular, 16S rRNA gene sequencing is a powerful technique to identify and quantify (in relative terms) the taxonomic composition of the rumen microbial population and also could be possible to predict metabolic functions using database of microbial genes functional annotations (Biscarini *et al.*, 2018).

Microbial identification through the 16S rRNA gene sequencing it is possible because in bacteria this gene contains highly variable and conserved regions (Wirth *et al.*, 2018). 16S rRNA-based studies are extraordinarily valuable because they can be used to document unexplored biodiversity and the ecological characteristics of either whole communities or individual microbial taxa (Caporaso *et al.*, 2011). In addition, this analysis provides valuable phylogenetic information for the comparison of microbial diversity in environmental samples such as rumen (Takahashi *et al.*, 2014).

2.7 Next generation sequencing

Next generation sequencing (NGS) is defined as technology allowing us to determine in a single experiment the sequence of a DNA molecule(s) with total size significantly larger than 1 million base pairs (1 million bp or 1 Mb) (Płoski, 2016).

The development of NGS technologies has permitted analyses of different types of samples at a deeper level than could be impossible with standard molecular techniques. One of the most important features of NGS is that allow high coverage characterization of hundreds of samples simultaneously, amplifying one specific hypervariable region of 16S rRNA gene, instead of the entire gene (Ziesemer *et al.*, 2015). The 16S rRNA gene has been widely used because (1) is ubiquitous in archaea and bacteria, (2) its sequence is highly conserved, allowing designing specific or universal primers, and (3) have nine regions with variable evolutionary rates that enable deciphering phylogenetic relationships (Ortiz-Estrada *et al.*, 2019).

Applications of NGS Technologies are huge, diverse, and has enabled researchers to study and understand the world of microorganisms and genes from broader and deeper perspectives. Some of those applications cover areas such as food microbiome studies (Cao *et al.*, 2017; Cancino-Padilla *et al.*, 2018), clinical bacteriology (Motro and Moran-gilad, 2017), agriculture sciences (Esposito *et al.*, 2016), aquaculture (Ramírez and Romero, 2017), and even cancer research (Ma *et al.*, 2017).

In case of rumen microbiome, NGS approach has been extensively used to study different conditions: effect of supplements (Sandri *et al.*, 2014), impact of feed restriction (McCabe *et al.*, 2015), effect of DNA extraction and sample preservation (Fliegerova *et al.*, 2014), physiological status (Pitta *et al.*, 2016), methane emissions (Denman *et al.*, 2015), among other utilization.

It is indubitable that NGS has increased our appreciation for the diversity and complexity of the ruminal microbial community (Auer *et al.*, 2017) and its application could provide the knowledge to solve current and future ruminant livestock challenges (Huws *et al.*, 2018). Therefore, the utilization of this approach to elucidate the effect of olive oil and hydrogenated vegetable oil supplementation on rumen bacterial community throughout a relative long-term exposure, is suitable to accomplish the objectives of this thesis and the data obtained could be used for decision making related to dairy production, nutritional value of dairy products and even environmental reasons.

Chapter 2

3. Material and methods

The Animal Care Committee of the Pontificia Universidad Católica de Chile approved all the experimental procedures, in accordance with their animal care guidelines, performed at the Estación Experimental Pirque of the Fundación AgroUC (33°38'28"S, 70°34'27"W). Cows were housed in individual stalls, had continuous access to water, and were milked three times a day.

3.1 Animals and treatments

Fifteen multiparous Holstein cows averaging 189 ± 28 days in milk at the beginning of the study were divided in three treatment groups. The experiment was conducted for 63 days, divided in three experimental periods of 21 days each. At the beginning of the study cows were assigned to treatment groups based on body condition score (BCS; scored on a five-point scale where 1 = emaciated to 5 = overly fat; Wildman et al., 1982), in an attempt to achieve homogeneous groups (Table 1).

Dietary treatments (Table 1) were formulated to meet the protein and energy requirements of a 650 kg dairy cow in mid-lactation consuming 26.5 kg DM daily (NRC, 2001). All cows received a basal diet containing 65% forage (corn silage, fresh alfalfa and alfalfa hay) and 35% concentrate (malt distillers, corn grain, wheat

bran, soybean grain and rapeseed meal). Treatments were isocaloric (NEL=1.6 Mcal kg⁻¹ DM).

Cows were individually fed at a fixed rate and did not show feed refusal. Treatments included a control or basal diet with no added lipid (n = 5 cows), and fat-supplemented diets containing OO (n = 5 cows; unrefined oil manufactured from olive oil; 30 g/kg DM) and HVO (n = 5 cows; manufactured from palm oil; 30 g/kg DM).

Oils were administrated separately and mixed manually into the daily ration for each cow. Dietary oils had distinct differences in their main fatty acids (FA) contents: olive oil contained (in g 100 g⁻¹ total FAs) 14 g of C16:0 and 74 g of C18:1 cis-9, whereas HVO contained (in g 100 g⁻¹ total FAs) 58 g of C16:0 and 40 g of C18:0. Treatment diets were sampled every 14 days and stored at -20°C for later chemical analyses.

Table 1. Ingredients and chemical composition of Control, hydrogenated vegetable oil (HVO) and olive (OO) dietary treatments¹.

Component	Treatments		
	Control	HVO	OO
<i>Ingredient composition (%DM)</i>			
Fresh alfalfa	28.9	28.9	28.9
Corn silage	27.0	27.0	27.0
Malt distillers	23.1	23.1	23.1
Corn grain	8.3	8.3	8.3
Wheat bran	6.2	6.2	6.2
Alfalfa hay	2.6	2.6	2.6
Soybean grain	2.0	2.0	2.0
Rapeseed meal	1.5	1.5	1.5
Vitamin and mineral premix	0.4	0.4	0.4
Olive oil	0	0	3.0
Hydrogenated vegetable oil	0	3.0	0
<i>Chemical composition (%DM)</i>			
Dry matter	38.4	38.4	38.4
Crude protein	14.4	14.3	13.4
Ether extract	4.6	7.1	7.7
Neutral detergent fibre	33.5	33.4	31.1
Acid detergent fibre	19.8	19.4	23.1
Lignin	4.2	4.5	4.5
Ash	6.2	6.0	5.0
<i>Fatty acid composition (g 100g⁻¹ FA)</i>			
C6:0	0.9	nd	0.1
C10:0	0.8	nd	0.1
C12:0	1.1	nd	0.2
C14:0	3.7	0.3	0.2
C16:0	23.7	39.2	12
C18:0	32.3	30.8	26.3
C18:1 cis-9	1.0	nd	32.8
C18:2 cis-9, cis-12	26.3	20.0	19.0
C18:3 cis-6, cis-9, cis-12	0.5	0.4	0.2
C18:3 cis-9, cis-12, cis-15	9.7	9.3	9.1

¹Vitamin and mineral premix contained (per kg): 25 g P; 80 g Ca; 25 gMg; 1.6 g S; 300 000 IU vitamin A; 50 000 IU vitamin D3 and 1600 IU vitamin E.

* nd, not detected. From Vargas-Bello-Pérez *et al.* (2018)

3.2 Milk yield and milk composition

Cows were milked daily at 07:00, 15:00 and 22:00 h. Milk yields were recorded electronically at each milking time and individual milk samples were taken as previously reported by (Vargas-Bello-Pérez *et al.*, 2018) on days 21, 42 and 63. Milk samples were analyzed for fat, protein, and somatic cell count (Table 2) by using an infrared analyzer (Milko-Scan CombiFoss 6000; Foss Electric, Hillerød, Denmark).

Table 2. Milk composition from cows fed control, olive oil (OO), and vegetable hydrogenated oil (HVO) dietary treatments¹

Parameter	Treatment			SEM	<i>P</i> -value	
	Control	HVO	OO		Diet	Time
<i>Production</i>						
Dry matter intake (kg DM day ⁻¹)	26,5	26,5	26,5	-	-	-
Milk yield, kg day ⁻¹	31,1 ^b	31,8 ^b	34,9 ^a	3,13	0,004	<0,001
Fat, kg day	1,02 ^a	1,04 ^a	0,88 ^b	0,12	0,05	0,57
Protein, kg day ⁻¹	1,05	1,08	0,97	0,25	0,58	0,48
<i>Milk composition, g 100 g⁻¹</i>						
Fat	3,28 ^a	3,28 ^a	2,83 ^b	0,31	0,04	0,94
Protein	3,39	3,41	3,16	0,36	0,29	<0,001
Somatic cell count, x 10 ³ mL ⁻¹	358 ^a	254 ^b	145 ^c	82,0	0,02	0,62
Body weight, kg	662	700	636	79,0	0,23	0,07
Body condition score	2,97	2,98	2,77	0,33	0,34	0,04

¹ Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil; SEM, standard error of the mean; Means in the same row with different superscript letters are significantly different (diet P < 0.05).

3.3 Fatty acid analysis

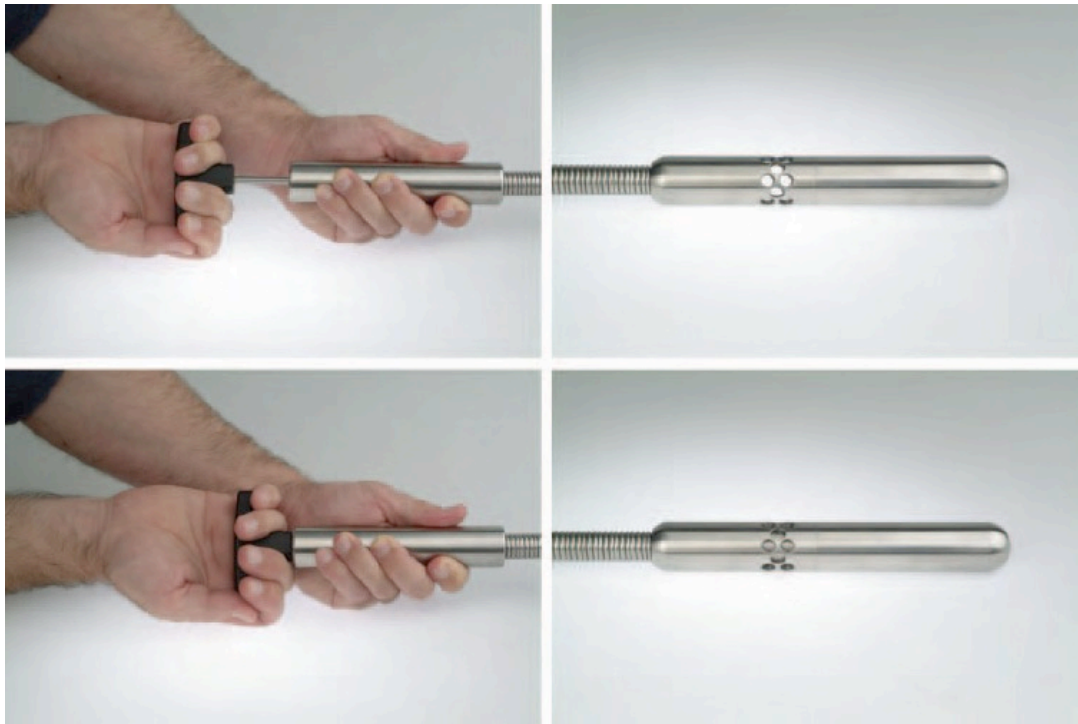
Lipid extraction of milk fat was performed by the procedure of Feng *et al.* (2004), carried out using the non-solvent method. Milk fatty acids were trans esterified with sodium methoxide according to the method of Christie (1982). Fatty acid composition was determined using a Shimadzu GC 2010 gas chromatograph equipped with a 100 m column (Rtx column 100 m x 0.32 mm x 0.20 mm). The gas chromatograph conditions were performed as the described by Vargas-Bello-Pérez *et al.* (2018).

3.4 Ruminal samples

Rumen samples were taken at the onset of the experiment (period 0), and then every 21 days for 63 days. Samples were collected using a single rumen scoop (FLORA) after morning milking and before feeding. The esophageal tube was inserted in mouth with proper subjection of the head and according to the technique described by Geishauser *et al.* (2012), reaching the ventral region of the rumen in order to avoid contamination of the sample with saliva . The head of the rumen scoop was opened pulling the handle for 25 seconds to facilitate the entry of fluid and fibrous material (Figure 1). After this time, the tube was closed releasing the grip and removed gently from the rumen. Samples were deposited into sterile 50 ml Falcon tubes.

Rumen fluid pH was determined immediately after sampling with a pH meter and 5 ml was used for NH₃-N analysis (Bal *et al.*, 2000) and another 2 ml was preserved with 100 µl 25% metaphosphoric acid for volatile fatty acid (VFA) determination. Samples for rumen microbiome were aliquoted in Eppendorf tubes. Ruminal

samples were maintained at 4°C and subsequently stored at -80°C for further analysis.



FLORA - rumen scoop (Wittibreut, Germany). Taken from Geishauser *et al.*, (2012).

The VFA measurement was performed by gas chromatograph (GC-2010) equipped with a 100-m wall-coated open tubular-fused silica capillary column (Stabilwax-DA; 30 m × 0.32 mm i.d., 0.25 µm film thickness, Restek, Bellefonte, PA). Samples were centrifuged at 10.000 x *g* for 5 min three times (supernatant each time) in order to obtain liquid fraction free of protein. Oven temperature were programmed for 145°C for 2 min and then increased from 145 to 220°C at 4°C/min. The injector and flame-ionization detector was 250 and 300°C, respectively.

3.5 DNA Extraction, PCR Amplification, and High-Throughput Sequencing

Ruminal samples were homogenized with vortex and weighed in 1,5 ml Eppendorf tubes. Later, 150 μ l of PBS (Phosphate-buffered saline) were added to 250 mg of each sample in order to performed cell lysis with lysozyme incubation at 37°C for 60 min. DNA was extracted using the UltraClean Fecal DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol.

Next generation sequencing approach was used to characterize the microbial populations present in the rumen for the control and treatment periods. The extracted DNA was amplified using the bacterial-specific primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (Caporaso *et al.*, 2011), to amplify the V4 region of 16S rRNA gene. Variable region 4 was selected because sequencing and taxonomic assignment using this region was associated with a low error rate and minimum loss of taxonomic resolution (Lokesh and Kiron, 2016). These primers have shown to be ideal to amplify the V4 region with high coverage, and the amplicons (read length) are suitable for the Illumina Miseq sequencing platform.

Polymerase chain reaction was performed using the following conditions: an initial denaturing cycle of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, annealing at 56°C for 30 s, an elongation at 68°C for 45 s. After V4 region amplification, PCR products were purified through QIAquick PCR Purification kit (Qiagen, Valencia, CA). Later, the purified products were measured fluorometrically using the High Sensitivity

(HS) kit on the Qubit Fluorometer 3.0 (Invitrogen Co., Carlsbad, USA). Finally, DNA sequencing were performed by CD Genomics (New York, USA) Illumina platform.

3.6 Bioinformatics processing

16S rRNA gene paired end short read sequence data generated on the Illumina Miseq was quality checked with FASTQC and analyzed using DADA2 and Phyloseq R package version 3.5.1 (Callahan *et al.*, 2016). De-multiplexed paired end sequences were first merged prior to sequence quality filtering, followed by denoising (error correction) and chimera checking and clustering of sequences to operational taxonomic units (OTUs).

After visualization of the quality profiles of the reads, sequences were trimmed to 270 bp (forward) and 220 bp (reverse). The paired-end Illumina reads were assembled into Amplicon Sequences Variants (ASV) using DADA2 pipeline. Taxonomy assignation was performed using the Silva training dataset version 132 and sequences corresponding to Eukaryota, Crenarchaeota, Euryarchaeota, Chloroflexi, Planctomycetes, Cyanobacteria and uncharacterized at phylum level were removed. OTUs with phylum-level classification to Cyanobacteria were removed as no photosynthesis occurs in the rumen and these assignments are likely due to the high homology of plant chloroplast rRNA to the Cyanobacteria 16S rRNA gene (Anderson *et al.*, 2016).

3.7 Statistical analysis

A model including diet, time, and diet \times time as fixed effects and cow within treatment as random effect was used to determine differences in animal performance and ruminal parameters.

Group means were calculated for rumen pH, VFA, ammonia and milk composition. Analysis of variance (ANOVA) and post hoc Tukey analysis were performed using GraphPad Prism version 6.00 for Mac OS to analyzed differences between means. A probability of $P < 0.05$ was considered to indicate a significant difference, and a tendency towards significance was considered at $0.05 < P < 0.1$.

Alpha and beta diversity were estimated from the complete OTU table (at the OTU level). Alpha (within-sample diversity) and Beta diversity (between-sample diversity) measures for samples grouped by dietary treatments and experimental periods were analyzed using phyloseq package in R (McMurdie and Holmes, 2013). Microbial diversity was determined according to the Shannon Index (combines richness or the total number of taxa and evenness, the relative abundance of each taxa), dominance was presented as the Simpson index and richness of samples were calculated based on the Chao1 index and observed species.

Beta-diversity among samples was calculated using the UniFrac metric and principal coordinates analyses (PCoAs) were conducted by evaluating both weighted (quantitative) and unweighted (qualitative) Unifrac distances, in order to highlight eventual clusters of similar groups of samples depending on the diet supplementation. In addition, PERMANOVA was used to elucidate the differences in microbial communities between the three different treatments.

Significant shifts in bacterial community composition were identified using LEfSe, which implements a Kruskal–Wallis sum-rank test followed by linear discriminate analysis (LDA) effect size to identify significantly different features with biological relevance (Segata *et al.*, 2011). This analysis was performed in the online-based platform Galaxy (<http://huttenhower.sph.harvard.edu/lefse/>). The threshold of LDA score was set at default value 2.0, with a P-value <0.05.

4. Results

4.1 *Animals and treatments*

Feed intake, body condition score (BCS) and body weight was not affected by dietary treatments. Basal diets were formulated to be isocaloric and isonitrogenous, and feed was given in equal portions once a day. Animals supplemented with OO tended to have lower BCS (2.77) and body weight (636 kg) compared with the Control (2.97 and 662, respectively). In case of HVO, cows showed higher BCS (2.98) and body weight (700 kg).

The fatty acid (FA) profile of the treatments were affected by the lipid composition of the vegetable oil supplements. HVO contained mainly C16:0 and C18:0 FAs and OO was composed predominantly by C18:0 and C18:1 cis-9 FAs.

4.2 *Milk yield and milk composition*

Milk yield with OO supplementation increased 3.8 kg day⁻¹ (12.2%) compared with the Control and 3.1 kg day⁻¹ (9.7%) compared with HVO (Table 3). In addition, milk yield showed a time effect ($P < 0.001$) in OO treatment, specifically in the third experimental period.

In case of milk composition, OO resulted in a 13.7% decrease in milk fat and 7.6% reduction in milk protein yield, meanwhile HVO increased fat and protein content in 2% and 2.9%, respectively, related to Control diet.

Table 3. Milk composition during the complete sampling period.

Parameter	Treatment			SEM	P-value	
	Control	HVO	OO		Diet	Time
<i>Production</i>						
Milk yield, kg day ⁻¹	31.1 ^b	31.8 ^b	34.9 ^a	3.13	0.004	<0.001
Fat, kg day	1.02 ^a	1.04 ^a	0.88 ^b	0.12	0.05	0.57
Protein, kg day ⁻¹	1.05	1.08	0.97	0.25	0.58	0.48
<i>Milk composition, g 100 g⁻¹</i>						
Fat	3.28 ^a	3.28 ^a	2.83 ^b	0.31	0.04	0.94
Protein	3.39	3.41	3.16	0.36	0.29	<0.001

¹ Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil; SEM, standard error of the mean; Means in the same row with different superscript letters are significantly different (diet P < 0.05). From Vargas-Bello-Pérez et al. (2018).

4.3 Milk fatty acids composition

Results of milk FA composition are shown in Table 4. Feeding OO in diets of dairy cows decreased milk fat content of short and medium chain FA (8:0 to 16:0), where C8:0, C11:0 and C12:0 FAs shows significant reduction (P < 0.05). OO also increased the proportion of most 18 carbon FA in milk fat (P < 0.05), such as C18:1 cis-9 and C18:3 cis-9, cis-12, cis-15 FAs in milk, whereas HVO increased (P < 0.05) C18:2 cis-9, trans-11 and had a tendency to increment C18:3 cis-6, cis-9, cis-12; C18:2 trans-9, trans-12 and C18:1 cis-9. In summary, OO increased (P < 0.05) total monounsaturated FAs (MUFAs) and total polyunsaturated FAs (PUFAs) and reduced (P < 0.05) total saturated FAs (SFAs) in milk. In addition, OO decreased (P < 0.05) AI and TI in milk. Also, there was a time effect on the following milk FAs (their contents were higher in the third experimental period): C15:0, C15:1 iso; C17:1 cis-9; C18:1 trans-10; C18:1 trans-11; C18:2 trans-9, trans-12; C18:2 cis-9, cis-12; C18:3 cis-6, cis-9, cis-12 and C18:2 cis-9, trans-11.

Table 4. Milk fatty acid profile from cows fed control, olive oil (OO), and hydrogenated vegetable oil (HVO) dietary treatments¹.

Fatty acid	Diet			SEM	<i>P</i> -value		
	Control	HVO	OO		Diet	Time	Diet x Time
C4:0	4.52	4.24	4.06	0.28	0.27	0.93	0.75
C6:0	2.82	2.93	2.49	0.26	0.24	0.57	0.98
C8:0	2.00 ^b	1.68 ^b	1.31 ^a	0.23	0.02	0.41	0.68
C10:0	4.20	4.40	3.44	0.48	0.12	0.59	0.86
C11:0	0.26 ^a	0.27 ^a	0.17 ^b	0.02	<0.001	0.87	0.99
C12:0	5.27 ^a	5.06 ^a	4.14 ^b	0.34	<0.001	0.29	0.92
C13:0	0.16	0.10	0.11	0.04	0.38	0.67	0.45
C14:0	15.8	15.3	15.1	0.50	0.57	0.12	0.57
C14:1 cis-9	0.85	0.82	0.96	0.13	0.50	0.48	0.70
C15:0	0.95	0.67	0.73	0.17	0.22	<0.001	0.06
C15:1 iso	0.91	0.68	0.72	0.16	0.34	<0.001	0.45
C16:0	40.7	40.6	40.1	1.53	0.92	0.65	0.42
C17:0	0.61	0.84	0.43	0.19	0.10	0.11	0.64
C17:1 cis-9	0.41	0.41	0.47	0.06	0.50	0.02	0.10
C18:0	5.05	4.20	3.70	0.81	0.25	0.18	0.41
C18:1 trans-10	0.59 ^b	0.54 ^b	0.73 ^a	0.11	0.25	0.05	0.06
C18:1 trans-11 (vaccenic acid)	1.86 ^b	1.16 ^b	2.51 ^a	0.37	0.03	0.02	0.45
C18:1 cis-9 (oleic acid)	8.39 ^b	8.89 ^b	12.2 ^a	1.22	0.04	0.25	0.79
C18:2 trans-9, trans-12	0.58	1.07	1.12	0.25	0.07	<0.001	0.06
C18:2 cis-9, cis-12	0.23	0.35	0.35	0.19	0.75	<0.001	0.08
C18:3 cis-9, cis-12, cis-15	0.95 ^b	0.72 ^b	1.21 ^a	0.19	0.05	0.61	0.91
C18:3 cis-6, cis-9, cis-12	0.13	0.20	0.35	0.13	0.24	0.51	0.76
C18:2 cis-9, trans-11	0.11 ^b	0.42 ^a	0.37 ^a	0.08	0.03	0.01	0.12
C20:1 n-9	0.11	0.12	0.05	0.09	0.71	0.07	0.40
C20:2	0.02	nd	nd	0.01	0.30	0.28	0.28
C20:3n-3	0.16	0.29	0.29	0.06	0.07	0.65	0.16
C20:3n-6	0.22	0.35	0.39	0.11	0.28	0.99	0.19
Σ Saturated fatty acids	82.6	82.3	77.4	2.92	0.20	0.76	0.62
Σ Monounsaturated fatty acids	12.2 ^b	12.2 ^b	16.2 ^a	1.24	0.03	0.20	0.83
Σ Polyunsaturated fatty acids	5.17 ^b	5.06 ^b	6.43 ^a	0.78	0.01	0.09	0.25
Atherogenicity index	4.29 ^a	4.86 ^a	3.41 ^b	0.42	<0.001	0.82	0.69
Thrombogenicity index	4.70 ^a	5.04 ^a	3.90 ^b	0.38	0.02	0.89	0.95
C14:1 cis-9/C14:0	0.05	0.06	0.06	0.01	0.35	0.19	0.98
C18:2 cis-9,trans-11/C18:1 trans-11	0.20	1.96	1.04	0.77	0.110	0.21	0.65

¹FA values are in g 100 g⁻¹ fatty acid; means in the same row with different superscript letter are significantly different (Diet P < 0.05). Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. SEM: Standard error of the mean; nd, not detected. From Vargas-Bello-Pérez et al. (2018).

4.4 Ruminal parameters

The results of ruminal pH, NH₃-N and VFA concentration are presented in Table 5. The mean of ruminal pH ranged between 7.11 (Control), 6.98 (OO) and 7.00 (HVO), and was not affected by the experimental period. Moreover, different treatment diets had no significant effect on ruminal pH. However, Control diet tended to have a higher rumen pH value than the other groups.

NH₃-N (mg/dL) was higher in Control diet compared with HVO and OO, except after 42 days supplementation where ammonia concentration increased significantly (P < 0.05) to 9.12 mg/dL with OO.

Total VFA concentration and proportions of individual VFA were not affected by treatments. However total VFA concentration tended to be higher in Control diet, meanwhile acetate, propionate and butyrate proportions showed mainly higher concentrations under lipid supplementation at different experimental period. Acetate had a tendency lower proportion with OO through all the supplementation, whereas OO tended to increase propionate at 21 days and 63 days, and increased butyrate only after 63 days of lipid addition.

Table 5. Effect of hydrogenated vegetable oil (HVO) and olive (OO) on Ruminant parameters

Item	Treatments			<i>P</i> -value
	Control	HVO	OO	
pH				
0 days	7.05	6.97	7.08	0.337
21 days	7.22	6.93	6.93	0.064
42 days	7.06	7.02	6.89	0.582
63 days	7.11	7.00	6.97	0.530
NH ₃ -N, mg/dL				
0 days	5.68	4.25	4.62	0.010
21 days	5.13	4.61	4.37	0.715
42 days	5.48	3.45	9.12	0.015
63 days	5.40	5.26	5.52	0.848
VFAs, molar % of Total VFA				
Acetate,				
0 days	67.24	67.21	67.48	0.76
21 days	67.39	66.03	64.79	0.39
42 days	65.14	63.85	63.25	0.58
63 days	65.38	66.15	62.07	0.37
Propionate				
0 days	16.41	17.83	15.23	0.29
21 days	17.34	18.41	19.96	0.46
42 days	20.42	22.58	22.51	0.76
63 days	20.14	18.34	22.34	0.35
Butyrate				
0 days	9.93	9.44	9.99	0.50
21 days	10.00	9.88	9.64	0.97
42 days	9.79	8.79	6.81	0.13
63 days	8.75	8.05	8.95	0.99
Total VFA				
0 days	92.64	51.76	57.62	0.18
21 days	110.15	102.76	82.63	0.08
42 days	105.48	110.75	110.09	0.95
63 days	75.95	46.85	59.55	0.14

¹Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Results were declared significant at *P*<0.05.

4.5 Sequencing

Sequencing the V3-V4 regions of the bacterial 16S rRNA gene produced a total of 4.606.204 reads (joined R1-R2 paired-end reads). After data filtering, quality control, and chimera removal, a total of 2.104.912 V4 16S rRNA sequence reads from the 48 samples were generated, with a mean of 43.852 sequence reads for each sample (minimum, 12.845; maximum, 79.204).

The mean length of the sequence reads was 367 bp. The ASVs identified based on V4 region analysis reached in total 43.515 sequences in Control, while in HVO and OO were 44.460 and 43.583, respectively. A total of 8.167 ASV were identified from the bacterial 16S rRNA transcript profile which were grouped taxonomically from the phylum to genus level (phylum, class, order, family, genus).

4.6 General ruminal community composition

At the phylum level (Figure 1a), 24 phyla were identified in the ruminal samples. Independently of the treatment and experimental period, the most abundant phyla for all combined samples were phylum Firmicutes (71,5%), followed by Bacteroidetes (26,2 %) and Actinobacteria (1%). The less abundant phyla were grouped as Others. The proportion of these three phyla account for the 98.7% of the total. However, the relative abundance and composition of these predominant phyla varied among the dietary groups and days of supplementation, tended to be higher in Control and at 63 days of supplementation (Table 6).

Table 6. Relative abundance of rumen bacterial community in Control, HVO and OO dietary treatments at phylum level and different supplementation periods.

Phylum	Treatments			
	Control	HVO	OO	SEM
0 days				
Firmicutes	0.598	0.636	0.708	0.032
Bacteroidetes	0.389	0.348	0.273	0.034
Actinobacteria	0.004	0.001	0.006	0.001
Proteobacteria	0.001	0.002	0.003	0.000
Others	0.007	0.013	0.010	0.002
21 days				
Firmicutes	0.602	0.828	0.729	0.065
Bacteroidetes	0.379	0.150	0.238	0.066
Actinobacteria	0.008	0.008	0.022	0.005
Proteobacteria	0.003	0.004	0.005	0.001
Others	0.008	0.010	0.006	0.001
42 days				
Firmicutes	0.660	0.747	0.648	0.031
Bacteroidetes	0.316	0.223	0.321	0.032
Actinobacteria	0.010	0.014	0.017	0.002
Proteobacteria	0.006	0.006	0.004	0.001
Others	0.660	0.747	0.648	0.031
63 days				
Firmicutes	0.812	0.849	0.874	0.018
Bacteroidetes	0.164	0.126	0.096	0.020
Actinobacteria	0.016	0.011	0.022	0.003
Proteobacteria	0.003	0.004	0.004	0.001
Others	0.006	0.010	0.003	0.002

¹Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Results were declared significant at P<0.05.

At the bacterial family level (Figure 1b), the most abundant bacterial family in all samples were Ruminococcaceae (22.8%), Lachnospiraceae (21.3%) and Prevotellaceae (19.7%), belonging to the phylum Firmicutes and Bacteroidetes respectively. In particular, Ruminococcaceae had higher relative abundance in HVO treatment, whereas Lachnospiraceae with OO and Prevotellaceae was more abundant in Control diet.

There were 143 bacterial taxa identified at the genus level through analysis of microbiota compositions (Figure 1c), and 70 of these genera showed a relative abundance of $\geq 1\%$ in all samples across the different groups. Among these genera, *Prevotella* and *Succiniclasicum* were dominant and had relatively higher abundance, at mean abundance levels of 19.5% and 16.6% respectively. Following, were observed four most abundant genus; Ruminococcaceae_NK4A214_group (11%), Christensenellaceae_R-7_group (8.6%), Ruminococcaceae_UCG-014 (6.4%), Lachnospiraceae_NK3A20_group (5.5%), and the less abundant genus were accounted in Others (32.4%). Genera *Prevotella* (phylum Bacteroidetes) showed greater relative abundance in Control diet meanwhile *Succiniclasicum* (phylum Firmicutes) with HVO supplementation (Table 7).

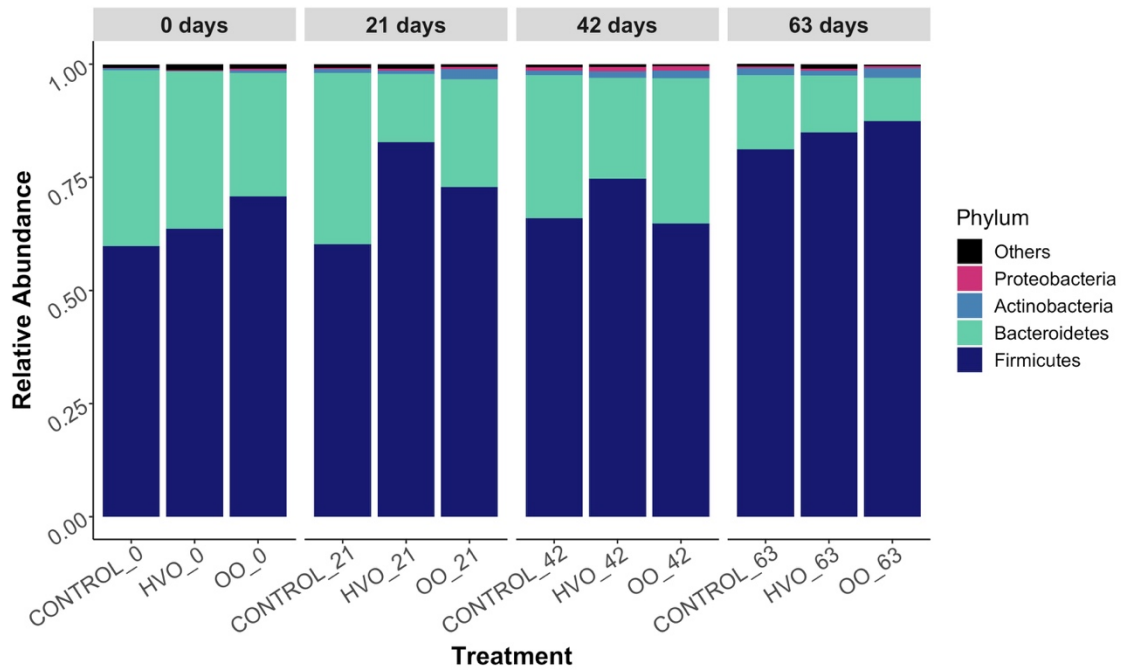
Table 7. Relative abundance of rumen bacterial community in Control, HVO and OO dietary treatments at genus level and different supplementation periods.

Genus	Treatments			
	Control	HVO	OO	SEM
0 days				
<i>Prevotella_1</i>	0.332	0.273	0.216	0.033
<i>Succiniclasicum</i>	0.127	0.108	0.068	0.017
<i>Ruminococcaceae_NK4A214_group</i>	0.094	0.103	0.124	0.009
<i>Christensenellaceae_R-7_group</i>	0.096	0.095	0.093	0.001
<i>Ruminococcaceae_UCG-014</i>	0.060	0.071	0.105	0.013
<i>Lachnospiraceae_NK3A20_group</i>	0.022	0.018	0.054	0.011
Others	0.268	0.331	0.339	0.022
21 days				
<i>Prevotella_1</i>	0.293	0.087	0.180	0.059
<i>Succiniclasicum</i>	0.192	0.215	0.132	0.025
<i>Ruminococcaceae_NK4A214_group</i>	0.092	0.137	0.117	0.013
<i>Christensenellaceae_R-7_group</i>	0.079	0.105	0.066	0.012
<i>Ruminococcaceae_UCG-014</i>	0.053	0.093	0.062	0.012
Others	0.262	0.315	0.382	0.035
42 days				
<i>Prevotella_1</i>	0.251	0.163	0.272	0.033
<i>Succiniclasicum</i>	0.212	0.209	0.163	0.016
<i>Ruminococcaceae_NK4A214_group</i>	0.079	0.101	0.083	0.007
<i>Ruminococcaceae_UCG-014</i>	0.041	0.086	0.043	0.015
<i>Lachnospiraceae_NK3A20_group</i>	0.046	0.063	0.053	0.005
Others	0.298	0.298	0.338	0.013
63 days				
<i>Prevotella_1</i>	0.121	0.085	0.070	0.015
<i>Succiniclasicum</i>	0.188	0.212	0.172	0.012
<i>Ruminococcaceae_NK4A214_group</i>	0.114	0.121	0.159	0.014
<i>Christensenellaceae_R-7_group</i>	0.110	0.122	0.064	0.017
<i>Ruminococcaceae_UCG-014</i>	0.047	0.048	0.054	0.002
<i>Lachnospiraceae_NK3A20_group</i>	0.078	0.063	0.116	0.016
Others	0.343	0.350	0.364	0.006

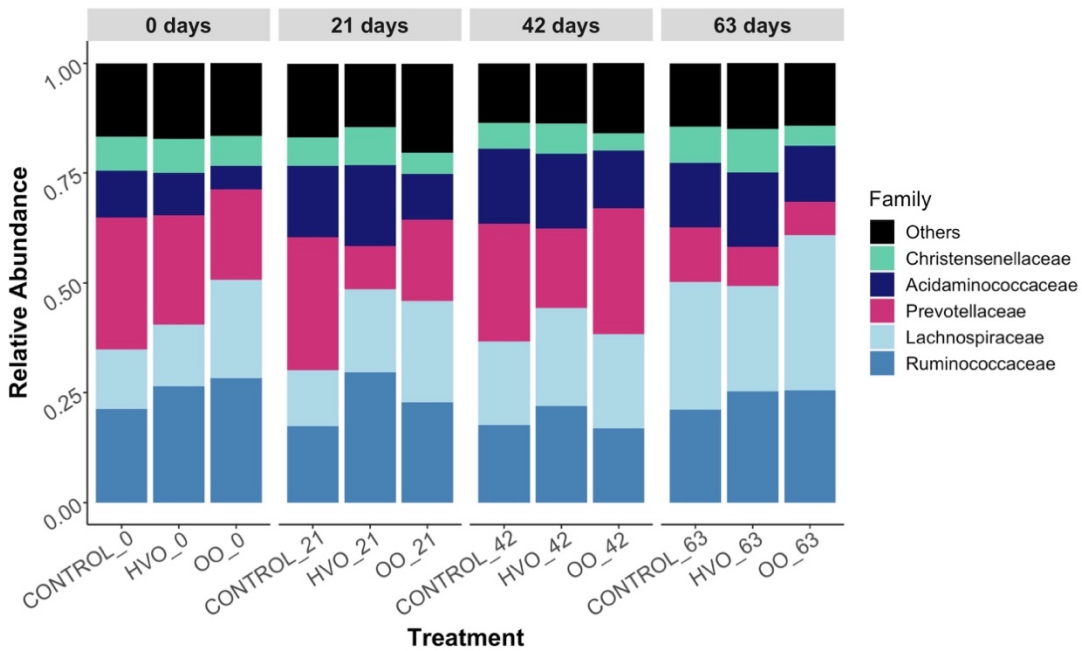
¹Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Results were declared significant at P<0.05.

Figure 1. Relative abundance at (a) Phylum, (b) Family and (c) Genus level grouped by days of supplementation.

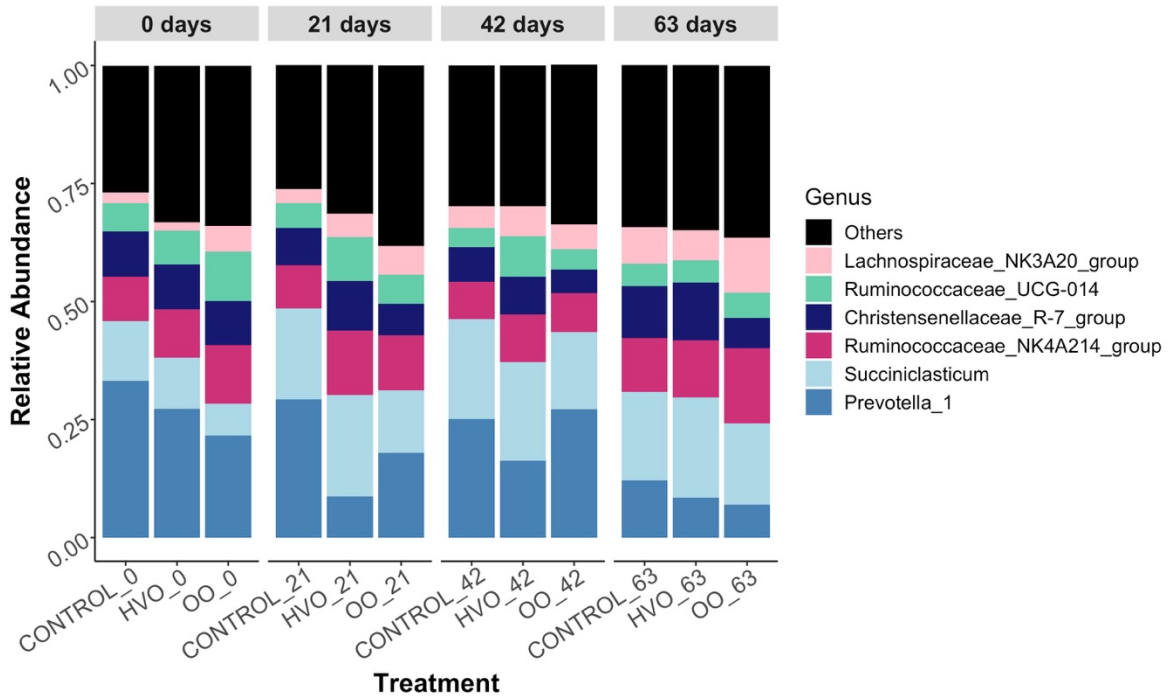
(a)



(b)



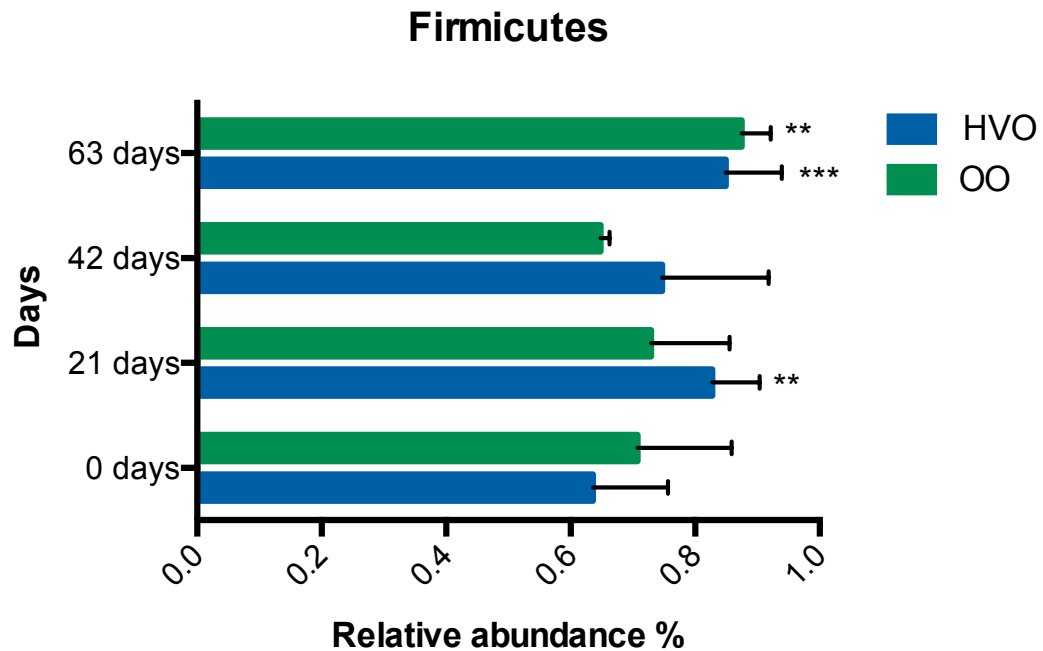
(c)



Effect of dietary treatment and supplementation time on relative abundance of two dominant phyla and genus in rumen microbiota are shown in Figure 2 and Figure 3. Variations in relative abundance were determined by Two-way ANOVA and Dunnett's multiple comparisons test. Results showed that phylum Firmicutes (Figure 2a) increased significantly their abundance with HVO at 21 days ($P \leq 0.01$) and 63 days ($P \leq 0.001$) of supplementation, meanwhile OO significant increasing was observed only at 63 days ($P \leq 0.01$). In the case of Bacteroidetes (Figure 2b), the relative abundance of this phylum decreased over time. HVO supplementation reduced significantly at 21 days ($P \leq 0.01$) and 63 days ($P \leq 0.001$), whereas OO addition shows a significant effect in relative abundance at 63 days ($P \leq 0.01$).

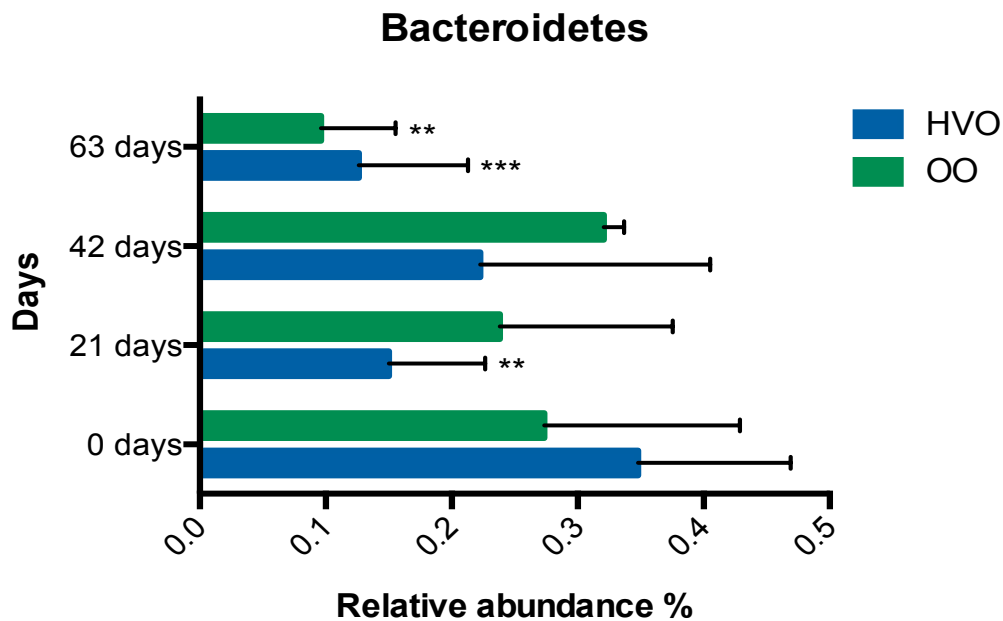
Figure 2. Effect of lipid supplementation and experimental time on relative abundance of the two dominant phyla in rumen microbiota, (a) Firmicutes and (b) Bacteroidetes

(a)



HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

(b)

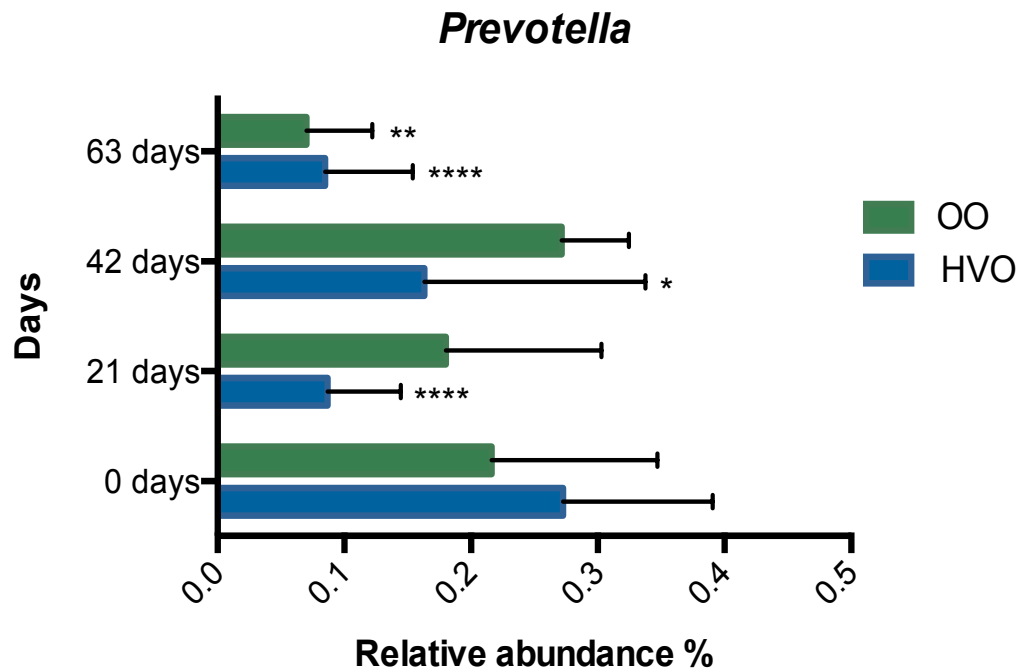


HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

Relative abundance of predominant genus in rumen revealed differences in time and type of lipid inclusion in diet of dairy cows. *Prevotella* relative abundance (Figure 3a) decrease on time, presenting significant results at 21 days ($P \leq 0.0001$), 42 days ($P \leq 0.05$) and 63 days ($P \leq 0.0001$) in HVO, and at 63 days ($P \leq 0.01$) with OO. While abundance of *Succiniclasicum* (Figure 3b) was higher in lipid supplementation throughout the experimental periods. At 21 days ($P \leq 0.05$), 42 days ($P \leq 0.05$) and 63 days ($P \leq 0.05$) *Succiniclasicum* increased with HVO, whereas there were no significant differences in OO supplementation.

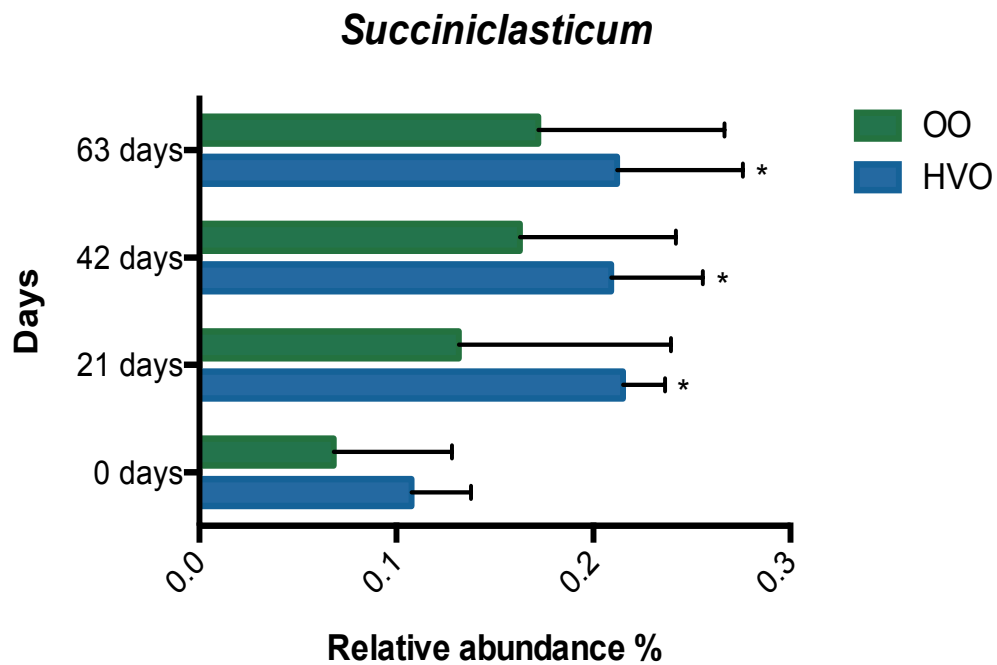
Figure 3. Effect of dietary treatment and supplementation time on relative abundance of the dominant genus in rumen microbiota, (a) *Prevotella* and (b) *Succiniclasticum*.

(a)



HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

(b)



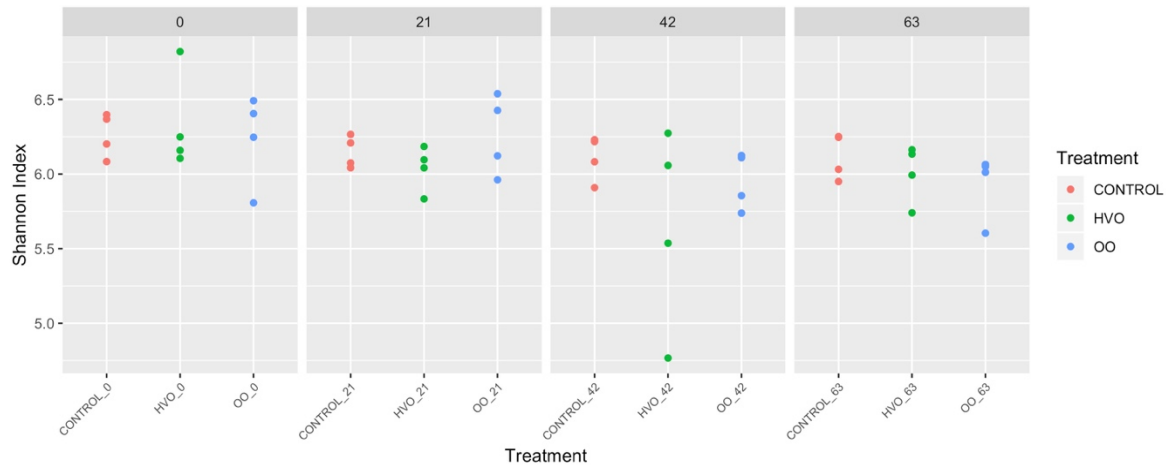
HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

4.7 Diversity and richness of microbial communities

The estimated alpha diversity indices for describing the richness and diversity of the rumen microbiota in the three experimental groups are reported in Figure 4. The alpha diversity indexes, Shannon (Figure 4a), Chao1 (Figure 4b) and Simpson (Figure 4c), were found not to be significantly between each dietary treatment.

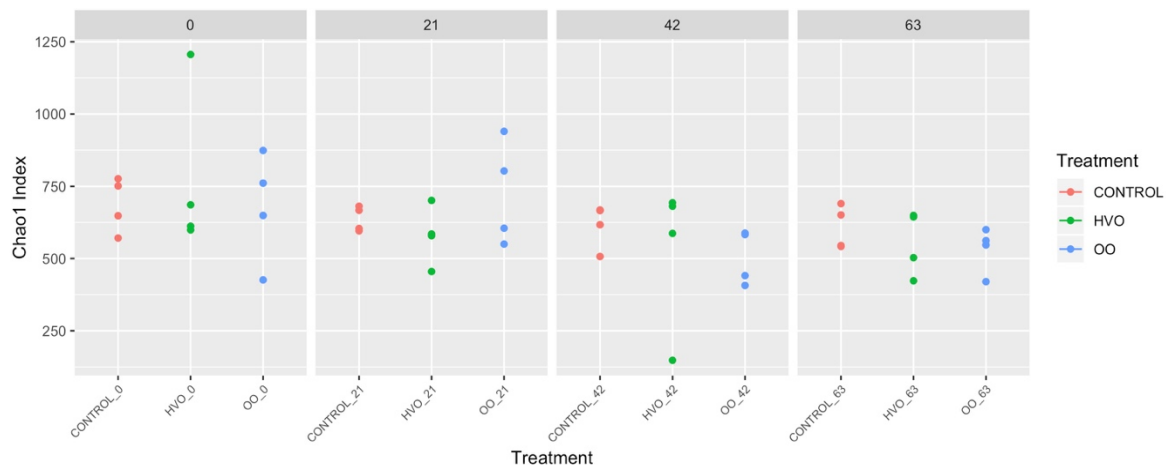
Figure 4. Ruminal microbial richness and diversity with lipid supplementation throughout the experimental periods. Bacterial diversity estimated by (a) Shannon Index and (b) Simpson Index, and bacterial richness estimated by the (c) Chao1 value.

(a)



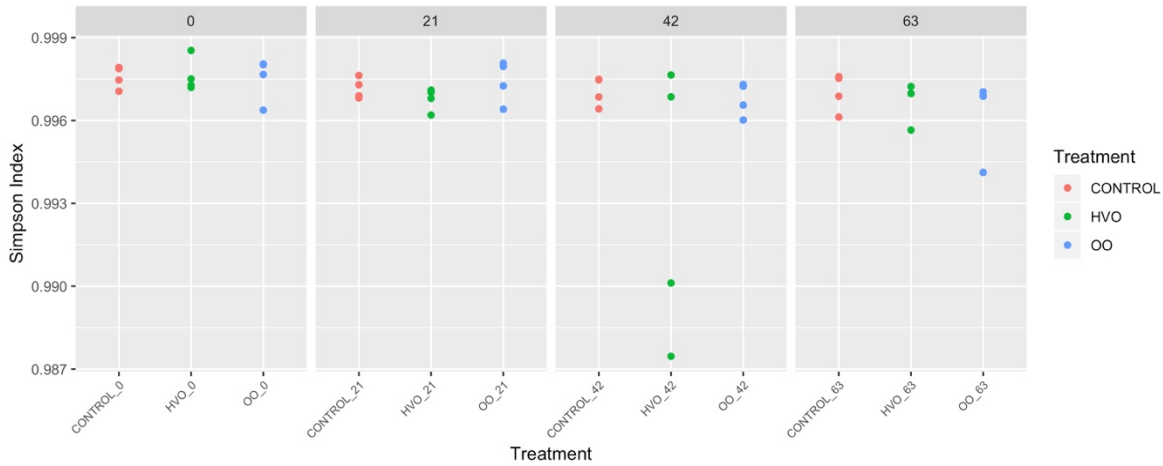
Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days.

(b)



Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days.

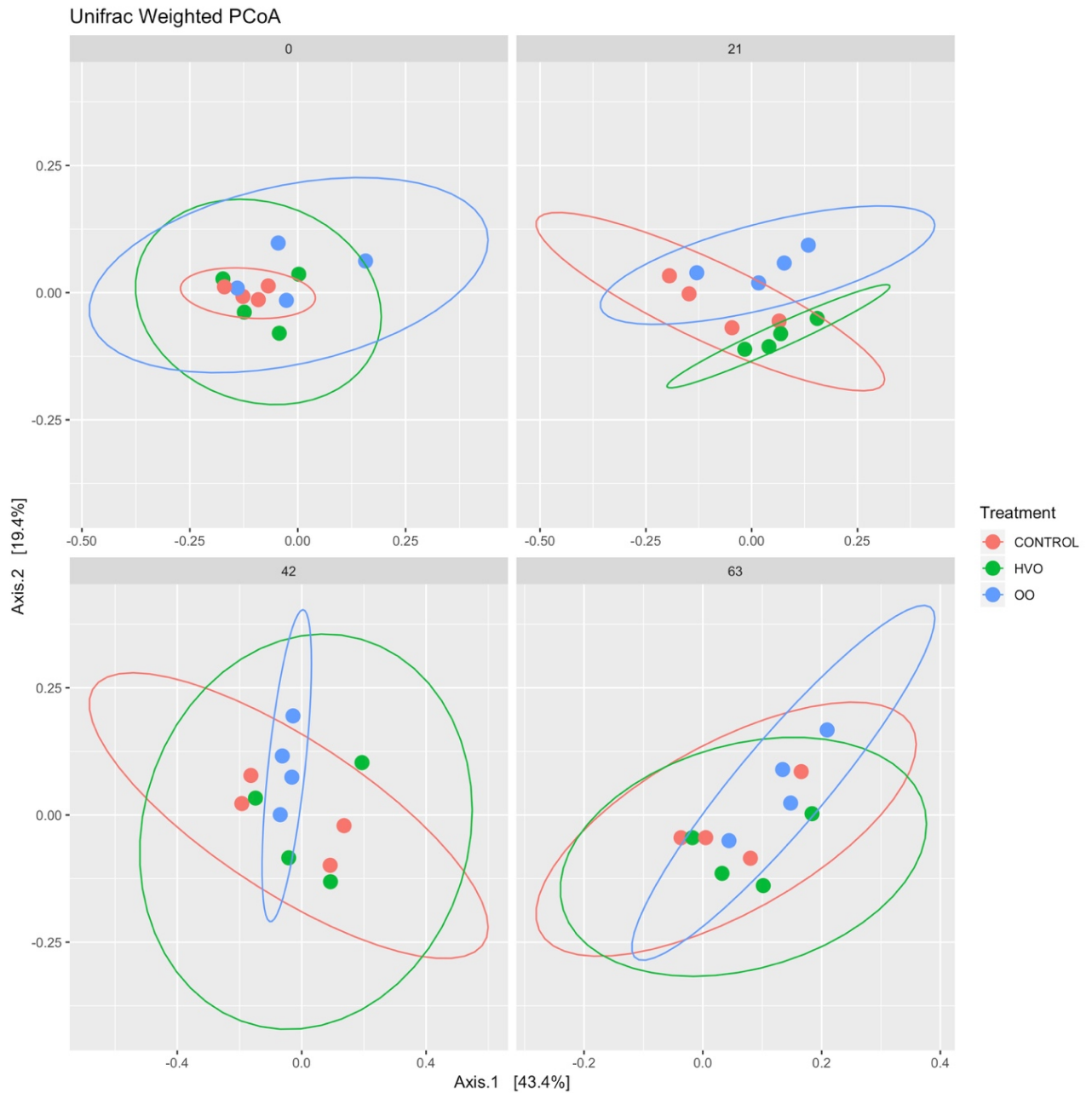
(c)



Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days.

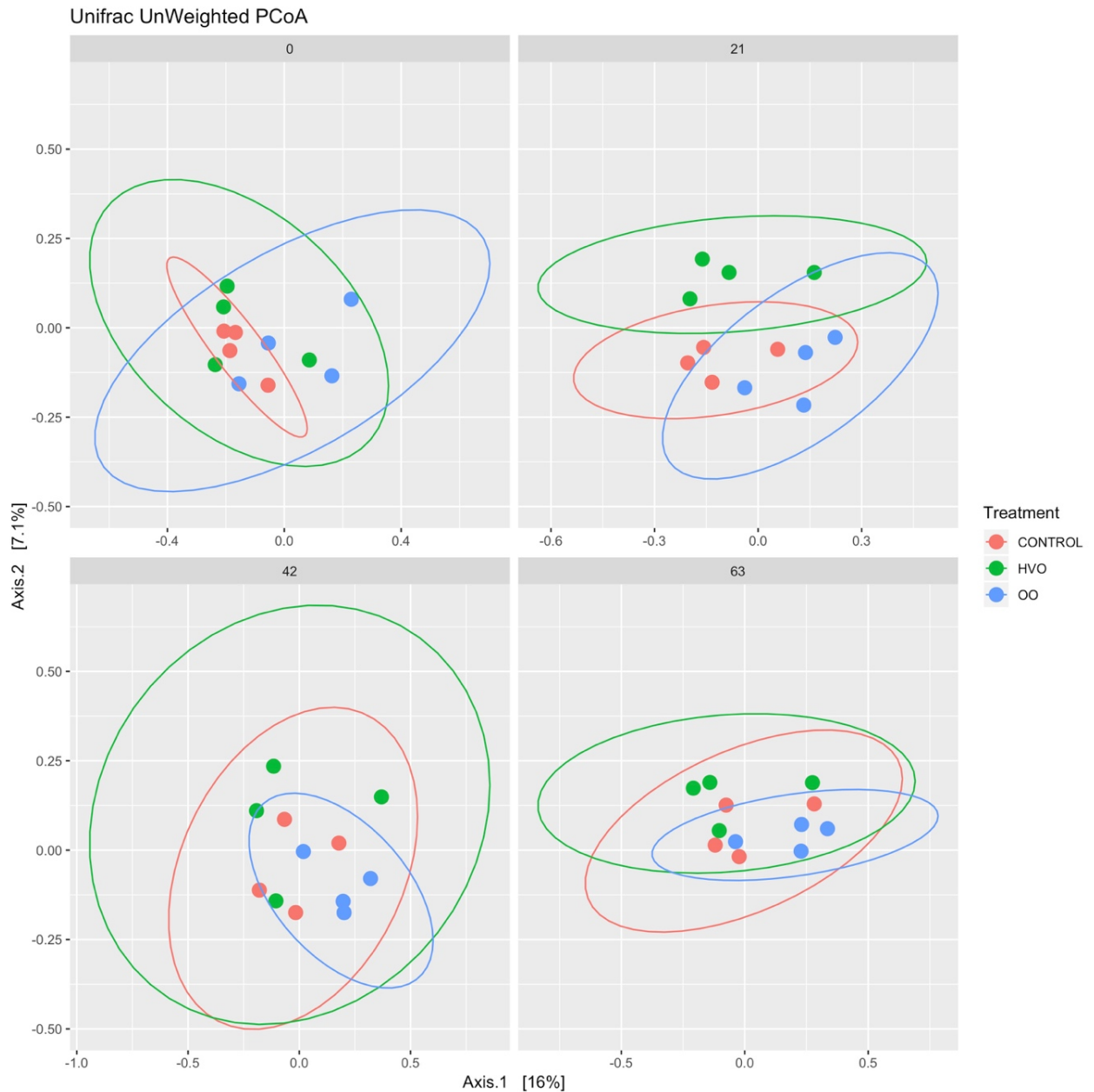
Beta diversity measure takes the phylogenetic divergence between the ASVs into account aiding in identification of differences among microbial communities. To estimate the beta diversity between the samples at four different time points during lipid supplementation, the Principal coordinate analysis (PCoA) was performed. In both the weighted (Figure 5) and unweighted (Figure 6) UniFrac distances, the closer positions of the samples indicate similar microbial composition between them, showing not differentiation among bacterial communities and therefore revealed not shift of community diversity. PERMANOVA confirmed the absence of significant differences in the composition of the rumen microbiota in weighted (Table 8) and unweighted (Table 9) results.

Figure 5. Principal coordinate analysis (PCoA) of bacterial community structures of the ruminal microbiota in the Control (red points), HVO (green points) and OO (blue points), constructed using the Weighted UniFrac method.



Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days.

Figure 6. Principal coordinate analysis (PCoA) of bacterial community structures of the ruminal microbiota in the Control (red points), HVO (green points) and OO (blue points), constructed using the UnWeighted UniFrac method.



Control, no fat supplement; HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days.

Table 8. PERMANOVA (Weighted) analysis of the effect of dietary treatments on rumen bacterial diversity. P-values were calculated based on 9999 possible permutations.

Treatment	Period	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Control-HVO	0 days	0.017	0.017	0.781	0.115	0.611
Control-HVO	21 days	0.063	0.063	2.812	0.319	0.052
Control-HVO	42 days	0.023	0.023	0.450	0.069	0.76
Control-HVO	63 days	0.006	0.006	0.598	0.091	0.658
Control-OO	0 days	0.044	0.044	1.860	0.237	0.158
Control-OO	21 days	0.022	0.022	1.717	0.222	0.229
Control-OO	42 days	0.017	0.018	1.247	0.172	0.32
Control-OO	63 days	0.015	0.015	1.505	0.200	0.255

SumOfSqs, sum of squares; MeanSqs, mean sum of squares; F, F value by permutation; Statistical significance with $P < 0.05$; P-value based on 999 permutations.

Table 9. PERMANOVA (UnWeighted) analysis of the effect of dietary treatments on rumen bacterial diversity. P-values were calculated based on 9999 possible permutations.

Treatment	Period	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Control-HVO	0 days	0.179	0.179	0.984	0.141	0.567
Control-HVO	21 days	0.248	0.248	1.341	0.183	0.049
Control-HVO	42 days	0.216	0.216	0.937	0.135	0.492
Control-HVO	63 days	0.167	0.167	0.867	0.126	0.548
Control-OO	0 days	0.224	0.224	1.282	0.176	0.129
Control-OO	21 days	0.264	0.264	1.507	0.200	0.062
Control-OO	42 days	0.252	0.252	1.439	0.193	0.062
Control-OO	63 days	0.214	0.214	1.188	0.165	0.223

SumOfSqs, sum of squares; MeanSqs, mean sum of squares; F, F value by permutation; Statistical significance with $P < 0.05$; P-value based on 999 permutations.

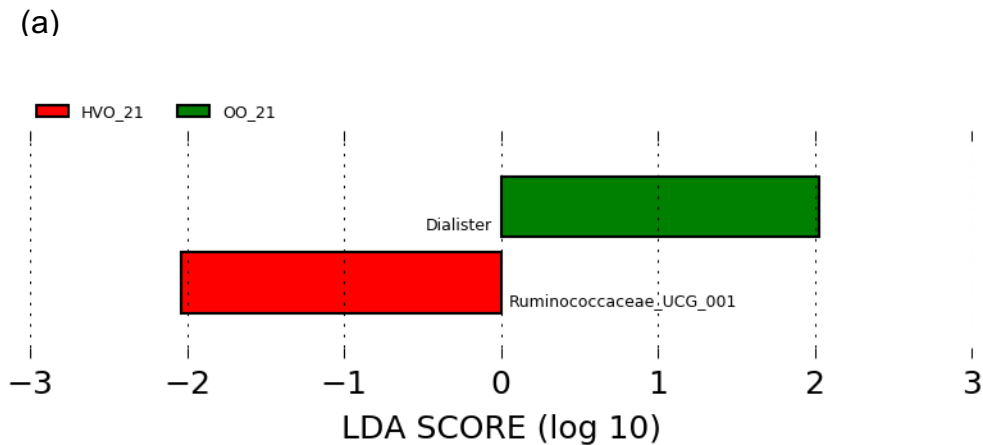
4.8 Differential rumen bacteria abundance

Bacteria differentially and significantly represented between Control (blue color), HVO (red color) or OO (green color) were identified by Linear discriminant analysis (LDA) combined with effect size measurements (LEfSe). This approach revealed a list of features that enable discrimination between treatments in ruminal samples and was performed with the pooled data to identify specific taxa that varied in abundance consistently with lipid addition to the diet of dairy cows.

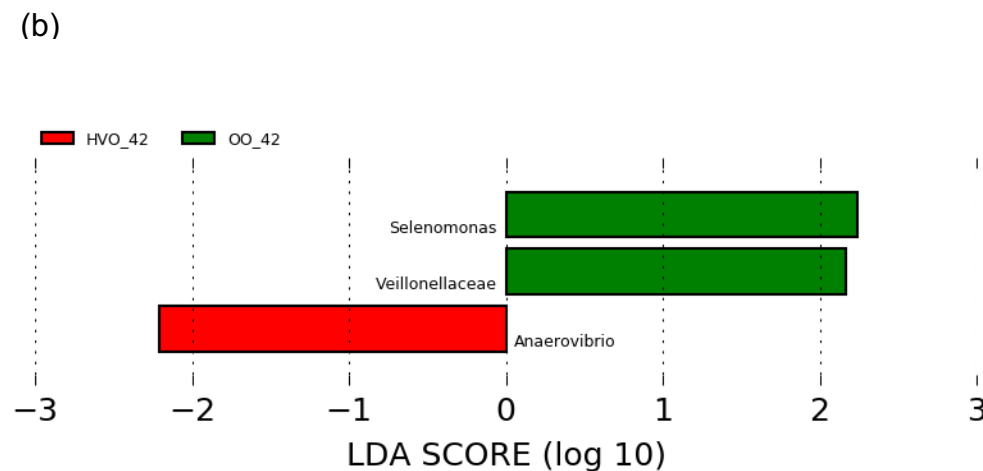
Histogram showing the significant abundant bacteria are shown in Figure 7, which are organized according to the supplementation time. The horizontal straight line in the panel indicates the group means, and the dotted line indicates the group medians.

LEfSe analysis revealed that at genus level (Figure 6a), *Dialister* was significantly more abundant in rumen samples from dairy cows fed 21 days with OO, whereas *Ruminococcaceae_UCG_001* was predominantly identified in HVO supplementation. Figure 6b shows significant differences at 42 days of lipid inclusion. In this case, genera *Selenomonas* and family Veillonellaceae were the most abundant during OO addition, meanwhile *Anaerovibrio* was significantly prevalent with HVO.

Figure 6. The LDA effect size (LEfSe) analysis of bacterial taxa between HVO and OO dietary treatments in ruminal samples of dairy cows. Histogram shows LDA score of lipid inclusion at (a) 21 days and (b) 42 days supplementation. Significant differences are defined as $P < 0.05$ and LDA score > 2.0 .



HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days. Significant differences are defined as $P < 0.05$ and LDA score > 2.0 .



HVO, supplemented with 30 g kg⁻¹ DM hydrogenated vegetable oil; OO, supplemented with 30 g kg⁻¹ DM olive oil. Supplementation periods of 0, 21, 42 and 63 days. Significant differences are defined as $P < 0.05$ and LDA score > 2.0 .

5. Discussion

To our knowledge, this is the first report to characterize the effect of two different lipid sources, palm oil and olive oil, on the composition of rumen bacterial community in dairy cows throughout relative long-term exposure of dietary supplementation. Rumen bacterial communities play a key role in the production performance and health of the host, and diet is the most important factor influencing the rumen microbiome (Henderson *et al.*, 2015). Therefore, the aim of this study was to investigate through Illumina Miseq sequencing approach, the effect of 3% lipid supplementation on the rumen bacterial community of high-producing dairy cows.

5. 1 *Physiological parameter*

For high-producing dairy cows it is difficult to reach the required energy level in the diet without adding fat. Diets with high energy contents elicited an increase in milk yield and milk components in dairy cows (Zhang *et al.*, 2015), and it has been suggested that diets formulated to be low in fat content are limited in either fat or energy, resulting in lower milk yield (Stoffel *et al.*, 2014).

Usually, consumption of lipids by ruminants is low because most forages contain limited amounts of fat. Also, it has been described that the rumen microbial population is intolerant to high dietary fat levels. Therefore, lipids should be included in diet normally at no more than 5% to 7% of the total diet DM (Szumacher-Strabel *et al.*, 2002). In the current study, basal diets were similar in composition, nutrients

and energy concentration, with the exception of the lipid supplement whose inclusion was 3% of the DM.

In our research feed intake (DMI), body condition score (BCS) and body weight was not affected by dietary treatments, which agrees with other studies focused in vegetal lipids supplementation (Altenhofer *et al.*, 2014; Chamberlain and DePeters, 2017; Bougouin *et al.*, 2018). The possible explanation of this result could be related to the proposed by Bayat *et al.* (2017), who indicated that fatty acid carbon length rather than the degree of unsaturation has a negative effect in DMI. Moreover, high levels of dietary oil supplements (≥ 50 g of oil/kg of DM) are typically associated with lower DMI, and the lipid inclusion in this study was only 30 g/kg DM of pail oil and olive oil in each treatment. Furthermore, Rabiee *et al.* (2012), found that a longer period of feeding fat resulted in a higher DMI. Finally, consequences of fat in DMI has been attributed to the adverse effects of unsaturated lipids on ruminal microbial communities, and therefore lower fiber digestion (Weld and Armentano, 2017).

Unsaturated lipids are toxic to rumen microorganisms, which may reduce the intake and digestibility of dry matter and nutrients modifying the ruminal fermentation. This reduction of intake and ruminal fermentation can reduce the rate of digestion and consequently the flow of nutrients to the mammary gland, which in turn could reduce milk production (Welter *et al.*, 2016).

Dairy producers are interested in improve milk yield and composition of their dairy cows, because economic outputs as payments are based on milk fat and protein contents (Bougouin *et al.*, 2018), especially fat due to it represents the major energy cost in the production of milk components (Bauman and Griinari, 2001). In addition, it has been stablished that nutrition is the main factor affecting milk yield and composition. However, it could be possible an undesirable resulting effect would be, such as reduction in the milk yield or components. Ruminant diets typically contain between 20 g and 40 g of lipid/ kg DM, therefore in this research we performed 30 g/kg DM of lipid inclusion, in order to avoid alterations in fed digestion and rumen homeostasis. Even though the diet contains a high proportion of unsaturated fatty acids, ruminant milk contain much higher levels of saturated fatty acids due in part, to extensive biohydrogenation of dietary unsaturated fatty acids in the rumen (Shingfield *et al.*, 2010).

In the present experiment, adding OO to dairy cow diets increased milk yield in 12.2% compared with Control and was 9.7% higher related to HVO. This result agree with Gómez-Cortés *et al.* (2008) findings who supplemented the diet of ewes OO and increased milk yield, probably due to a greater energy content of diet. In addition, Mach *et al.* (2013) reported 10% greater ($p < 0.05$) milk yield when dairy cows are supplementing with linseed (rich in 18:3, cis-9, cis-12, cis-15; ALA). Moreover, addition of sunflower seeds oil to the diet of goats increased milk production, probably due to the increased total VFA concentration in rumen, increased efficiency of N utilization and increased conversion and availability of nutrients for milk synthesis (Morsy *et al.*, 2015). This result differ from the reported by Altenhofer *et al.*

(2014), who found that rapeseed and soybean oil supplementation did not affect milk yield. In case of HVO, milk yield was not affected by lipid supplement. This is in agreement with Chamberlain and DePeters, (2017) who supplemented dairy cows with 20 g of palm oil/kg DM in the TMR and did not observed changes in milk production. However, it is important consider that milk and fat yields also could be affected by individual animal factors such as breeds, individuality features, parity number, stage of lactation and milk production level (Samková *et al.*, 2012), which should contemplated in order to obtain proper conclusions.

Related to Control, OO decreased milk fat content in 13.7% and milk protein yield in 7.6%, whereas HVO increased fat and protein content in 2% and 2.9%, respectively. Reduction in milk fat content and yield is commonly denominated milk fat depression (MFD) (Bauman and Griinari, 2001). This phenomenon typically occur within a few days, and in severe cases milk fat yield can be reduced by more than 50%, with little or no change in the yields of milk, milk protein or lactose (Shingfield *et al.*, 2010). In our study, milk fat reduction did not reach extremely lower values and otherwise milk yield increased 3.8 kg day⁻¹ with OO dietary treatment. However under OO supplementation milk fat composition shifts toward reduced proportions of short (4 to 8 carbons) and medium (10 to 14 carbons) chain fatty acids and greater concentrations of longer chain (>16 carbons) fatty acids, comparable with the observed by Bauman and Griinari (2003).

Moreover, Pitta *et al.* (2018) proposed that milk fat depression is consequence of the interactions between dietary nutrients, gastrointestinal microbiota and the tissue physiology, and it may be the result of alterations in microbial populations or their

activities (shift in biohydrogenation pathways), especially species involved in lipid metabolism (Weimer *et al.*, 2010).

In case of milk fatty acid profile, vegetal oil supplements (rich in C18:1 and C18:2 fatty acids) have been shown to alter milk fatty acid composition and improve nutritional quality of milk (Bayat *et al.*, 2017), associated with a lower amount of saturated fat and an increase of unsaturated fat.

In the current trial feeding OO in diets of dairy cows decreased milk fat content of short and medium chain FA (8:0 to 16:0), and increased significantly monounsaturated FAs (MUFAs) and total polyunsaturated FAs (PUFAs) in milk, such as C18:1 cis-9 and C18:3 cis-9, cis-12, cis-15 FAs in milk, which is characteristic in dairy cows fed dietary plant oil supplements, and it is considered a favorable change, because consumption of these fatty acids has been shown to increase risk of cardiovascular disease (Bayat *et al.*, 2017).

On the contrary, HVO increased ($P < 0.05$) C18:2 cis-9, trans-11 and had a tendency to increment C18:3 cis-6, cis-9, cis-12; C18:2 trans-9, trans-12 and C18:1 cis-9. Due to polyunsaturated FA are not synthesized by ruminants, their concentration in milk depends on the amount of PUFA absorbed from the intestines (Morsy *et al.*, 2015). In addition, the supplementation of unsaturated lipids in the diet of dairy cows may reduce milk fat content due to the production of trans fatty acids by incomplete ruminal biohydrogenation of dietary unsaturated fatty acids. The “de novo” synthesis is responsible for the formation of fatty acids of up to 16 carbons, reducing the milk fat concentrations of short and medium chain fatty acids (Welter *et al.*, 2016).

5.2 Ruminal fermentation parameters

Lipid supplementation did not affect ruminal pH. Results obtained were within normal biological range (minimum, 6.98; maximum, 7.11; Grünberg and Constable, 2009), which could indicate an unaffected cellulolytic processes of fiber digestion and microbial adaptation to the diet. Nur Atikah *et al.* (2018) suggested that an adequate roughage supply in the diet reduced the negative effect of dietary oil on rumen fermentation because the fiber fraction creates a supporting environment for rumen microbes to hydrolyze the dietary oils. In addition, it is important to consider the several factors that influence the diurnal pH, such as the timing of individual feeding and rumination bouts after feed presentation; the rate of passage of digesta and VFA from the rumen, the effectiveness of VFA absorption from the rumen; and the effectiveness of buffering from salivation into the rumen (Palmonari *et al.*, 2010).

OO treatment increased significantly $\text{NH}_3\text{-N}$ after 42 days of supplementation ($P < 0.05$) reaching 9.12 mg/dL. Ammonia concentration could increase as a result of the action of protozoa, due they are involved in protein degradation and deamination (Males and Purser, 1970), increased rate of proteolysis and amino acid metabolism (Bi *et al.*, 2018), reduction in protozoal predation toward rumen bacteria thus, reducing the recycling of bacteria protein in the rumen (Nur Atikah *et al.* 2018). The higher ammonia concentration on day 42 of OO inclusion could be related to a positive effect on protozoa, however the current study did not perform analysis in this microbial community, or the result of an inability to use ammonia, rather than higher production (Males and Purser, 1970).

In addition, a low microbial growth and microbial ammonia utilization in the rumen (by cause of lipid supplementation for example) might contribute to higher rumen $\text{NH}_3\text{-N}$ concentration, but without significant effects on total microbial population (Bayat *et al.*, 2017). Moreover, the $\text{NH}_3\text{-N}$ concentration in rumen fluid can reflect the balance of protein degradation and synthesis. It is well known that $\text{NH}_3\text{-N}$ is an intermediate product of feed protein, non-protein nitrogen degradation and microbial protein synthesis, and it is mainly affected by feed protein degradation, rumen wall absorption, microorganism utilization and rumen chyme outflow rate (Tong *et al.*, 2018). The main sources of the ammonia are nitrogen from the diet, which is transferred from the blood to the rumen via saliva or the rumen epithelium. Also nitrogen is released during the autolysis of microorganisms and the ammonia levels have a nutritional importance because bacteria use ammonia as a nitrogen source (Weston and Hogan, 1968).

Total VFA concentration and proportions of individual VFA were not affected by treatments. These results were similar to that obtained by Benchaar *et al.* (2012) who not found differences ruminal liquor pH and total VFA concentrations in dairy cows fed with 3% of linseed oil. They established that the lack of changes in these ruminal parameters suggested that the level of lipid supplementation was not high enough to affect ruminal metabolism. In addition, influence of dietary lipid supplements on molar VFA proportions may depend on composition of the basal diet, inclusion rate, and amount of lipid in the basal diet (Bayat *et al.*, 2017). Lyons *et al.* (2017), observed no significant differences in butyric, propionic and acetic acid in ruminal

samples from lambs supplemented with linseed oil, possibly due the high level of redundancy of the rumen microbiome. The diet induce changes in rumen microbial community structure that are related to changes in ruminal VFA synthesis or methane production (Tapio *et al.*, 2017a). The increased level of the total VFA concentration indicates the efficiency of nutrient digestion (Nur Atikah *et al.*, 2018).

5.3 Metataxonomic analysis of the rumen bacterial diversity

We used next generation sequencing approach to characterize changes in the rumen bacterial community of dairy cows throughout 63 days exposure of lipid supplementation (saturated and unsaturated source). This research is interesting from the microbiological point of view because polyunsaturated fatty acids (PUFA) have been associated with toxic effect on rumen bacteria due to the formation of complexes between fatty acids and cell walls of rumen microbes, depressing the growth of bacterial populations (Bayat *et al.*, 2017; Pitta *et al.*, 2018) and causing impediment in the nutrient passage (Enjalbert *et al.*, 2017), probably because double bonds of PUFA alter the shape of the molecule and disrupt the lipid bilayer structure of the bacteria (Maia *et al.*, 2007). Despite this knowledge about the action of unsaturated fatty acids, supplementing the diet of dairy cows with PUFA also has been widely employed to improve milk quality, changing the saturated fatty acid profile to an unsaturated which is beneficial for human health (Jenkins and McGuire, 2006; Lanier and Corl, 2015; Toral *et al.*, 2018). Therefore, it is relevant to study and understand the consequences of lipid supplementation on rumen bacterial community and performance parameters, in order to nutritionally benefit consumers, the through a proper management of animal nutrition.

Despite the inherent biases of 16S-based methods, such as unequal amplification in PCR, this type of approaches proved to be useful for metagenomic analysis (Ross *et al.*, 2013; Cancino-Padilla *et al.*, 2018). To date, several metagenomic studies have been focused on the effect of different dietary interventions in rumen microbiome, including manage of concentrate in diet (Petri *et al.*, 2013; Pitta *et al.*, 2016a) or supplementation with diverse ingredients (Belanche *et al.*, 2016; Welch *et al.*, 2017; Cremonesi *et al.*, 2018), confirming to be successful in the identification of differences in microbiomes as a result of different diets. These techniques have contributed to understand the reasons for production improvements, but at a molecular level which could be useful to raise more efficient animals and obtain economic profit associated with this issue, and also solve current environmental problems such as global warming, through the reduction of methane emissions.

In case of fat supplementation, vegetable oils (Huws *et al.*, 2015; Vargas-Bello-Pérez *et al.*, 2016; Wang *et al.*, 2017) and marine lipids (Patra and Yu, 2013; Castro-Carrera *et al.*, 2014) have been widely used. However, the use of crude olive oil throughout long-term supplementation in dairy cows have not been reported in the literature, thus more studies are needed about this topic. Furthermore, rumen community diversity still remain unknown and it have been suggested that culturable microbes in the rumen account for less than 1% of the total microbial species (McCabe *et al.*, 2015), which expose the lack of knowledge related to rumen microbiome.

5.4 Rumen bacterial composition

The main objective of the experiment was to investigate differences in the rumen microbiota arising as a consequence of dietary supplementation. Consistent with previous reports based in rumen microbiome of dairy cows (Kim *et al.*, 2011; Jewell *et al.*, 2015; Zhu *et al.*, 2018), Bacteroidetes and Firmicutes were the most abundant phyla and the sum of these two phyla accounted for the 97.7% of the microorganisms in the rumen samples in this study (Figure 1a). Relevance of both phyla in ruminal bacterial community rely in their function associated with degradation of fiber and polysaccharides (Cheng *et al.*, 2017), and their recognition as members of the core microbiome of cattle through members such as genera *Ruminococcus* and *Butyrivibrio* (Firmicutes) and *Prevotella* (Bacteroidetes) (Weimer, 2015).

In particular, Looor *et al.* (2016) proposed that Bacteroidetes is dominant in the rumen from the 6 week of life, and this predominance would exist independently from sampling time, diet (Cremonesi *et al.*, 2018) and dietary treatment (McCann *et al.*, 2014; Paz *et al.*, 2018). We observed variations in relative abundance of Firmicutes and Bacteroidetes depending on the treatment and experimental period.

Results showed that phylum Firmicutes (Figure 2a) increased their abundance with HVO at 21 days and 63 days of supplementation, meanwhile with OO increment was observed only at 63 days. Matthews *et al.* (2019) proposed that higher percentages of Firmicutes compensated for the lower abundances of Bacteroidetes which could explain the data obtained in this research. In addition, the authors hypothesized that a decreased abundance of Bacteroidetes in comparison to Firmicutes resulted in

increased milk fat, but this is in opposition with our findings because we evidence reduction in content of fat in milk with OO, while HVO did not present difference related to Control treatment.

In case of Bacteroidetes (Figure 2b), decreased in abundance over time, especially at 21 days and 63 days with HVO supplementation, whereas decreased after 63 days of supplementation with OO. This findings agree with Pitta *et al.* (2018), who established that without regard to the source of oil supplements used, higher concentrations of PUFA can be detrimental to Bacteroidetes, and it is not completely understood why these populations change with increasing doses of dietary PUFA or with other dietary nutrients. Further, a decrease in gut Bacteroidetes could indicate an increase in fat concentrations in blood and tissues (Pitta *et al.*, 2018), which was not observed in this study due to body condition score (BCS) and body weight was not affected by dietary treatments.

From a productive point of view, Jami *et al.* (2014), noticed that the ratio of Firmicutes to Bacteroidetes is positively correlated with milk fat percentage, and the increase in Firmicutes and Proteobacteria at the expense of Bacteroidetes in older dairy cows is probably associated with a higher milk production in multiparous cows (Pitta *et al.*, 2016b). It is possible that changes in ratio Firmicutes: Bacteroidetes in this study was insufficient to produce modifications in the content of solids in milk, but nevertheless lipid supplementation was able to increase milk yield (probably through energy improvement of the diet) and modify milk profile with OO inclusion.

The analysis at Genus level (Figure 1c) showed that *Prevotella* and *Succiniclasicum* were dominant across all the samples and dietary treatments. This result is in concordance with observed by Pitta *et al.* (2010) and Wirth *et al.* (2018), who informed that *Prevotella* is the most predominant ruminal genus accounting for 42% to 60% of the bacterial 16S rRNA gene sequences and in higher numbers in liquid fractions of ruminal samples.

Prevotella is considered part of the ruminal core of cows under all dietary interventions (Lyons *et al.*, 2017; Koringa *et al.*, 2018), and Henderson *et al.* (2015), identified this genus in a large selection of ruminants in their research (such as bovines, caprids, cervids, and camelids). These bacterial species are capable of utilizing starches, non-cellulosic polysaccharides, and simple sugars as energy sources to produce succinate as the major fermentation end product (Bi *et al.*, 2018), relevant functions in rumen physiology.

We observed differences in relative abundance of predominant genus over time and depending of the lipid source. *Prevotella* (Figure 3a) decrease their abundance at day 21, 42 and 63 of HVO supplementation, while OO showed an effect only at day 63. This result is in accordance with finding related to the phyla level due to the reduction in relative abundance of phylum Bacteroidetes during lipid supplementation. In specific, *Prevotella ruminicola* (as member of phylum Bacteroidetes) has been described sensitive to higher PUFA and starch concentrations (Pitta *et al.*, 2018), and with significantly negative correlation with

milk-fat yield, explaining the negative correlation of Bacterioidetes to this parameter (Jami *et al.*, 2014).

In particular, *Succiniclasicum* (Figure 3b) showed higher abundance during lipid supplementation throughout the experimental periods. At 21 days, 42 days and 63 days this genus increased with HVO, whereas there were no significant differences in OO supplementation. Members of this bacterial group apparently differed in quantity between hay diet- and high grain diet and have been described as starch degraders (Wirth *et al.*, 2018), producing propionate through succinate decarboxylation. Jin *et al.* (2018), observed high abundance of *Succiniclasicum* in rumen cow feeding wheat straw. They related this finding with the production of large amounts of succinate and thus higher concentration of propionate. However, we did not obtain differences in VFA concentration compared with the Control treatment. Also, Bi *et al.* (2018) founded that the relative abundances of the genera *Succiniclasicum* significantly increased with the increase in dietary energy levels. This last statement could explain our results because lipid supplementation (saturated or unsaturated source) increased energy content in the dietary treatments.

In addition, a higher abundance of *Succiniclasicum* may divert H₂ away from methanogenesis reducing methane emissions (Myer *et al.*, 2015), which is relevant in dairy cattle production due to methane production involve an energy lost for the animals. Nevertheless, this parameter was not analyzed in this research, therefore more information is needed to ensure and prove this hypothesis.

Microbial diversity is considered as a function of the number of different classes (richness) and the relative distribution of individual elements among these classes (evenness) (Rajendhran and Gunasekaran, 2011).

In the current study, to detect the specific bacteria associated with each dietary group, the linear discriminant analysis (LDA) effect size (LEfSe) value was used to determine the taxonomic biomarkers. LEfSe identified five bacterial genera showing biologically significant differences (Figure 6a and Figure 6b) with an LDA score higher than 2.0 score among the dietary groups.

In Figure 6a, *Dialister* (Firmicutes) was abundant in rumen samples from dairy cows fed 21 days with OO, whereas *Ruminococcaceae_UCG_001* (Firmicutes) was predominantly identified in HVO supplementation. In case of *Dialister*, it has been positively correlated with total VFAs and propionate, indicating that this genera may be involved in VFA metabolism and starch degradation due to observed increases in amylase and carboxymethyl cellulase activities (Meale *et al.*, 2017). This result is in agreement with findings in abundance in Firmicutes with OO supplementation. Furthermore, it is desirable obtain higher abundance of *Dialister* in rumen because bigger concentration of this genus has been negatively associated with methane emissions (Zheng *et al.*, 2018). Therefore, we could speculate that supplementation with OO improved efficiency in dairy cows and would be recommended to decrease environment pollution. Respecting to *Ruminococcaceae_UCG_001*, this increased significantly their abundance with HVO, a saturated lipid source, which is in

concordance with findings of Popova *et al.* (2017) about negative effect of linseed oil (unsaturated source) in this bacterial group.

Figure 6b shows significant differences at 42 days of lipid inclusion. In this case, genera *Selenomonas* (Firmicutes) and family Veillonellaceae were the most abundant during OO addition, meanwhile *Anaerovibrio* was significantly prevalent with HVO. Maczulak *et al.* (1981), observed that growth of *Selenomonas ruminantium* was stimulated by oleic acid, which agrees with increased abundance of this genus under OO addition, also Maia *et al.* (2007) and Potu *et al.* (2011) found that these bacteria were insensitive to PUFA. Presence of *Selenomonas ruminantium* in rumen is desirable because have been identified as a low hydrogen producer (Wang *et al.*, 2016), which is beneficial to decrease methane emissions. Regarding to family Veillonellaceae (propionate producers), Lyons *et al.* (2017) obtained higher relative abundance of this group in the lambs fed with 40 g kg⁻¹ DM of linseed oil, and also associated with low methane emitting cattle (Wallace *et al.*, 2015).

Finally, *Anaerovibrio* is recognized as one of the major species involved in lipid hydrolysis in ruminants (Huws *et al.*, 2010; Vargas-Bello-Pérez *et al.*, 2016) and was found predominantly in HVO treatment. Also, increased concentration of PUFAs in rumen may be linked to the lower abundance of the *Anaerovibrio* (Mannelli *et al.*, 2018). Conversely, Potu *et al.* (2011) reported no effect of lipid supplementation on the DNA concentrations of *Anaerovibrio lipolytica*.

5.5 Rumen diversity

In the current research we estimated alpha diversity indices Figure 4 to describe the richness and diversity of the rumen microbiota in the three experimental groups. Diversity indexes Shannon (Figure 4a), Chao1 (Figure 4b) and Simpson (Figure 4c), were found not to be significantly between each dietary treatment. This result is in disagreement with the established by Bayat *et al.* (2017), who proposed that lipid supplements altered diversity of rumen microbial communities and relative abundances of some less common taxa. Huws *et al.* (2015) and Pitta *et al.* (2018) also founded that the number of bacterial populations (species richness) and their distribution (diversity) were under different oil supplements enriched in PUFA. On the other hand, Nur Atikah *et al.* (2018) founded that supplementation with vegetables oils did not alter the fiber degrading bacteria and total microbial populations, indicating that these microorganisms are not sensitive to dietary oils supplementation.

Furthermore, AlZahal *et al.* (2017) declared that the bacterial community composition rely not only in the type of diet, but also by the obtention of the sample. Related to this, Pitta *et al.* (2016b) did not observed difference in microbial profiles between rumen fluid collected using stomach tube or via a cannula. Despite oral tubing can retrieve only finely digested particles which may result in a bacterial underestimation, except for a few members the overall microbial community structure has been found to be similar between the two sampling methods. In addition, the use of a stomach tube offers the advantage of sampling a greater

number of intact animals that have been selected for desirable traits which is less expensive and time consuming.

The lack of differences observed between dietary treatments at the level of diversity analyses may simply indicate that the important variation in microbial communities lie at a finer resolution (Myer *et al.*, 2015). In addition, despite a high functional stability due to functional redundancy and the resilience of microbial ecosystem (Xie *et al.*, 2018), the rumen microbiota exhibits individual variation and can be disturbed by abrupt or major dietary changes (Enjalbert *et al.*, 2017). Moreover, Lyons *et al.* (2017) proposed that due to the redundancy and resilience present in the rumen microbiome, dietary manipulations of rumen microbiomes in later life typically only last days to weeks.

While it is clear that hosting microbial communities can be functionally beneficial or even necessary to an animal, the extent to which hosting a diverse microbial community is necessary for this phenomenon is still uncertain. A host may wish to limit diversity because not all microbes are beneficial, whether because they are pathogens or because they are just cheating strains that avoid providing function to the host. Furthermore, increasing diversity has been shown to actually reduce stability in gut communities (Reese and Dunn, 2018).

6. Conclusion

Overall, 3% of olive oil inclusion can be safely supplemented to a 65% forage-based diet of dairy cows, with the objective to improve milk with potential health-beneficial FA without causing any detrimental effect on rumen function, rumen bacterial communities or animal performance.

To our knowledge, this project will be the first to provide a comprehensive evaluation of the long-term effects of different dietary lipids: monounsaturated (unrefined olive oil) and saturated (hydrogenated palm oil) fatty acids on rumen microbiota from mid-lactating dairy cows using NGS approach.

Understanding how lipid supplements modify the whole rumen microbial consortium is essential to understand better its effect on rumen fermentation and host metabolism in general. Also, deeper understanding of lipid supplementation effects may allow us to modulate the rumen microbiome for better agricultural yield through bacterial community design, because it is apparent that the ruminal microbiome in cows continues to mature over time in a predictable fashion and becomes more diverse as the cow adapts to changing diets, metabolism and the environment. It is crucial further research in identifying unknown strains of microorganisms and understand the microbial dynamics in response to changing dietary and physiological factors in dairy cows, in order to clarifying their functional role in rumen and gain insights into improving both yield and composition of milk.

7. References

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