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# Diversity and composition of the bacterial community in the feces of cattle and goats

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# ABSTRACT

The present study aimed to assess and compare the diversity and composition of the bacterial community of feces of cattle and goats, using advanced method of molecular biology.

A total of 17 female goats, Bionda dell'Adamello and 16 female cows, Original "Bruna" (Brown Swiss), were selected for the study, they were fed pasture and hay and they had free access to water. The collection of fresh fecal samples was carried out immediately after defecation and all of the samples were freeze-dried and kept in the fridge until needed for the analysis. The DNA extraction and the amplification of the bacterial variable V4-V5 region of 16S rRNA was performed. The bacterial structure was studied using high-throughput sequencing. The analysis of bacterial diversity was evaluated through alpha diversity, and beta diversity and the taxonomical composition was analyzed using QIIME2 platform.

Alpha diversity measurements of the bacterial community revealed significant difference in Pielou Evenness index showing a higher species evenness in cows compared to goats. Other alpha diversity indices showed no statistical differences between the two groups of animals, indicating a similar richness of bacterial species. Beta diversity measurements revealed statistical differences between animals indicating dissimilarities and distinct clustering of bacterial composition of feces between goats and cows. Regarding the taxonomical composition, Firmicutes was dominant phylum both in goats and cows, followed by Bacteroidetes, Proteobacteria and Spirochaetes. Linear discriminant analysis effect size (LEfSe) showed a total of 63 significantly different taxa between goats and cows. Mainly, the relative abundance of Spirochaetales, *Christensenellaceae*, *Spirochaetaceae*, *Treponema* and *Ruminococcus* was higher in goats compared to cows. In contrary, the relative abundance of Bacillales, *Paraprevotellaceae*, *p-2534-18B5*, *Peptostreptococcaceae*, *Planococcaceae* and *CF231* was higher in cows compared to goats. In conclusion, our findings suggest that host's species has a significant influence on the composition of fecal bacterial community.

# Introduction

Domestic ruminants (cattle, buffaloes, goats and sheep) are essential breeds of animals because they contribute to humans' nutrition and well-being. They are widely utilized to provide milk, dairy products, meat, wool and skin (Hodgson and Harlow, 1979; Mazinani and Rude, 2020). The global population of ruminants is constantly increasing as well as their importance in food production (Sauer *et al.,* 2012; FAO, 2019; Miller and Lu, 2019). The livestock industry is one of the most dynamic sectors. In fact, the demand for animal's products is rapidly rising as the quality-of-life improves (Thornton, 2010; Cao and Li, 2013).

Ruminants' particular capacity to utilize complex carbohydrates from plants and convert them into valuable products is a main factor of their involvement in agricultural production. Their polygastric digestive system is intended to ferment fibrous plant materials. The largest and most important part of a four-chambered stomach, rumen, plays the key role in biomass fermentation (Parish *et al.,* 2017). Rumen is inhabited by anaerobic microbes which are capable of digesting (hemi)cellulose substances (Matthews *et al.,* 2019). Rumen microbes are divided into three domains: *Archaea, Bacteria* and *Eukarya* which include *protozoa* and *fungi* (Woese *et al.,* 1990). Bacteria and protozoa make up the majority of microbial biomass, followed by anaerobic fungi (Zhu, 2016). Methanogenic archaea account for less than 4% of the rumen's microbes, however they are the only microorganisms which generate methane (Matthews *et al.,* 2019). Methane emission can lead not only to air pollution, but represents energy loss for animal. This is one of the main reasons for intensive studies of ruminant microbiome, the improvement of animals' nutrition and husbandry and using of genetic selection in order to reduce $CH_4$ production (Shibata and Terada, 2010).

Rumen microorganisms form a complex microbial ecosystem that has a symbiotic relationship with the host. Ruminants depend on these microbes because mammals are not capable to split β-1-4 glycosidic linkages between hexose and pentose monomers in cellulose and hemicellulose chains, respectively. Only microorganisms have the ability to decompose β- glycosidic bonds and therefore are responsible for rumen fermentation of biomass, they digest plant material to provide the energy for host animal. Microorganisms produce volatile fatty acids (acetate, butyrate and propionate) as end fermentation products, which are a major

source of energy for the host animal (70%). They greatly contribute to ruminant production and can determine the fat and protein content of milk (Moran, 2005).

On the other hand, microbes use the feed ingested by the host to generate energy for themselves (Choudhury *et al.,* 2015). Interactions among microbes also exist, such as the competition for the same nutrient (Mosoni *et al.,* 1997) and symbiotic relationships as cross-feeding (Choudhury *et al.,* 2015).

Several factors may influence composition and diversity of rumen microbes, including diet, ruminant species, age, geographical location, type of production system and host's genotype. Microbial colonization of rumen begins immediately after birth and then continues rapidly during the first few months, till weaning (Taschuk and Griebel, 2012). Animal diet is the major determinant of ruminal microbial composition (Henderson *et al.,* 2015; Gruninger *et al.*, 2019). Economic aspects of meat and milk production induced feeding systems for adding substantial portions of grains into diets to saturate the nutritional demands of animals. The high-grain diet can satisfy the ruminant's energy and proteins needs, in order to achieve a better performance, but, on the other hand, it can cause alterations in the rumen microbiome (Mao *et al.,* 2013; Nagata *et al.,* 2018), leading to metabolic disorders (González *et al.,* 2012; Khiaosa-ard and Zebeli, 2018). The host animals may also have an influence on rumen microbiome as a result of the specific animal's physiological and behavioral responses to the environmental conditions (Hofmann, 1989). Recent research found that host's genotype can also affect rumen microorganisms since some traits are heritable and may be influenced by the ruminant's genetics (Li *et al.,* 2019; Difford *et al.,* 2018).

As ruminants are largely dependent on rumen fermentation, developing strategies to modulate the rumen microbial community and improve fermentation efficiency is essential for increasing animals' productivity. Therefore, examining rumen microbes can determine the effects of different factors on the microbiome in order to improve quality products, increase profitability and decrease environmental impacts (Matthews *et al.,* 2019).

Since bacteria are the most prevalent microorganisms in the rumen and can reflect the profile of microbial population, information about their density and diversity is highly important.

In the present study, the composition of bacterial community was explored in the feces of cattle and goats fed high fibrous diet using advanced method of molecular biology.

# 1. GLOBAL SITUATION AND DIGESTIVE SYSTEM OF CATTLE AND GOATS

## 1.1 Current status of global cattle and goat populations and their productivity

Ruminants have a great importance for human society as they play a crucial role in food production (Sauer *et al.,* 2012). In fact, cows have made a significant contribution to human welfare, they are the most effective in dairy and meat production, they also provide hides, fuel and some other products (Hodgson and Harlow, 1979). Goats are also very important, they are commonly used worldwide for the production of wool, meat, milk and skins, but they are especially important for food production in arid and semiarid parts of world. In Europe, the majority of goats are used for milk and cheese production, in the Mediterranean countries, goats are often used for meat production (Dubeuf, 2002).

Cattle population has increased considerably in the last 50 years. In Asia, Africa, America and Oceania, the numbers increased significantly, while in Europe the numbers decreased slightly. The recent estimation of world cattle population is 1.51 milliard animals. The greatest number is found in America (527 million), followed by Asia (470 million), Africa (361 million), Europe takes the fourth place (117 million) and finally Oceania (35 million). The worldwide distribution of cattle in 2019 is summarized in the table 1.1-1 (FAO, 2019). Among European Countries, Italy takes the seventh place - after Russian Federation (18 million), France (18 million), Germany (11 million), United Kingdom (9.7 million), Spain (6.6 million) and Ireland (6.5 million) - with 6,377,230 heads. Cattle distribution in these Countries is summarized in the table 1.1-2 (FAO, 2019). Concerning milk and meat production, Italy produces 779,820 tons of meat and 12,494,400 tons of milk. Production and producing animals are summarized in the table 1.1-3 (FAO, 2019). In 2015, Italy was the third largest contributor (11.4%) to total cattle meat production in Europe (Cozzi, 2007).

Goats' population has also increased significantly in the last 50 years. In Asia, Africa and Oceania the numbers increased considerably, in America, the numbers increased slightly, while in Europe the numbers decreased slightly. This is due to environmental conditions and socio-economic situation. The recent estimation of world goat population is 1.09 milliard animals. The greatest number is found in Asia (575 million), followed by Africa (458 million), America (39 million), Europe takes the fourth place (16 million) and finally Oceania (4 million). The worldwide distribution of goats in 2019 is summarized in the table 1.1-4 (FAO, 2019). Among European Countries, Italy takes the sixth place - after Greece (3.5 million), Spain (2.6 million), Russian Federation (1.9 million), Romania (1.5 million) and France (1.2 million) - with 1,058,720 goats. Goats' distribution in these countries is summarized in the table 1.1-5 (FAO, 2019). Concerning milk and meat production of goats, Italy produces 2,290 tons of meat and 62,340 tons of milk. Production and producing animals are summarized in the table 1.1-6 (FAO, 2019). However, the unitary production of sheep and goats' milk in Italy is important. Thus, Italy is the world leader with 83 liters of milk per head. In contrary, meat and wool production is less important (Pulina, 2000). The production of milk is important especially for cheese manufacturing (Sandrucci *et al.,* 2019). The population of Italian goats in 2013 was mainly located on the two main Italian islands: Sardinia and Sicily (AIA 2014). Farming systems in Italy show a large variability ranging from intensive indoor breeding adopting advanced tools to semi-extensive and extensive traditional systems (Sandrucci *et al.,* 2019).

Accordingly, the following statistics confirm the increasing demand for livestock products. In fact, over the last 50 years, the worldwide production of foods from animal sources has more than tripled (FAO, 2018) and the consumption of meat and dairy products is on the rise (Heinke *et al.,* 2020). Due to the great importance of livestock sector, the study of the rumen microbes is very important as they play a crucial role in ruminants' production (Sauer *et al.,* 2012).

**Table 1.1-1: Worldwide distribution of cattle (FAO, 2019)**

| Rank | Region | Number of Cattle |
|---|---|---|
| **1** | America | 527,009,141 |
| **2** | Asia | 470,014,051 |
| **3** | Africa | 361,282,309 |
| **4** | Europe | 117,256,993 |
| **5** | Oceania | 35,458,581 |

**Table 1.1-2: Cattle distribution in the European Countries (FAO, 2019)**

| Rank | Country | Number of Cattle |
|---|---|---|
| **1** | Russian Federation | 18,151,394 |
| **2** | France | 18,150,620 |
| **3** | Germany | 11,639,530 |
| **4** | United Kingdom | 9,738,913 |
| **5** | Spain | 6,600,330 |
| **6** | Ireland | 6,559,650 |
| **7** | Italy | 6,377,230 |

**Table 1.1-3: Cattle production and producing animals in Italy (FAO, 2019)**

| | | |
|---|---|---|
| **Producing Animals/Slaughtered** | Meat, cattle | 2,729,600 (head) |
| **Production** | Meat, cattle | 779,820 (tones) |
| **Milk animals** | Milk, whole fresh cattle | 1,875,720 (head) |
| **Milk** | Milk, whole fresh cattle | 12,494,400 (tones) |

**Table 1.1-4: Worldwide distribution of goats (FAO, 2019)**

| Rank | Region | Number of Goats |
|---|---|---|
| **1** | Asia | 575,537,616 |
| **2** | Africa | 458,815,721 |
| **3** | America | 39,248,509 |
| **4** | Europe | 16,139,476 |
| **5** | Oceania | 4,326,973 |

**Table 1.1-5: Goats distribution in the European Countries (FAO, 2019)**

| Rank | Country | Number of Goats |
|---|---|---|
| **1** | Greece | 3,580,000 |
| **2** | Spain | 2,659,110 |
| **3** | Russian Federation | 1,992,896 |
| **4** | Romania | 1,598,800 |
| **5** | France | 1,242,000 |
| **6** | Italy | 1,058,720 |

**Table 1.1-6: Goats' production and producing animals in Italy (FAO, 2019)**

| | | |
|---|---|---|
| **Producing Animals/Slaughtered** | Meat, goat | 152,940 (heads) |
| **Production** | Meat, goat | 2,290 (tones) |
| **Milk animals** | Milk, whole fresh goat | 827,420 (heads) |
| **Milk** | Milk, whole fresh goat | 62,340 (tones) |

## 1.2 Ruminants' digestive anatomy and physiology

Ruminant livestock species include cows, buffaloes, sheep and goats. They have a polygastric digestive system composed by a four-chambered stomach. It is designed to ferment plant-based food to acquire nutrients and energy for the animal (Parish *et al.,* 2017). The ruminant digestive system is composed by the mouth, esophagus, four-compartment stomach (rumen, reticulum, omasum and abomasum), small intestine (duodenum, jejunum and ileum) and large intestines (cecum, colon and rectum). The core of the stomach is heavily expanded to form the fermentation compartments, but also compartment that correspond to the basic stomach of non-ruminants. Extensive microbial fermentation of consumed feed takes place within rumen and reticulum. After grazing, regurgitation occurs. The rumen-containing regurgitate bolus is chewed and swallowed. This is called the rumination process (Harfoot, 1978).

### 1.2.1 Mouth

The mouth, the very first part of digestive system contains the lips, the teeth and the tongue, which are the principal prehensile structures. The mouth is involved in physical processes such as mastication and mixing of feed with saliva (Luginbuhl, 1983). Saliva moistens the ingested feed to assist in mastication and swallowing. It plays a role in maintaining the pH of the fermenting digesta and affects feed and water intake as well as the rate of nutrient removal from the rumen (Bartley, 1976).

### 1.2.2 Esophagus

The esophagus is a muscular tube about 90 to 105 cm in length that connects the pharynx with the forestomach. Its main role is to move the feed from the mouth to the forestomach and vice versa during the rumination (Habel, 1975).

### 1.2.3 Rumen

The rumen represents the largest section of the four-compartment stomach, it is divided into dorsal and ventral sacs. The majority of microbial fermentative activity and nutrient assimilation takes place in the reticulo-rumen (Luginbuhl, 1983).

### 1.2.4 Reticulum

The reticulum is the most anterior section of the four compartments. It is disconnected from the rumen by a rigid tissue, but that separation doesn't extend to the right lateral wall of the stomach enabling a large communication between these two compartments (Harfoot, 1978).

### 1.2.5 Omasum

The omasum is located at the right side of the stomach. It is connected to the reticulum via the reticulo-omasal orifice and it is also connected to the abomasum via the omaso-abomasal orifice (Harfoot, 1978). Its main role is the absorption of water and the transport of small feed particles to the abomasum (Luginbuhl, 1983).

### 1.2.6 Abomasum

The abomasum is a tube-shaped compartment that links the omasum to the small intestine (Harfoot, 1978). It is granular and its main role is the digestion of proteins. The epithelium is provided with secretory cells producing pepsin and hydrochloric acid (Luginbuhl, 1983).

### 1.2.7 Duodenum

The duodenum is the first section of the small intestine, it provides bile and pancreatic secretion for chemical digestion via a common tube for both endocrine organs (Harfoot, 1978).

### 1.2.8 Jejunum

The jejunum is the longest section, located in the center of the small intestine. It is vaguely detached from the duodenum but is connected to the ileum, which stops at the ileo-cecal orifice. Its main role is the absorption of essential nutrients (Luginbuhl, 1983).

### 1.2.9 Ileum

The ileum is the last section of the small intestine. It is connected to the cecum throughout the ileo-cecal orifice. Its main role is the reabsorption of bile acids and the absorption of vitamin B12 (Luginbuhl, 1983).

### 1.2.10 Cecum

The cecum is tube-shaped blind pouch, it is the first section of the large intestine. The cecum seems to serve little function in a ruminant, but water absorption takes place here (Harfoot, 1978).

### 1.2.11 Colon

The colon is the second section of the large intestine, it is divided into ascending, transverse and descending segments. It is the essential site of water absorption (Moran, 2005).

### 1.2.12 Rectum

The rectum is the latest section of the large intestine, it attaches the colon to the anus. Its main function is to excrete the unabsorbed substances in the form of feces (Moran, 2005).

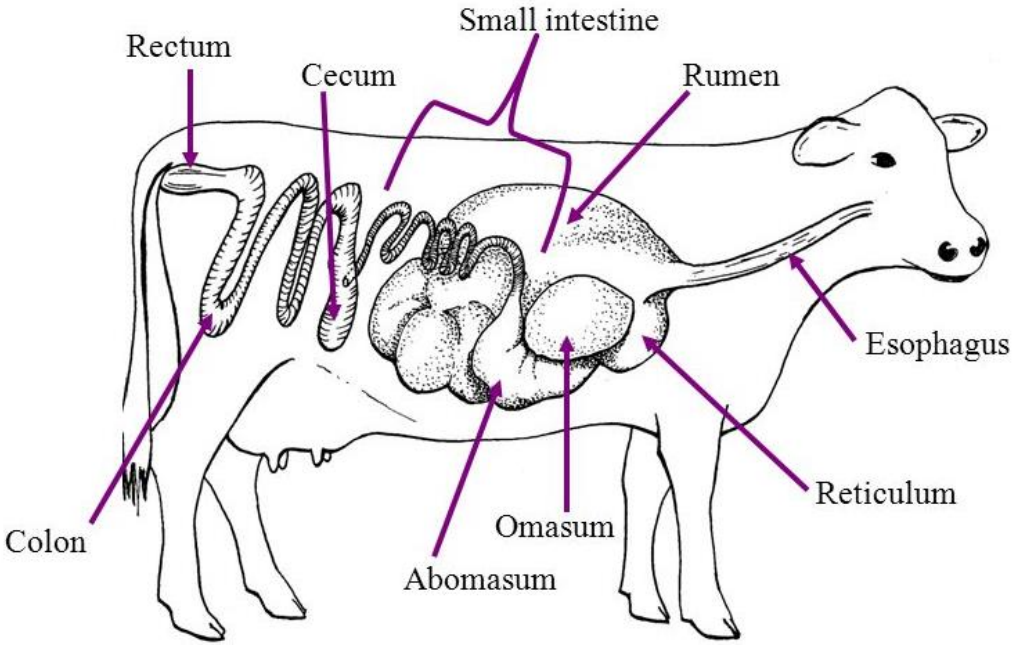



**Figure 1.2-1: Anatomy of ruminants' digestive tract**

## 1.3 Rumen physiology and function

The rumen is the biggest and the most important compartment for diet fermentation. It has an oval shape and is horizontally compressed. It is internally separated into ventral and dorsal pouches. The inner surface is coated by papillae, which are distributed in an asymmetrical way. The ventral and dorsal regions are heavily papillated, in contrary to the edges of the pillars which are slightly papillated. The papillae amplify greatly the surface of absorption (Harfoot, 1978). The rumen is connected to the outside environment in a way that the material flows continuously in and out the rumen (Choudhury *et al.,* 2015). The rumen is not fully developed at birth, both physically and metabolically. Consequent physical development is divided into two categories: the increase in rumen size and the growth of the papillae. Then, metabolic maturation needs the establishment of ruminal microbiome for fermentation activity and the synthesis of short-chain fatty acids (Baldwin and Connor, 2017). Information about chemical, physical and microbiological characteristics of the rumen is summarized in the table 1.3-1.

The rumen is a large anaerobic fermentation pouch containing microbes that can degrade (hemi)cellulose substances of plant biomass as grass, straw, silage and hay. Rumen microbial community is characterized by a great density, an important diversity and complex interactions. These microbes are anaerobic or facultative anaerobic, they produce substances which can be used directly by the animal or by other microbes as energy and nutrients (Matthews *et al.,* 2019). They usually cooperate together in a synergistic way to generate lignocellulolytic enzymes which support the diet digestion. Volatile fatty acids, formic acid, hydrogen, carbon dioxide and methane are produced as a result of this process (Krause *et al.,* 2003). Carbon dioxide and methane constitute the major proportion of the produced gases (Hoover and Miller, 1991). The composition of gases in the rumen is summarized in the table 1.3-2.

Rumen papillae are a site of absorption for certain nutrients. Volatile fatty acids, ammonia and some minerals as sodium, magnesium and zinc are absorbed throughout the rumen wall (Hoover and Miller, 1991).

Ruminant's diet has major influence on microorganisms and may alter the rumen microbiome community composition. The manipulation of rumen fermentation is crucial to enhance animal production. In order to increase productivity, an optimal growth and development of these microbes is essential and a healthy and balanced diet is needed (Matthews *et al.,* 2019).

**Table 1.3-1: Physical, chemical and microbiological parameters of the rumen (Mackie *et al.,* 1999; Castro- Montoya *et al.,* 2011)**

| ***Physical properties*** | |
|---|---|
| Dry matter (%) | 10–18 |
| Osmolality | 250–350 mOsmol/$Kg^{-1}$ |
| pH | 5.5–6.9 (Mean 6.4) |
| Redox potential | −350 to −400 mV |
| Temperature | 38–41 °C |
| ***Chemical properties*** | |
| Amino acids and oligopeptides | <1 mmol.$L^{-1}$ present 2–3 h post feeding |
| Ammonia | 2–12 mmol.$L^{-1}$ |
| Dietary (cellulose, hemicelluloses, pectin) component | Always present |
| Endogenous (mucopolysaccharides) | Always present |
| Growth factors | Good supply; branched-chain fatty acids, long-chain, fatty acids, purines, pyrimidines, other unknown |
| Lignin | Always present |
| Minerals | High Na; generally good supply |
| Nonvolatile acids (mmol.$L^{-1}$) | Lactate <10 |
| Soluble carbohydrates | <1 mmol.$L^{-1}$ present 2–3 h post feeding |
| Trace elements/vitamins | Always present; good supply of B vitamins |
| Volatile fatty acids (mmol.$L^{-1}$) | Acetate 60–90, propionate 15–30, butyrate 10–25, branched chain and higher 2–5 |
| ***Microbiological properties*** | |
| Anaerobic fungi | $10^{3–5}$ $g^{-1}$ (20 genera) |
| Bacteria | $10^{10–11}$ $g^{-1}$ (>2000 species) |
| Bacteriophage | $10^{7–9}$ $g^{-1}$ particles $ml^{-1}$ |
| Ciliate protozoa | $10^{4–6}$ $g^{-1}$ (25 genera) |

**Table 1.3-2: Rumen gases composition (Hoover and Miller, 1991)**

| **Gases** | **Percentages (%)** |
|---|---|
| Hydrogen | 0.2 |
| Oxygen | 0.5 |
| Nitrogen | 7 |
| Methane | 26.8 |
| Carbone dioxide | 65.5 |

# 2. RUMEN MICROBIOME OF CATTLE AND GOATS

## 2.1 Rumen microbiome community and functions

Ruminants' digestive tract holds a complex microbial community, which comprise several types of microorganisms (Peng *et al.,* 2015). The rumen is inhabited by bacteria, protozoa, archaea, fungi (Mizrahi *et al.,* 2021) and also viruses (bacteriophages) have been detected in rumen bacteria (Gilbert *et al.,* 2017).

These microbes are anaerobic or facultatively anaerobic, since the rumen functions under strict anaerobic conditions (Clarke, 1977). Their main role is the production of hydrolytic enzymes which can degrade plant material consumed by the animal and generation of end fermentation products, volatile fatty acids, which represent energy source for host animals. These microorganisms can interact with each other enhancing the ruminant's digestion (Krause *et al.,* 2003).

The study of rumen microbes is crucial since the manipulation of microbial ecology can help enhancing ruminant's production and health. Although, a disturbing of the rumen ecosystem can lead to negative consequences affecting ruminant's productivity and health (Choudhury *et al.,* 2015).

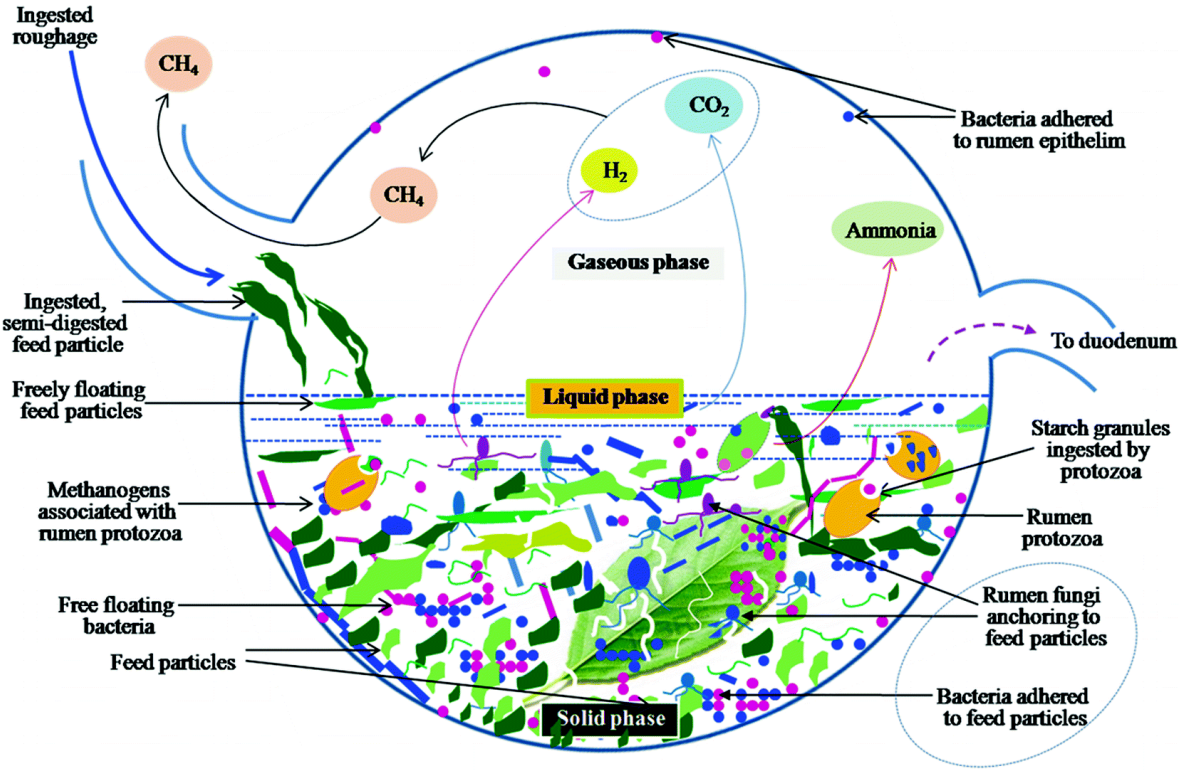



**Figure 2.1-1: Scheme of rumen ecosystem (Singh *et al.*, 2019)**

### 2.1.1 Bacteria

Bacteria are the most common microorganisms present in the ruminants' gut, with more than 200 species and amount of $10^{10}$ - $10^{11}$ cells/ml (McSweeney and Mackie, 2012). Their main role is to digest plant fibrous material by hydrolytic enzymes. Together with anaerobic fungi, rumen bacteria are responsible for the initial degradation of the ingested feed (Matthews *et al.,* 2019). The major fermentation end products are volatile fatty acids (VFAs) such as acetate, butyrate and propionate. Other components are lactic acid, succinic acid, formic acid, ethanol, carbon dioxide and hydrogen. Some of these products can be rapidly used by other bacteria (Ratti *et al.,* 2014).

The capacity of rumen bacteria to adhere to solid surfaces is an important feature for survival in the rumen and for the digestion of feed (Minato *et al.,* 1966). Generally, they are classified into four categories according to their association, as free-floating in the liquid phase, adhered to feed material, attached to rumen epithelium and associated to protozoa and fungi (Choudhury *et al.,* 2015). The adherence to feed material can be loose or tight (Larue *et al.,*

2005). Rumen bacteria have been classified into eleven groups according to their function: acetogens, acid utilizers, cellulolytic, hemicellulolytic, lipolytic, pectinolytic, proteolytic, amylolytic, saccharolytic, tanninolytic and ureolytic (Choudhury *et al.,* 2015) as shown in table 2.1-1. Since cellulose is the principal component of plants' cell wall, cellulolytic bacteria are essential components (Russell *et al.,* 2009). Their capacity to digest cellulose is mostly determined by the type of feed and the bacterial species (Fondevila and Dehority 1996). On the other hand, lactate-degrading bacteria are crucial for the degradation of lactic acid and the prevention of its accumulation, which prevents acidosis (Mackie and Heath, 1979). Pectinolytic bacteria play an important role because pectin constitutes around 10 to 20% of carbohydrates in the animal's feed (Castillo-González *et al.,* 2014). Regarding proteins and structural polysaccharides, they are digested in range between 50 to 70% in the rumen. Proteolysis occurs as a result of proteolytic bacteria producing enzymes leading to protein degradation processes (Cotta and Hespell, 1986). Finally, lipids are also fermented in the rumen by lipolytic bacteria, the unsaturated fatty acids are converted to saturated fatty acids (Jenkins *et al.,* 2008).

Recent studies have revealed that the dominant bacterial phyla are: Firmicutes, Bacteroidetes, Proteobacteria and Spirochaetes (Firkins and Yu, 2006) as shown in table 2.1.1-1. After determining the DNA sequences, it has been shown that the dominant genera were mainly cellulose degraders *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* and hemicellulose degraders *Prevotella*, *Butyrivibrio* and *Pseudobutyrivibrio* (Henderson *et al.,* 2015; Mizrahi *et al.,* 2021). All of these bacteria belong to core genera which were found in almost all ruminants (Jami and Mizrahi, 2012; Henderson *et al.,* 2015).

Bacterial community composition has a direct influence on animal's production. For instance, Firmicutes/Bacteroidetes ratio is correlated to milk fat percentage. An elevation in the amount of Firmicutes results in the decrease in the amount of Bacteroidetes. Milk fat yield increases with the reduction of the quantity of Bacteroidetes compared to Firmicutes (Jami *et al.,* 2014). This suggests that the microbes present in the rumen play a main role in determining the ruminant's physiological parameters.

The constitution and diversity of rumen's bacteria are influenced by several parameters such as diet, animal species, nutritional requirements and resistance to particular end-products potentially harmful to other microorganisms (Castillo-González *et al.,* 2014).

**Table 2.1.1-1: Rumen bacterial community composition on phylum level**

| Study | Ruminant | Country | Phylum | |
|---|---|---|---|---|
| Tajima *et al.,* 1999 | Cows | Japan | Liquid phase | Firmicutes: 52.4%<br>Bacteroidetes: 38.1%<br>Proteobacteria: 4.7%<br>Spirochaetes: 2.4% |
| | | | Solid associated | Firmicutes: 71.4%<br>Bacteroidetes: 26.2%<br>Spirochaetes: 2.4% |
| An *et al.,* 2005 | Cattle | China | Bacteroidetes: 39.59%<br>Firmicutes: 22.34%<br>Proteobacteria: 26.9%<br>Fibrobacteres: 3.55%<br>Spirochaetes: 0.51% | |
| Pandya *et al.,* 2010 | Buffaloes | India | Firmicutes: 26.19%<br>Bacteroidetes: 16.66%<br>Spirochaetes: 9.52% | |
| Cunha *et al.,* 2011 | Goats | Brazil | Liquid phase | Firmicutes: 56.3%<br>Bacteroidetes: 37.9%<br>Proteobacteria: 0.5% |
| | | | Solid associated | Firmicutes: 39.8%<br>Bacteroidetes: 38.8% |
| Peng *et al.,* 2015 | Bovines | China | Bacteroidetes: 57%<br>Firmicutes: 35%<br>Proteobacteria: 3.9%<br>Spirochaetes: 1.6% | |
| Noel *et al.,* 2017 | Dairy cows | New Zealand | Firmicutes: 82.1%<br>Bacteroidetes: 11.8% | |
| Zhu *et al.,* 2017 | Dairy cows | Denmark | Prepartum | Firmicutes: 57%<br>Bacteroidetes: 22%<br>Proteobacteria: 7% |
| | | | Postpartum | Firmicutes: 35%<br>Bacteroidetes: 18%<br>Proteobacteria: 32% |

### 2.1.2 Protozoa

Rumen protozoa are the second most common microorganisms present in the ruminants' gut (Patel and Ambalam, 2018). They represent around 50% of the viable microbes. Most of them are ciliates and little are flagellates. They are unicellular, motile and do not attach to plant fragments. Their size can vary between 20 and 200 μm. Around 250 species of ciliate have been identified in the different ruminants. They are present in rumen fluid at a density of $10^4$ - $10^6$ cells/ml (Choudhury *et al.,* 2015).

Ciliate protozoa are divided into two groups based on their morphological aspects:

1. Holotrich protozoa, characterized by 15 genera including mainly *Dasytricha*, *Charonina*, *Isotricha* and *Buetschlia.*
2. Entodiniomorphid protozoa, characterized by several genera including *Diplodinium*, *Metadinium*, *Eudiplodinium*, *Eremoplastron*, *Epidinium*, *Elytroplastron*, *Entodinium*, *Ostracodinium.*

Based on their substrate preferences, they can be defined as lignocellulose hydrolysers, starch degraders and soluble sugar utilizers (Patel and Ambalam, 2018). They ingest feedstuff, degrade proteins, carbohydrates and fat. They play some role in the digestion of fibers and are important protein degraders (Williams and Coleman, 1992).

Regarding their enzymatic profile, holotrich protozoa have invertase, polygalacturonase, amylase and pectin esterase, therefore, they can digest pectin, sugars and starch. They can also secrete cellulase and hemicellulase, but the amount is minor relatively to the amount secrete by entodiniomorphid protozoa (Williams and Coleman, 1992).

Concerning end products, rumen protozoa generate amino acids, ammonia, butyrate, acetate and $H_2$ (Choudhury *et al.,* 2015). In the matter of methane, protozoa don't generate methane directly, but due to the production of hydrogen they stimulate the growth of methanogenic microorganisms and thus the production of $CH_4$ increases (Patel and Ambalam, 2018). Methanogenic microorganisms can bind to the surface of protozoa to access $H_2$.

Animal's diet affects protozoa colonization. In high grain diet, the *Entodinium* genus is dominant and quickly digests starch (McSweeney and Mackie, 2012). On the other hand, low roughage diets minimize fiber retention, which lowers the number of protozoa in the rumen (Choudhury *et al.,* 2015).

### 2.1.3 Methanogens

Methanogenic archaea are the oldest microorganisms emerged on earth (Singh and Sirohi, 2012). They have a wide range of different types of metabolisms allowing them to thrive in different environmental conditions. They represent less than 4% of the rumen viable biomass, around $10^6$ - $10^8$ cells/ml and they are strictly anaerobic (Matthews *et al.,* 2019).

Methanogenic archaea produce methane by three major pathways:

- Hydrogenotrophic pathway: Hydrogen and carbon dioxide generated by protozoa, fungi and bacteria are converted to methane (Martin *et al.,* 2010). Formate, which may be utilized by the most common ruminal methanogenic archaea is part of the hydrogenotrophic category (Janssen and Kirs, 2008).
- Aceticlastic pathway: Acetate is split and the carbonyl group oxidized to carbon dioxide while the methyl group is reduced to methane (Ferry, 2011; Lyu *et al.,* 2018).
- Methylotrophic pathway: Generally, methyl groups are considered as methanogenesis substrates, they are mainly found in methanol and methylamine. The hydrolysis of methanolic side-groups in plant polymers produces methanol, on the other hand, methylamines originate from choline and glycine betaine (Tapio *et al.,* 2017; Lyu *et al.,* 2018).

The most prevalent hydrogenotrophic methanogens are the genera *of Methanobrevibacter*, which is divided into two groups:

- SGMT clade: *Methanobrevibacter smithii, Methanobrevibacter gottschalkii, Methanobrevibacter Millerae* and *Methanobrevibacter thaueri.*
- RO clade: *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae.*

Other genera include: *Methanosphaera, Methanimicrococcus* and *Methanobacterium*. Finally, methylotrophic methanogens are *Methanosphaera*, *Methanomassiliicoccaceae* and *Methanosarcinales*, they are the less abundant (Tapio *et al.,* 2017).

Around 90% of methanogenic archaea in rumen correspond to the following genera: *Methanobrevibacter* (63.2%), *Methanomicrobium* (7.7%), *Methanosphaera* (9.8%), *Thermoplasma* (7.4%) and *Methanobacterium* (1.2%) (Jassen and Kirs, 2008).

The figure 2.1.3-1 represents methane production in ruminants.

Methanogenic archaea are located at the base of the trophic chain (Morgavi *et al.,* 2011), because they use the $H_2$ and $CO_2$ generated by the bacteria, anaerobic fungi and protozoa as substrate for methane generation and ATP synthesis (Albers *et al.,* 2007). They are the only microbes in rumen which produce $CH_4$ (Matthews *et al.,* 2019).
Several studies have confirmed that there is no correlation between the abundance of methanogenic archaea and methane emission (Danielsson *et al.,* 2012; Morgavi *et al.,* 2011; Zhou *et al.,* 2011), however the composition of methanogenic community can have an influence on methane emission, mainly the relative abundance of *Methanobrevibacter* from SGMT clade is directly correlated to methane emissions (Shi *et al.,* 2014; Danielsson *et al.,* 2012).
The existence of a symbiotic association between rumen methanogens and $H_2$ producers should be noted. It is usually accomplished by a fixation or flocculation. This symbiotic relationship can produce around 37% of rumen methane emission (Finlay *et al.,* 1994).

Methane production reduces rumen hydrogen concentrations and allow the fermentation to be more effective. Nevertheless, the emission of methane can lead to the loss of energy for the ruminant maintenance, development, pregnancy and lactation, it can also lead to air pollution.

Methane production varies according to several factors. First of all, methane emission usually rises with the increase of feed intake. In fact, the digestibility and the amount of feeding are positively correlated to the rate of $CH_4$ production (Kirchgessner *et al.,* 1991; Shibata *et al.,* 1993). Nevertheless, methane production depends as well on the diet's composition and the quality of forage. For instance, $CH_4$ emission decreases when the protein ratio in the diet

increases, on the contrary, it increases when the fiber ratio in the diet increases (Shibata *et al.,* 1992; Sekine *et al.,* 1986). Environmental temperature can affect the production of methane. The emission of $CH_4$ decreases under high temperature because the feed intake of animals is reduced. However, when temperatures exceeded 26°C, $CH_4$ production per dry matter intake increased by about 10% (Kurihara *et al.,* 1995).

The most effective way to decrease methane emission from ruminants is the improvement of animals' nutrition and husbandry, as well as the use of genetic selection (Shibata and Terada, 2010). Feeding high protein / low fiber diets, especially with greater concentrate and adding unsaturated fatty acids can lower $CH_4$ generation (Holter and Young 1992, Czerkawski *et al.,* 1966). Also, the use of ionophore and some chemicals such as lasalocid, monensin and salinomycin may reduce $CH_4$ emission levels (Sauer *et al.,* 1998), however an EU-wide ban on the use of antibiotics as growth promoters in animal feed entered into effect on January 1, 2006 and antibiotics and growth promoter mention above are forbidden as feed additives to help fatten livestock. Monensin is however still used in Africa and Asia in animal production to promote animal growth and health. In Europe some other substances, e.g. bacteriocin nisin, are investigated in *in vitro* experiments using rumen microbiota showing suppression of amino acid deamination and methanogenesis without negative impact on DM digestibility and VFA production (Sar *et al.,* 2005; Shen *et al.,* 2016). Also, the genetic and genomic selection is now on the rise, because it has been described that $CH_4$ emission is a repeatable and heritable trait (Pickering *et al.,* 2015, Wallace *et al.,* 2019).

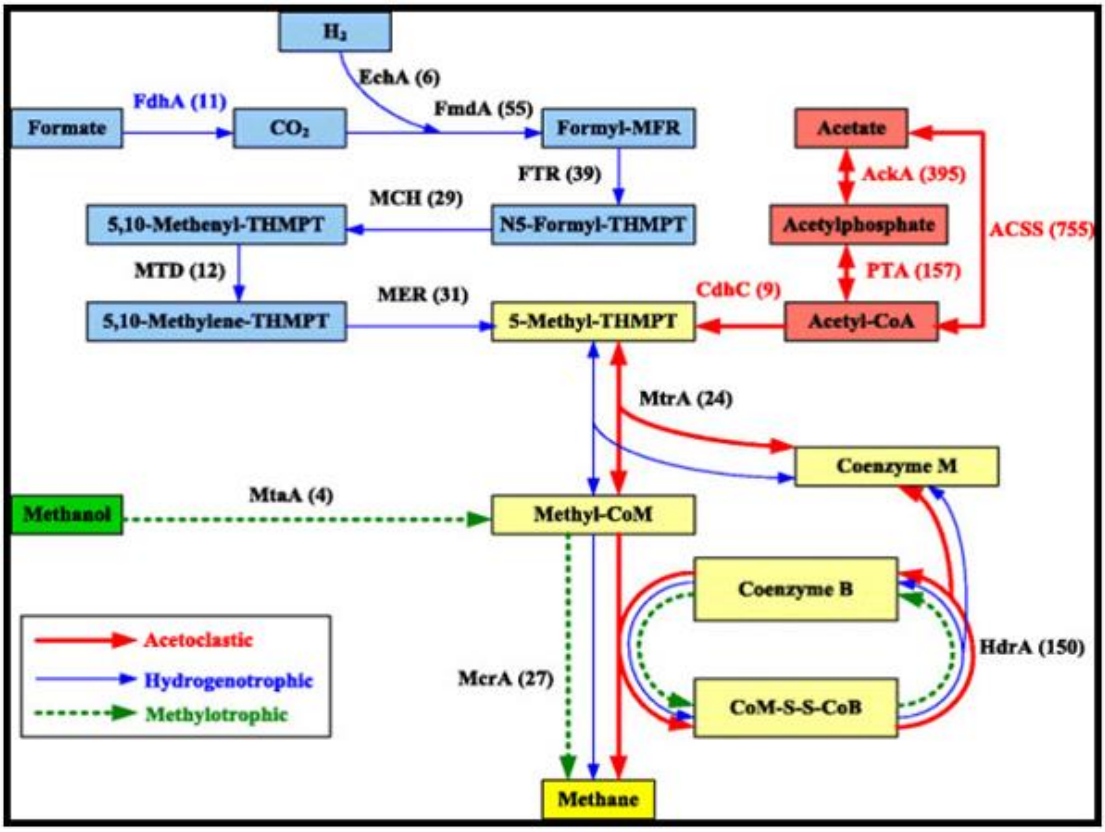



**Figure 2.1.3-1: Methane production in the rumen**

### 2.1.4 Anaerobic Fungi

Anaerobic fungi present in the rumen ($10^3$– $10^6$ zoospores/ml) constitute about 8-12% of viable microorganisms, but this percentage varies depending on the animal's diet (Krause *et al.,* 2013). They have an important role in the digestion of lignocellulosic materials and are considered the most effective degraders of fiber (Choudhury *et al.,* 2015), generating high amounts of cellulases and hemicellulases for the breakdown of cellulose and xylan (Akin and Borneman, 1990). They fall in the class Neocallimastigomycetes that consists of 20 genera generally classified according to their morphology (Hess *et al.,* 2020; Hanafy *et al.,* 2021; Stabel *et al.,* 2020) as follows:

- The monocentric rhizoidal: *Piromyces, Buwchfawromyces, Agriosomyces, Neocallimastix, Pecoramyces, Oontomyces, Feramyces, Tahromyces, Liebetanzomyces, Joblinomyces, Capellomyces, Khoyollomyces, Ghazallomyces*, *Aklioshbomyces* and *Aestipascuomyces.*
- The polycentric rhizoidal: *Orpinomyces*, *Anaeromyces* and *Paucimyces.*
- The bulbous: *Cyllamyces* and *Caecomyces*.

The ruminal digestion process of plant material begins with the anaerobic fungi which are essential for feed utilization effectiveness and ruminant development (Kittelmann *et al.,* 2012).

These fungi are functioning in the rumen, but can be detected in all the different regions of the digestive tract (Davies *et al.,* 1993), they have a great cellulolytic activity and play crucial role in the digestion of lignified plant materials contributing to the metabolism of the ruminant (Akin and Borneman, 1990).

Rumen fungi can affect the methane production because they generate $H_2$, due to the possessing of hydrogenosomes instead of mitochondria. This $H_2$ can be utilized by methanogenic Archaea resulting in methane production (Hess *et al.,* 2020). The contribution of anaerobic fungi to methane production is difficult to evaluate. The greatest part of anaerobic fungi is retained in the solid phase of the rumen, while in the liquid phase some fungi may be present, because they may have bounded to some particles present there (Matthews *et al.,* 2019). The anaerobic fungi synthesize a variety of hydrolytic enzymes, including cellulases, xylanases, mannases, esterases, glucosidases and glucanases, which effectively hydrolyse plant biomass consisting mainly of cellulose and hemicellulose. Some of hydrolytic enzymes produced by gut fungi act individually and are free in solution, whereas others are constituents of large (hemi)cellulolytic multienzyme complexes called cellulosomes. The Neocallimastigomycetes is the only known member of the Fungi possessing cellulosomes and this can explain their cellulolytic superiority over aerobic cellulolytic fungi. These exocellular enzyme complexes are extremely active and can degrade both amorphous and crystalline cellulose (Bayer *et al.,* 2004).

### 2.1.5 Bacteriophages

Bacteriophages, usually found at >$10^9$ particles/mL, are only recently detected in the rumen microbial population (Choudhury *et al.,* 2015). Generally, most bacteriophages have a nucleic acid genome enclosed in protein capsid. Their morphologic shape consists of a head, small collar and long tail (Figure 2.1.5-1). Bacteriophages reproduce via lytic reproduction, infect bacteria and inject the viral genome into bacterial cells to produce new bacteriophages (Guttman *et al.,* 2005), which can result in the death of infected microbes. This effect is negative, but on the other hand, by breaking down bacterial cells, the protein becomes available to the ruminant as a supply of amino acids. Bacteriophages are specific for each bacteria and theoretically could be used to remove unwanted bacteria and methanogens form the ruminant (Klieve *et al.,* 1999).

Despite the lack of knowledge, the high abundances of bacteriophages indicate possible important role in the balance of the rumen microbial system. Viruses may be a driving element for the progress of different microbial systems, helping in horizontal gene transfer (Berg Miller *et al.,* 2012). For instance, the importance of viruses was shown in the transmission of glycoside hydrolase from rumen bacteria to rumen fungi (Garcia-Vallvé *et al.,* 2000). It was also shown in the transfer of gene encoding for plant digestion from rumen bacteria to rumen protozoa (Ricard *et al.,* 2006).

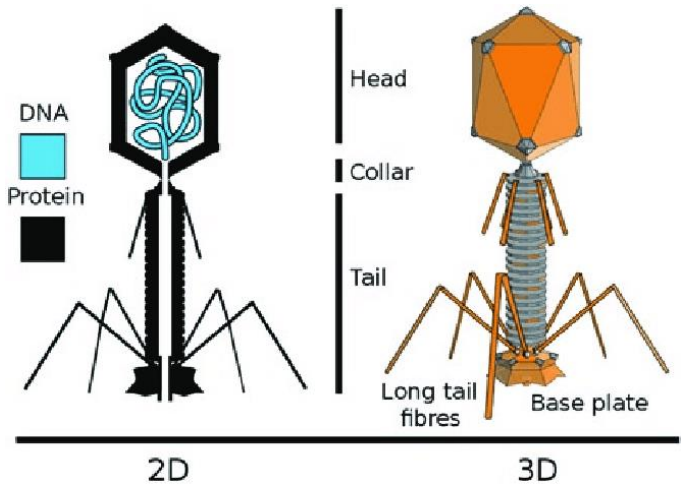



**Figure 2.1.5-1: Morphological structures of bacteriophages (El-Gazzar and Enan, 2020)**

**Table 2.1-1: Rumen microorganisms and functions (Choudhury *et al.*, 2015)**

| Microbial types | Important genera and species |
|---|---|
| **Bacteria** | |
| Acetogens | *Acetitomaculum ruminis, Eubacterium limosum* |
| Acid utilizers | *Megasphaera elsdeni, Wolinella succinogenes, Veillonella gazogene, Micrococcus lactolytica, Oxalobacter formigenes, Desulfovibrio desulfuricans, Desulfotomaculum ruminis, Succiniclasticum ruminis* |
| Cellulolytic | *Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium cellobioparum, Clostridium longisporum, Clostridium lochheadii, Eubacterium cellulosolvens* |
| Hemicellulolytic | *Prevotella ruminicola, Eubacterium xylanophilum, Eubacterium uniformis* |
| Lipolytic | *Anaerovibrio lipolytica* |
| Pectinolytic | *Treponema saccharophilum, Lachnospira multiparus* |
| Proteolytic | *Prevotella ruminicola, Ruminobacter amylophilus, Clostridium bifermentans* |
| Amylolytic | *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola* |
| Saccharolytic | *Succinivibrio dextrinosolvens, Succnivibrio amylolytica, Selenomonas ruminantium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus helveticus, Bifidobacterium globosum, Bifidobacterium longum, Bifidobacterium thermophilum, Bifidobacterium ruminale, Bifidobacterium ruminantium* |
| Tanninolytic | *Streptococcus caprinus, Eubacterium oxidoreducens* |
| Ureolytic | *Megasphaera elsdenii* |
| **Bacteriophages** | *Methanobacterium* phage Ψ M1, *Methanobacterium* phage Ψ M10, *Methanobacterium* phage Ψ M100, *Methanothermobacter* phage Ψ M100, *Methanobacterium* phage ΨM2 |

| | |
|---|---|
| **Fungi** | *Piromyces, Buwchfawromyces, Agriosomyces, Neocallimastix, Pecoramyces, Oontomyces, Feramyces, Tahromyces, Liebetanzomyces, Joblinomyces, Capellomyces, Khoyollomyces, Ghazallomyces*, *Aklioshbomyces* and *Aestipascuomyces. Orpinomyces*, *Anaeromyces* and *Paucimyces. Cyllamyces Caecomyces*. |
| **Methanogens** | *Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter ruminantium, Methanobrevibacter smithii, Methanomicrobium mobile, Methanosarcina barkeri, Methanoculleus olentangyi* |
| **Protozoa** | *Entodinium bovis, Entodinium bubalum, Entodinium bursa, Entodinium caudatum, Entodinium chatterjeei, Entodinium parvum, Entodinium longinucleatum, Entodinium dubardi, Entodinium exiguum, Epidinium caudatum, Isotricha prostoma, Isotricha intestinalis, Dasytricha ruminantium, Diplodinium dendatum, Diplodinium indicum, Oligoisotricha bubali, Polyplastron multivesiculatum, Eremoplastron asiaticus, Eremoplastron bubalus* |

## 2.2 Interactions in the rumen

The rumen is a complex ecosystem, this complexity is expressed by several interactions among microorganisms and between those microorganisms and the host. Ruminants are unable to degrade plant material by themselves, thus, they require the support of microbes. The animals rely on these microbes as they degrade the feed providing them energy and nutrients. On the other hand, microbes use plant material ingested by the animal to extract energy and nutrients for themselves, and the rumen thus, represents the most advanced symbiotic relationship between microbes and host (Choudhury *et al.,* 2015). Studies suggest the existence of a two-way communication between the host and the microbial community, implying that the host's endocrine condition might have a direct influence on the rumen microbial community (Sperandio *et al.,* 2003; Lyte and Freestone, 2009).
Concerning microbial populations, substantial interactions among microbes exist both inside and between niches. The microbial competition is likely to happen, since various species seek for the same substrate, such as cellulose. This can result also in a negative interaction between cellulolytic species (Mosoni *et al.,* 1997), however mutual interactions between microorganisms are prevalent and essential for fermentation processes in the rumen.

A large amount of microbes cooperates to catabolize substrates and to generate fermentative end-products in the rumen. Some microorganisms break down forage into simple sugars, transport and ferment them producing branched-chain fatty acids, cofactors and vitamins used by other bacteria which are in charge of digesting feed material (Allison *et al.,* 1962). This bacterial cross-feeding or fermentative end-product utilization increases energy use efficiency within microbial niches and promotes the host animal's general health (Choudhury *et al.,* 2015).

An example of bacterial cross-feeding involves *Ruminococcus albus*, a cellulolytic bacterium, which requires the presence of phenyl propanoic acid (PPA) to degrade cellulose, the absence of PPA prevent this species from adhering to cellulose. Conveniently some rumen microorganisms generate phenyl propanoic acid from the fermentation of phenolics, allowing *Ruminococcus albus* to digest cellulose (Stack and Hungate, 1984).

Syntrophic associations also include the process of $H_2$ transfer. In fact, the rise of rumen $H_2$ levels leads to various negative effects including a drop in rumen pH and an inhibition of biodegradation caused by the unfavorable balance (Ragsdale and Pierce, 2008). However, the relationship between $H_2$ producing microbes and $H_2$ utilizers restores the normal rumen balance. Moreover, in the absence of methanogens, *Ruminococcus albus*, a cellulolytic bacterial species, generates acetate, $H_2$, $CO_2$ and ethanol, while in the presence of methanogens, it generates greater levels of acetate and much less ethanol (Iannotti *et al.,* 1973). The connection results in enhanced growth and improved cellulose breakdown because acetate production leads to ATP generation. While an elevated level of methane production in the rumen is harmful for the environment, a specific level of $CH_4$ generation is required to eliminate some end-product accumulation, enabling thus the rumen fermentation to progress (Choudhury *et al.,* 2015).

The study of microbial interactions is therefore crucial for the improvement of animal production through the manipulation of the nutritional composition of the ingested feed and the alteration of microbial populations (Choudhury *et al.,* 2015).

## 2.3 Maturation of rumen microbiota

The colonization of microbes occurs in the rumen shortly after birth and changes dramatically over the first several months until weaning (Taschuk and Griebel, 2012). First of all, the rumen is not functional during the early days of life, when the animals are breastfed. The ingested milk skips the rumen due to a reflex closure of the esophageal groove (Van Soest, 1994). The size of the rumen is significantly lower compared to the mature rumen (Krishnamoorthy *et al.,* 2011) and the wall villi which are implicated in the absorption of nutrients are not established yet (Van Soest, 1994). The understanding of rumen microbial maturation from birth to weaning is crucial for ruminants to ensure that rumen microorganisms are capable of digesting feedstuffs after weaning (Yeoman *et al.,* 2018).

Some studies have shown that shortly after birth, aerobic and facultative anaerobic microbes colonize the rumen, but these species gradually decrease with time and are substituted by anaerobic microbes (Fonty *et al.,* 1987; Minato *et al.,* 1992). However, some species such as cellulose degrading bacteria begin to develop after birth before the introduction of plant-based diet (Fonty *et al.,* 1987; Minato *et al.,* 1992).

Concerning the modifications at the phylum level, Firmicutes were shown to be predominant at birth while the abundance of Bacteroidetes was low, but after a really short time, the level of Bacteroidetes increased to become the predominant bacterial population (Zhang *et al.,* 2019).

At the genus level, an alteration in the composition of Bacteroidetes phylum was observed. Generally, the *Prevotella* genus dominates this phylum in many ruminants (Stevenson and Weimer, 2007; Jami and Mizrahi, 2012). Nevertheless, during the early phase of growth, *Bacteroides* is shown to be the predominant genus among Bacteroidetes, but it is soon surpassed by *Prevotella* during the first two months. This major modification in the bacterial composition coincides with the animal's exposure to plant feed (Li *et al.,* 2012; Rey *et al.,* 2014). The changes during maturation of rumen microbiome involve also rumen anaerobic fungi (Zhang *et al.,* 2019), protozoa and methanogens (Skillman *et al.,* 2004; Friedman *et al.,* 2017; Thauer *et al.,* 2008; Poulsen *et al.,* 2013).

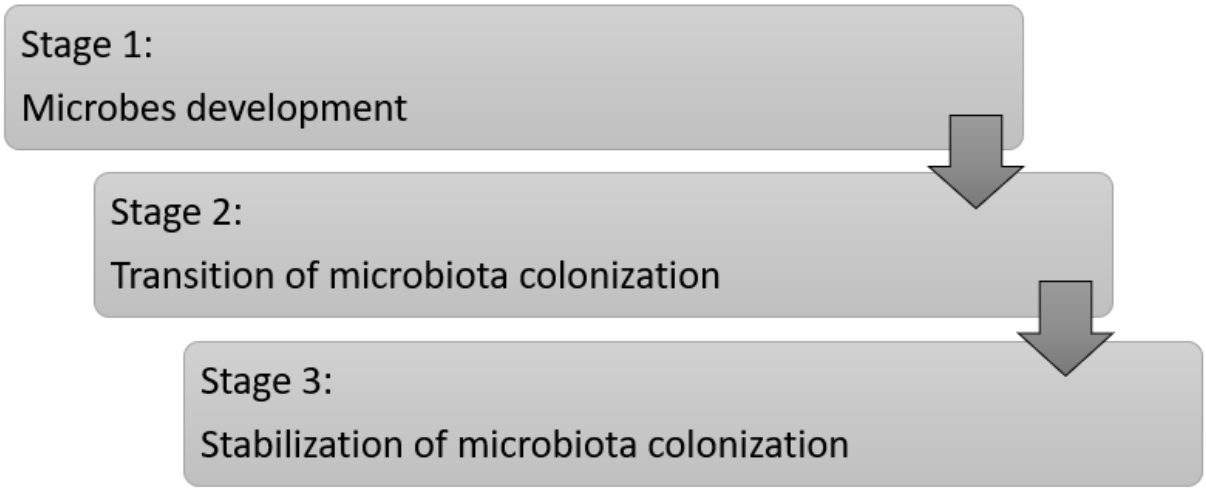



**Figure 2.3-1: Stages of microbial colonization**

## 2.4 Factors affecting rumen microbial composition and diversity

Rumen microbial composition can be influenced by several factors such as diet, ruminant species, host's genotype and physiological status (Zhu, 2016).

### 2.4.1 Host's species

The influence of host on rumen microbial population is linked to physiological and behavioral adaptations of the animal to the environmental factors (Hofmann, 1989). Ruminants living in high-altitude areas such as Tibetan sheep generate less methane and more VFA. In contrary, ruminants living in low-altitudes areas, such as cattle, are observed to have high methane emission and low VFA production. Microbial community composition and diversity differs significantly between these ruminants (Zhang *et al.,* 2016).

However, a large group of microbes is shown to be similar among ruminants. For instance, the dominant bacterial genera present in several ruminant species are *Prevotella*, *Ruminococcus*, *Butyrivibrio*, *Unclassified Lachnospiraceae*, *Bacteroidales*, *Clostridiales* and *Ruminococcaceae*. These bacteria might be termed as "core bacterial microbiome". Yet, this does not preclude the existence of variations in the microbiota of different ruminant species. *Fibrobacter* is noticed to be present in significantly higher levels in cattle compared to sheep and goats (Henderson *et al.,* 2015). This is consistent with another study reporting a greater amount of *Fibrobacter*, *Succiniclasticum* and *Ruminococcus* in the rumen fluid of dairy cows compared with the rumen of dairy goats (Zhang *et al.,* 2018). In addition, *Unclassified Veillonellaceae* and *Lactobacillus* are found to be higher in rumen's goats compared to cattle (Henderson *et al.,* 2015).

Concerning methanogenic archaea, relative abundances are shown to be similar between different ruminants. The two major groups are *Methanobrevibacter ruminantium* and *Methanobrevibacter gottschalkii* clades; they are found nearly in all ruminants (Henderson *et al.,* 2015).

Regarding protozoa, the variability of relative abundances is shown to be more significant than that of archaea and bacteria. Generally, the rumen protozoal population has a significant

host individuality (Weimer, 2015). The dominant genera present in almost all ruminant species are *Epidinium* and *Entodinium*. However, *Enoploplastron* and *Ophryoscolex* have a larger host distribution, they are primarily found in sheep and cattle, respectively (Henderson *et al.,* 2015).

Finally, ruminant species has less impact than diet on the variation in microbiota population composition and diversity (Henderson *et al.,* 2015).

### 2.4.2 Host's genotype

Host's genotype can also influence rumen microbes. A study comparing rumen microbial community and ruminant's digestibility in different breeds of cows revealed that e.g. the Jersey breed have a higher abundance of *Ruminococcus flavefaciens* compared to the other breeds, as well as a different microbial composition and a greater feed digestibility (Beecher, *et al.,* 2014).

Several studies suggest that some rumen microbial characteristics are heritable and can be affected by the animal's genetics. This shows the potential of genetic selection and breeding to obtain an efficient rumen microbiome in order to optimize ruminant's productivity and decrease methane emission (Li *et al.,* 2019; Difford *et al.,* 2018; Wallace *et al.,* 2019).

### 2.4.3 Host's Diet

The type of diet was found to have the decisive influence on microbial composition (Henderson *et al.,* 2015; Matthews *et al.,* 2019). Concerning bacterial community, Gram-negative bacteria are predominant when animals are served a high forage diet (Oetzel, 2003) and a rise in the levels of *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogene* is observed, as well as the levels of VFA and $H_2$ produced (Choudhury *et al.,* 2015). It induces a positive outcome because *Ruminococcus albus* and *Fibrobacter succinogene* are the best cellulose degraders. They have an increased capacity to hydrolyze cellulose compared to the other cellulolytic bacteria. This great ability is due to the

possession of numerous genes encrypting enzymes implied in fiber digestion and possessing cellulosomes (Koike and Kobayashi, 2009).

However, when the animals are served a high grain diet, an alteration in the bacterial composition is detected, as well as a reduction in community richness and diversity (Zhang *et al.,* 2019). In fact, at the phylum level an increase in Firmicutes and a decrease in Bacteroidetes has been noted (Mao *et al.,* 2016; Hua *et al.,* 2017). On the contrary, in goats a rise in Bacteroidetes level and a reduction in Firmicutes levels has been noted (Fliegerova *et al.,* 2021). At the genus level, a reduction of *Fibrobacter* has been observed (Zhang *et al.,* 2017), whereas an increase in gram positive bacteria such as *Enterococcus* and *Lactobacillus* has been detected (Oetzel, 2003; Huo *et al.,* 2014). Nevertheless, the abundance of certain species remains greatly variable, a decrease in the levels of *Prevotella* and *Butyrivibrio* has been detected in animals fed with a high grain diet (Metzler-Zebeli *et al.,* 2013; Grilli *et al.,* 2016), however other studies have observed a rise in the levels of *Prevotella* (Wetzels *et al.,* 2015, Fliegerova *et al.,* 2021) and a rise in the levels of *Butyrivibrio fibrisolvens* (Liu *et al.,* 2013) in animals fed a high grain diet.

The amylolytic bacterium, *Streptococcus bovis*, is generally present in low levels in ruminants, which are gradually adapted to a high grain diet. Yet, the level of this bacteria can increase due to a sudden shift to a high grain diet. *Streptococcus bovis* has an optimum growth in an acid environment. Its increase, due to the high grain diet, cause acidosis (Choudhury *et al.,* 2015), it can also lead to the rise of glucose levels and the loss of protozoa because the pH becomes low (Matthews *et al.,* 2019). In addition, *Streptococcus bovis* stimulates the production of lactic acid, which can result in ruminal acidosis. Ruminal acidosis is well known metabolic disorder of digestive origin, usually categorized in acute or sub-acute forms (Kleen *et al.,* 2003, González *et al.,* 2012), induced by consumption of readily fermentable carbohydrates, especially starch and occurring often as consequence of abrupt transition to a high-grain diet from a predominantly forage diet. In acute acidosis, pH reaches low levels (<5) due to accumulation of lactic acid. In sub-acute acidosis pH drops below 5.6 due to the accumulation of volatile fatty acids (VFA). Even if lactic acid is produced, lactate-fermenting bacteria (e.g. *Megasphaera elsdenii*) convert it into propionic

acid, which helps in stabilizing the rumen environment and maintaining the development of the rumen acid-intolerant fiber digesters (Choudhury *et al.,* 2015).

In some nutritional diets, citrus products, as citrus tissue, are commonly used and produce a large proportion of pectin compounds. Such by-products may be utilized as a substitute to extremely fermentable grains, thereby stopping the development of *Streptococcus bovis* and preventing acidosis. In addition, the quality of feed usage for milk output could be also increased (Matthews *et al.,* 2019). On the other hand, the consumption of straw treated with ammonia stimulates the development of *Eubacterium ruminantium* (Minato *et al.,* 1989), which stimulates the xylanase activity for the digestion of plant fiber, especially hemicellulose degradation (Taguchi *et al.,* 2004).

Concerning protozoa, a diet rich in grains has caused a rise in the level of Entodiniomorphid protozoa, while the number of Holotrich protozoa remains the same. However, at the development of sub-acute ruminal acidosis, the level of rumen protozoa greatly decreased (Metzler-Zebeli *et al.,* 2013; Liu *et al.,* 2013).

Regarding the methanogenic population, *Methanobrevibacter* and *Methanosphaera* are the most common regardless of the diet. However, a high grain diet has resulted in a considerable increase in the level of *Unclassified Thermoplasmatales* (Fliegerova *et al.,* 2021).

Finally, anaerobic fungi community is also influenced by the animal's diet. An increased level of Neocallimastigomycota has been observed in goats fed high grain diet (Fliegerova *et al.,* 2021). However, another study done on cows showed opposite results (Ishaq *et al.,* 2017). Furthermore, a decrease in the diversity of Neocallimastigomycota has been observed in cattle fed high grain diet (Denman *et al.,* 2008).

To summarize, the contradicting results shown between studies can be induced by several factors, as the microbiome vary according to animal species and host's genotype (Zhu, 2016).

## 2.5 Techniques for analysis of the rumen bacterial community

### 2.5.1 Culture-dependent techniques for bacteria isolation

Culture dependent approaches use a variety of enrichment culture conditions with the aim of stimulating a natural environment for the bacterial population. However, the cultivation of anaerobic microbes is usually difficult, because of the necessity of oxygen elimination, the presence of complex growth requirements and the slow growth of the microorganisms (Matthews *et al.,* 2019). A continuous culture system has been established to mimic the rumen environment in order to enumerate and identify the rumen microorganisms (Rufener *et al.,* 1963). When trying to cultivate microorganisms, the solid phase of the rumen content might cause some problems. Many microbes stick to particle matter and are hard to separate. However, methylcellulose is utilized to accelerate bacterium separation from feed by offering a source of nourishment (Fessenden, 2016). The conventional bacterial identification methods include the study of the shape, the morphology and the Gram staining, as well as the nutritional needs and fermentation end products (Matthews *et al.,* 2019). Roll tubes have been used instead of traditional agar plates to cultivate anaerobic microbes (Hungate, 1969). The agar medium is applied in a thin coat to the inner surface of the tube before being washed with an oxygen free gas. The tube does not require any special incubation device and may be inspected without any anaerobic precautions (Hungate, 1966). Eventually, the most frequently cultured rumen bacteria are species form the Firmicutes phylum and *Lachnospiraceae* family. Bacteriodetes have been accounted for a minor percentage of the strains detected, with only two taxa being represented (Creevey *et al.,* 2014). Although the culture techniques have detected the main bacterial strains which play an important role in rumen fermentation, they do not represent the whole diversity of the rumen microbiome (Matthews *et al.,* 2019).

### 2.5.2 Culture-independent techniques to study bacterial diversity

Culture independent approaches are usually DNA based techniques for microbes' identification. They allow the examination of bacterial population at a molecular level.

Metagenomics use high-throughput sequencing techniques to describe a diversity of microbiome. For bacterial population, 16S rRNA amplicon sequencing is used (Luton *et al.,* 2002). DNA sequencing techniques have radically changed the approach of microbes' identification and have overcome the limitations found in the culture dependent method. Several techniques are available to obtain the desired results, all of them are based on the same main methodology of template preparation and fragment amplification, followed by sequencing (Reuter *et al.,* 2015).

In order to identify bacterial species, 16S rRNA amplicon needs the use of specific primers which can recognize identical regions present in all bacteria, but in the same time, this region has to be different enough to identify bacteria on phylum, class, order, family and genera level ideally on species level. Therefore, it is important to have a reliable phylogenic marker and a vast database of sequences (Chaucheyras-Durand and Ossa, 2014). PCR amplification with specific primers is an important step, since improper PCR conditions, such as incorrect annealing temperatures may lead to a false amplification (Matthews *et al.,* 2019).

Culture independent methods use bioinformatics tools to compare various microbes in a specific ecosystem. They have the ability to identify cultivated as well as unculturable microorganisms. They can thus offer information which will enable the culture of species that have never been cultivated before. The bioinformatical programs frequently used include Humann2, Qiime and RDP (Matthews *et al.,* 2019).

# Objective

The objectives of the present thesis include the identification and comparison of the bacterial community composition of cattle and goats' feces using advanced molecular biology technique. The main purpose of this work is elucidating the influence of animal host species on the diversity and composition of intestinal bacterial ecosystem. Two economically important species of ruminants, dairy cattle and goats, fed the same diet, were included in this study, which aims to contribute in enlarging the knowledge about the factors affecting the microbiome of the ruminants in order to increase livestock productivity and improve animal's health.

# 3. MATERIALS AND METHODS

## 3.1 Animals and diets

The samples for this study were collected from Ceto (Latitude: 46°22'00''; Longitude: 10°21'09''), province of Brescia, in Lombardy, Italy, on 5 February 2021. A total of 17 female goats of a local breed, Bionda dell'Adamello and 16 female cows of the ancient breed, Original "Bruna" (Brown Swiss), were selected for the experiment. All the animals had free access to the pasture from the morning until afternoon (16:30-17:00) and free access to water both out-door and indoor. Hay was administrated indoor, 3 times every day, at 7:00/7:30, 17:00 and 18:00/18:30 for both cows and goats.

All the animals' information is summarized in the tables 3.1-1 and 3.1-2.

**Table 3.1-1: Characteristics of the goats (Bionda dell'Adamello breed) from Lombardy**

| Sample name | Age | Lactating | Pregnant |
|---|---|---|---|
| GN 1 | 4 years | Yes | No |
| GN 4 | 6 years | Yes | No |
| GN 6 | 4 years | Yes | No |
| GN 7 | 3 years | No | Yes |
| GN 14 | 3 years | No | Yes |
| GN 15 | 3 years | No | Yes |
| GN 16 | 4 years | No | Yes |
| GN 17 | 5 years | No | Yes |
| GN 21 | 3 years | No | Yes |
| GN 22 | 7 years | No | Yes |
| GN 23 | 5 years | No | Yes |
| GN 24 | 7 years | No | Yes |
| GN 25 | 2 years | No | Yes |
| GN 27 | 3 years | No | Yes |

| | | | |
|---|---|---|---|
| GN 28 | 7 years | No | Yes |
| GN 29 | 4 years | No | Yes |
| GN 30 | 6 years | No | Yes |

**Table 3.1-2: Characteristics of the cattle (Original Bruna, Brown Swiss) from Lombardy**

| Animal | Age | Lactating | Pregnant |
|---|---|---|---|
| CN 1 | 3 years | Yes | Yes |
| CN 2 | 3 years | Yes | Yes |
| CN 6 | 7 years | Yes | Yes |
| CN 9 | 7 years | Yes | Yes |
| CN 10 | 3 years | Yes | Yes |
| CN 15 | 4 years | No | Yes |
| CN 16 | 5 years | No | Yes |
| CN 17 | 8 years | No | Yes |
| CN 19 | 7 years | No | Yes |
| CN 20 | 7 years | No | Yes |
| CN 21 | 7 years | No | Yes |
| CN 22 | 7 years | No | Yes |
| CN 23 | 7 years | No | Yes |
| CN 24 | 8 years | No | Yes |
| CN 26 | 5 years | No | Yes |
| CN 28 | 9 years | No | Yes |

## 3.2 Collection of samples

The collection of fresh fecal samples was carried out immediately after defecation, the content was placed into a clean bag and transported on ice to the laboratory, where all of the samples were freeze-dried and kept in the fridge until needed for the analysis. The moisture

and dry matter contents of the samples were determined by weighing them before and after lyophilization. The samples were distinguished by letter GN (goats from Lombardy), CN (cattle from Lombardy).

## 3.3 DNA Extraction

The DNA extraction from fecal samples was carried out using the DNeasy® Plant Pro kit with preliminary step, which consisted in sample desintegration using liquid nitrogen, mortar and pestle. The detailed description of DNA isolation procedure is described below:

1. Add the dry faeces into the chilled mortar then disintegrate the sample into powder using a pestle and liquid nitrogen.
2. Weight proper amount of dried sample corresponding to 300 mg of fresh sample into the tubes provided with isolation kit and add 500 µl of Solution CD1 and 50 µl of Solution PS. Vortex briefly to mix.
3. Homogenize sample by vortexing using a Vortex Adapter for Vortex-Genie 2 (Scientific Industries, USA) and vortex at maximum speed for 10 min.
4. Centrifuge the sample in disruption tubes at 13,500 x g for 3:20 min.
5. Transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided with kit).
6. Add 200 µl Solution CD2 and vortex for 5 s.
7. Centrifuge at 13,500 x g for 2:20 min at room temperature. Avoiding the pellet, transfer the supernatant to a clean 1.5 ml microcentrifuge tube (provided).
8. Add 500 µl of Buffer APP and vortex for 5 s.
9. Load 600 µl lysate onto an MB Spin Column. Centrifuge at 12,000 x g for 1:20 min.
10. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB spin column.
11. Place the MB spin column into a clean 2 ml collection tube (provided).

12. Add 650 µl Buffer AW1 to the MB spin column. Centrifuge at 12,000 x g for 1:20 min. Discard the flow-through and place the MB spin column back into the same 2 ml collection tube.
13. Add 650 µl of Buffer AW2 to the MB spin column. Centrifuge at 12,000 x g for 1:20 min. Discard the flow-through and place the MB spin column into the same 2 ml collection tube.
14. Centrifuge at up to 13,500 x g for 3:20 min. Place the MB spin column into a new 1.5 ml elution tube (provided).
15. Add 100 µl of Buffer EB to the centre of the white filter membrane and wait for 5 min.
16. Centrifuge at 12,000 x g for 1:20 min. Discard the MB spin column and collect eluted DNA solution.

The concentration and quality of the nucleic acids were determined by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A) and the DNAs were stored at -20$^{o}$C until required.

## 3.4 PCR Amplification of 16S rRNA bacterial fragment

### 3.4.1 Primers and PCR Program

DNAs isolated from each sample were diluted 10 times in nuclease free $H_2O$ prior to PCR reaction and 2 µl of the diluted DNA solutions were used as template for PCR reaction. The bacterial variable V4-V5 region of 16S rRNA was amplified from each sample using the specific primer pair BactBF (GGATTAGATACCCTGGTAGT) and BactBR (CACGACACGAGCTGACG) according to Fliegerova et al. (2014). The PCR reaction was performed using EliZyme$^{TM}$ HS FAST MIX Red Master Mix (Elisabeth Pharmacon, Czech Republic). The enzyme mix includes buffer, enzyme, dNTPs and magnesium. Each 30 µl PCR mixture contained 15 µl of PCR Master Mix, 1 µl of each primer, 11 µl nuclease free $H_2O$ and 2 µl of template DNA (10-fold diluted). The following PCR assay was performed: 5 minutes of denaturation step at 95°C, 25 cycles consisting of 30 seconds at 95°C, 30 seconds

at 57°C, 30 seconds at 72°C and final elongation step at 72°C for 5 min. PCR reaction was carried on the thermocycler Biometra TAdvanced (Biometra, Germany). Each PCR amplicon was examined by agarose gel electrophoresis (1.5%) to evaluate the size and quality. The length of bacterial fragment 16S rRNA used in this study was approximately 300 bp.

### 3.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to examine the PCR amplicons. Typically, 1.5% agarose gels prepared in 1 X TBE buffer were used. The agarose was melted in the buffer by boiling, ethidium bromide (0.5 µg/ml) was directly incorporated into the gel for staining. The gels were poured onto plastic trays with a plastic comb attached to make sample wells, before setting. The gels were run in cleaver Scientific's horizontal gel electrophoresis with a running buffer of 1x TBE. A volume of 5 µl of 200-1500 kb DNA marker (Top-Bio, Czech Republic) was loaded in the first well as a molecular size marker, then 4 µl of the DNA samples were loaded in the following wells. The gels were run at 110 V for 30 minutes. The DNA was visualized using GelDoc Go Imaging System (Bio-Rad laboratories, USA).

### 3.4.3 Purification and concentration of the PCR products

The purification of the PCR products was done using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, USA). The detailed description of the procedure is described below:

Before starting:

Add 4 volumes of ethanol (> 95%) to one volume of DNA Wash Buffer.

Protocol:

1. Dilute the samples (x 5) with DNA Cleanup Binding Buffer, mix well by pipetting up and down or flicking the tube. Do not vortex.
2. Insert column into collection tube and load sample onto column and close the cap. Spin for 1 minute, then discard flow-through.
3. Re-insert column into collection tube. Add 200 µl of DNA Wash Buffer and spin for 1 minute. Discarding flow-through is optional.
4. Repeat washing step (Step 3).

5. Transfer column to a clean 1.5 ml microcentrifuge tube.
6. Add 20 µl of DNA Elution Buffer to the centre of the column. Wait for 1 minute, then spin for 1 minute to elute DNA.

The concentration and quality of the nucleic acids were determined by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, U.S.A).

## 3.5 Library preparation for high-throughput sequencing (HTS)

Library preparation is a fundamental process, in which the amplified fragment of DNA, 16S rDNA, is modified into a form that matches the sequencing system (Head *et al.,* 2014). In this study Nebnext® Fast DNA Library Prep Set (New England Biolabs, USA) for Ion Torrent™ was used.

### 3.5.1 End Repair

DNA amplicons are not blunt-ended, therefore end repair is required to ensure that each fragment is free of overhangs and includes 5′ phosphate and 3′ hydroxyl groups.

The mixture was set up using the reagents in the order shown in the following scheme:

| **Reagent** | **Volume** |
|---|---|
| NEBNext End Repair Reaction Buffer | 3 µl |
| NEBNext End Repair Enzyme Mix | 1.5 µl |
| Free nuclease $H_2O$ | 18.5 µl |
| DNA amplicons | 7 µl |
| Final volume | 30 µl |

The original reaction is performed in 60 µl, half volume was used.
End repair reaction was carried on the thermocycler Biometra TAdvanced (Biometra, Germany) using the following PCR conditions: 20 minutes at 25°C, followed by 10 minutes at 70°C, hold at 8°C.


**Figure 3.5.1-1: End repair reaction**

### 3.5.2 Adaptor ligation

Short adaptor sequences are attached to both ends of the DNA fragments by blunt-end ligation. These adapters incorporate specific sequences for fragment identification by the sequencer. P1 adaptor and different barcoded adaptors were used for each sample.

The mixture was set up using the reagents in the order shown in the following table:

| **Reagent (for each sample)** | **Volume** |
|---|---|
| T4 DNA Ligase Buffer for Ion Torrent | 5 µl |
| P1 adaptor | 0.5 µl |
| Bst 2.0 WarmStart DNA Polymerase | 0.5 µl |
| T4 DNA Ligase | 2.5 µl |
| Free nuclease $H_2O$ | 10.5 µl |
| Final volume | 19 µl |

A total of 19 µl of the mixture was added into each sample, along with 1 µl of the different diluted barcode adaptors.

Adaptor ligation reaction was carried on the thermocycler Biometra TAdvanced (Biometra, Germany) using the following conditions: 15 minutes at 25 °C, 5 minutes at 65 °C and hold at 8°C.

The formation of reaction products is illustrated below.

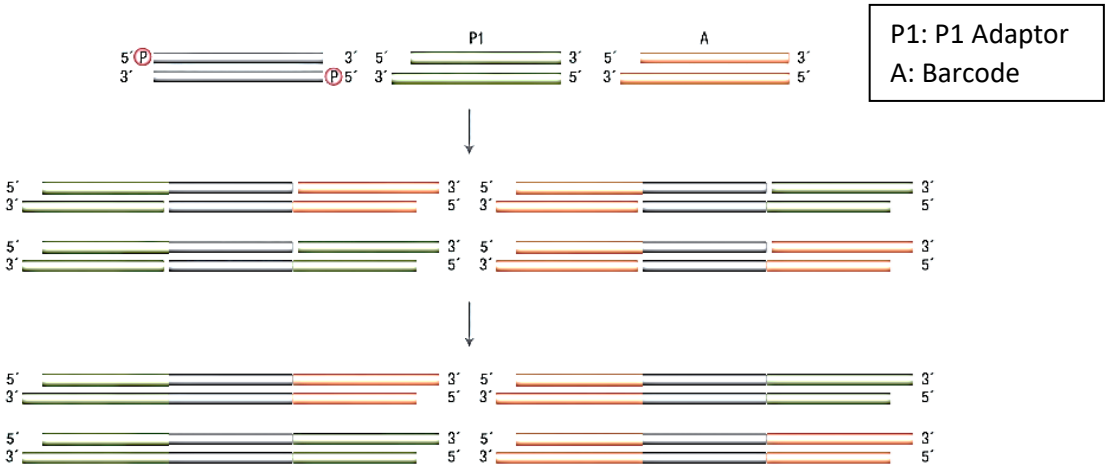



**Figure 3.5.2-1: Adaptor ligation reaction**

### 3.5.3 Clean-up of Adaptor Ligated DNA

DNA clean-up using the magnetic beads is targeted for the elimination of small fragments of DNA, such as primers, dimers and adaptors from DNA library.

The detailed description of the procedure is described below:

1. To each well add 1.2 x volume of Ampure beads AMPure XP (Beckman Coulter, USA) to the product (the total volume is 50 µl so add 60 µl).
2. Mix by pipetting up and down multiple times.
3. Incubate the plate at room temperature for 2- 5 minutes to bind DNA to the beads.
4. Place the plate on a magnet and incubate until the liquid will be clear (about 2 minutes).
5. Remove and discard the supernatant.
6. Keep the plate on the magnet, wash 2 times:
   (a) Add 180 µl of 70% ethanol.
   (b) Wait for 1 minute, then remove and discard the ethanol.
7. After the two washes, try to remove all ethanol without disturbing the beads.
8. Wait until all of the ethanol has evaporated (dry the beads 2 to 3 minutes).
9. Remove the plate from the magnet.

10. Add 50 µl of 10 mM Tris-HCl.
11. Mix by pipetting up and down.
12. Wait for 2 minutes to elute the DNA from the beads.
13. Place the plate on a magnet to separate the beads from the DNA.
14. Wait until the liquid will be clear (2 minutes).
15. Transfer the supernatant (DNA) to a new plate.

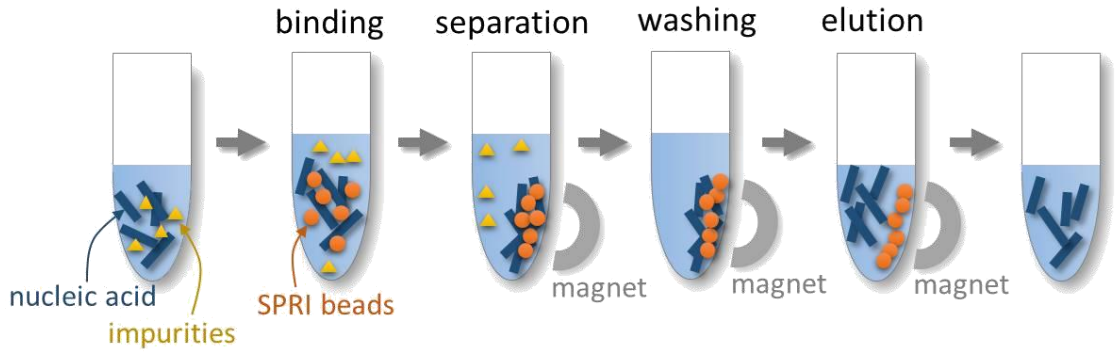



**Figure 3.5.3-1: DNA Purification with Ampure beads**

### 3.5.4 PCR Amplification for high-throughput sequencing

Amplification is carried out with a high-fidelity polymerase NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs, USA) in order to obtain an appropriate library suitable for sequencing.

This step has several purposes:

- Selecting molecules with the appropriate adaptors at both ends.
- Increasing the concentration of library.

The mixture was set up using the reagents in the order shown in the following table:

| **Reagent (for each sample)** | **Volume** |
|---|---|
| NEBlibF primer | 1 µl |

| | |
|---|---|
| NEBlibR primer | 1 µl |
| NEBNext® Ultra™ II Q5® Master Mix | 25 µl |
| Final volume | 27 µl |

A total of 27 µl of the mixture was added into the wells of a new 96 well plate, then 23 µl of the purified samples were added.

Amplification reaction was carried on the thermocycler Biometra TAdvanced (Biometra, Germany) using the following PCR conditions: 30 seconds at 98°C, 7 cycles consisting of 10 seconds at 98°C, 30 seconds at 58°C and 30 seconds at 72°C, then 5 minutes at 72°C and hold at 8°C.

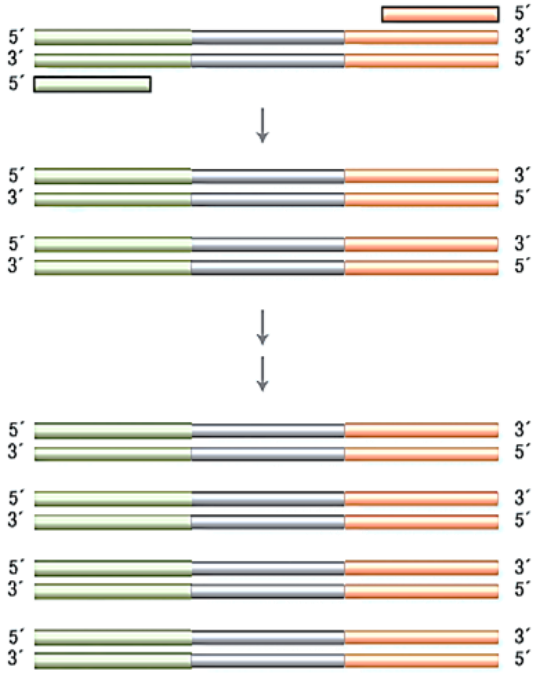



**Figure 3.5.4-1: Amplification reaction**

### 3.5.5 DNA Purification

DNA Libraries were purified using Ampure beads for the elimination of the impurities. The same procedure as the clean-up of adaptor ligated DNA was used.

### 3.5.6 Dilution of DNA libraries

Libraries must be diluted before quantification, in order to have the appropriate concentrations which fall within the range of the assay.

Several dilutions were done:

- Dilution 1:20 (5 µl of DNA library + 95 µl of 10 mM Tris-HCl)
- Dilution 1:1000 (2 µl of DNA from first dilution + 98 µl of 10 mM Tris-HCl)
- Dilution 1:4000 (25 µl of DNA from second dilution + 75 µl 10 mM Tris-HCl)

### 3.5.7 Sizing and analysis of DNA libraries

DNA libraries were analyzed using the 2100 Bioanalyzer instrument (Agilent, USA). The total DNA concentration in the samples must be in range from 100 pg/µl to 10 ng/µl. DNA libraries from the first dilution (1:20) were therefore used.

Agilent High Sensitivity DNA Kit components:

| **Agilent High Sensitivity DNA Chips** | **Agilent High Sensitivity DNA Reagents** |
|---|---|
| 10 High Sensitivity DNA Chips | High Sensitivity DNA Ladder |
| 1 Electrode Cleaner | High Sensitivity DNA Markers 35/10380 bp (4 vials) |
| Syringe Kit | High Sensitivity DNA Dye Concentrate 1 vial |
| 1 Syringe | High Sensitivity DNA Gel Matrix (2 vials) |
| | 2 Spin Filters |

The procedure is described in details below:

Preparing the Gel-Dye Mix

1. Allow High Sensitivity DNA dye concentrate and High Sensitivity DNA gel matrix to equilibrate to room temperature for 30 minutes.
2. Add 15 µl of High Sensitivity DNA dye concentrate to a High Sensitivity DNA gel matrix vial.
3. Vortex solution well and spin down. Transfer to spin filter.

4. Centrifuge at 2240 g ± 20 % for 15 minutes. Protect solution from light. Store at 4°C. Use prepared gel-dye mix within 6 weeks of preparation.

Checking the Chip Priming Station

1. Make sure the syringe is tightly connected to the Chip Priming Station as described in the document of the Syringe Kit.
2. Pull the plunger of the syringe to the 1.0 mL position (plunger pulled back).
3. Place an empty chip in the Chip Priming Station.
4. Close the Chip Priming Station and make sure to lock it by pressing the cover.
5. Press the plunger down until it is locked by the clip as shown in figure 3.5.7-1 (a).
6. Wait for 5 s and press the side of the clip to release the plunger as shown in figure 3.5.7-1 (b).

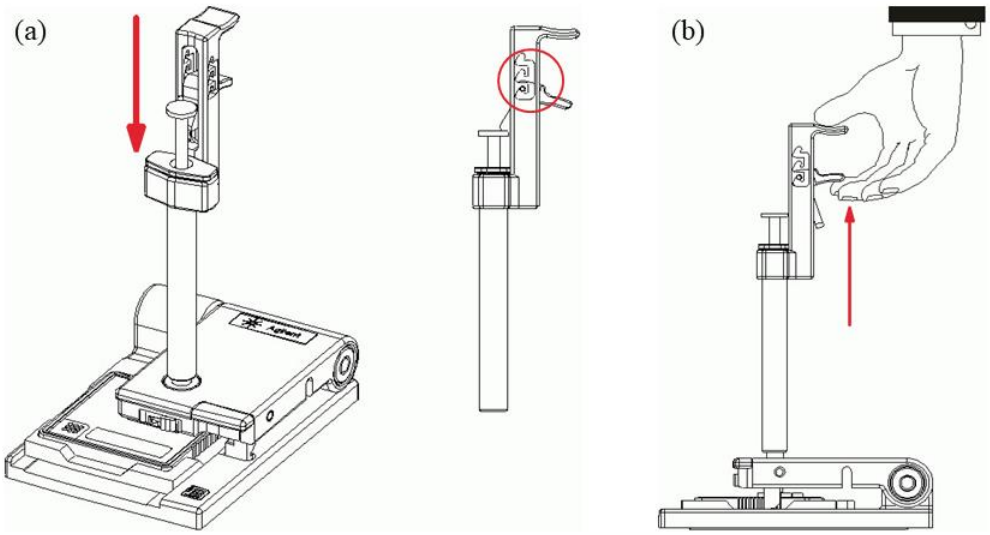



**Figure 3.5.7-1: Checking the Chip Priming Station**

Loading the Gel-Dye Mix

1. Allow the gel-dye mix to equilibrate to room temperature for 30 minutes before use.
2. Put a new High Sensitivity DNA chip on the chip priming station.
3. Pipette 9 µl of gel-dye mix in the well marked

4. Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly 60 seconds then release clip.
7. Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.
8. Open the chip priming station and pipette 9 µl of gel-dye mix in the wells marked 

Loading the Marker

1. Pipette 5 µl of marker in all sample and ladder wells. Do not leave any well empty.

Loading the Ladder and Samples

1. Pipette 1 µl of High Sensitivity DNA ladder in the well marked 
2. In each of the 11 sample wells pipette 1 µl of sample (used wells) or 1 µl of marker (unused wells).
3. Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
4. Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

Results

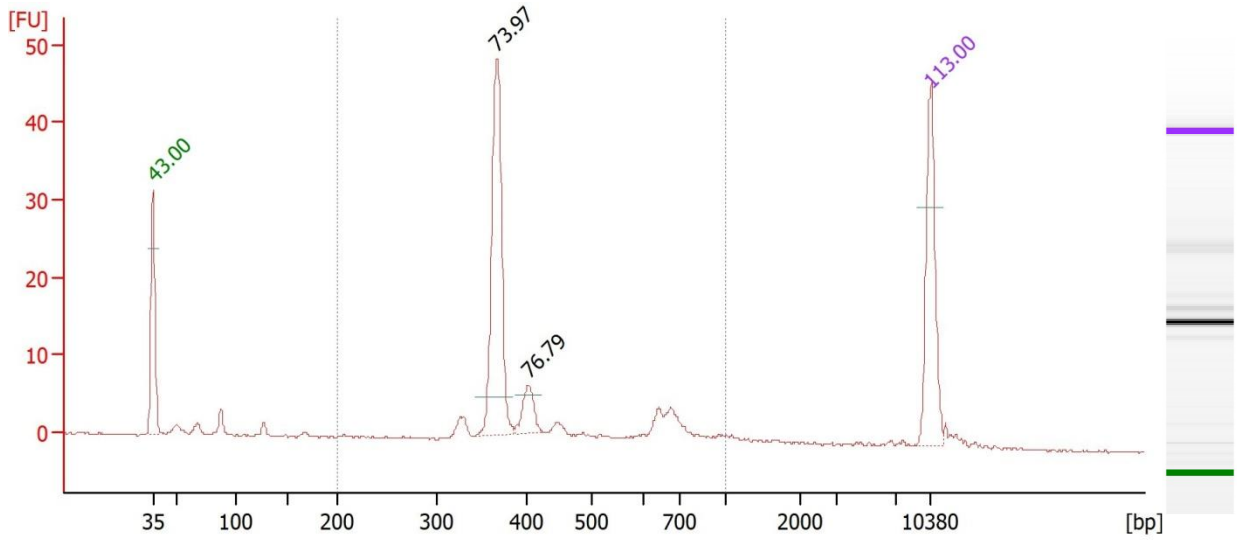



**Figure 3.5.7-2: DNA peaks of a successfully prepared sample**

**Overall Results for the sample:**

Number of peaks found: 2

Noise: 0.2

**Peak table:**

| Peak | Size [bp] | Conc. [pg/µl] | Molarity [pmol/l] | Observations |
|---|---|---|---|---|
| 1 | 35 | 125.00 | 5,411.3 | Lower Marker |
| 2 | 368 | 194.71 | 802.4 | |
| 3 | 404 | 26.06 | 97.8 | |
| 4 | 10,380 | 75.00 | 10.9 | Upper Marker |

The peaks at 43 and 113 seconds are size markers of 35 bp and 10380 bp, respectively. The peak at 73.97 seconds is the sample (368 bp, 194.71 pg/µl).

Major features for a successful High Sensitivity DNA sample run are:

- Sample peak appear between the lower and upper marker peaks.
- Baseline is flat.
- Baseline readings at least 5 fluorescence units.
- Marker readings at least 3 fluorescence units higher than baseline readings.

### 3.5.8 Quantification of DNA libraries

Quantification of the DNA libraries is important for the efficient utilization of the Ion Torrent high-throughput sequencing (HTS) platform. It is a critical step, because an overestimation of the concentrations can lead to an insufficient quantity of DNA-bearing beads causing a lower sequence reads generation and a reduction of the sequencing capacity. On the contrary, an underestimation of the concentrations can lead to multiple DNA templates per bead, which cannot be read (Zheng *et al.,* 2010).

The quantification was done using the KAPA Library Quantification Kit (Roche, USA).

The procedure is described in details below:

| Reagent (for each sample) | Volume |
|---|---|
| KAPA SYBR FAST qPCR Master Mix (2X) | 6 µl |
| Primer Premix (10X) | 1 µl |
| 50X ROX Low | 0.2 µl |
| Total volume | 6.2 µl |

1. Dispense 6.2 µl of the mixture into each well of the plate.
2. Add 4 µl of each dilution of libraries in the wells
3. Add 4 µl of nuclease free $H_2O$ to all negative control wells.
4. Add 4 µl of each DNA Standard into the appropriate wells, working from the most diluted (Standard 6) to the most concentrated (Standard 1).

| | |
|---|---|
| DNA Standard 1 | 83 pM |
| DNA Standard 2 | 83. pM |
| DNA Standard 3 | 0.83 pM |
| DNA Standard 4 | 0.083 pM |
| DNA Standard 5 | 0.0083 pM |
| DNA Standard 6 | 0.00083 pM |

5. Seal the PCR plate and transfer to the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA) to evaluate the sample concentration.
6. Perform qPCR with the following cycling protocol:

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 5 minutes | 1 |
| Denaturation | 95°C | 30 seconds | 35 |
| Annealing/Extension/ Data acquisition | 60°C | 45 seconds | |
| Melt curve analysis | 60-65-95°C | | |

For reliable library concentrations, standard curve should meet these criteria:

- The reaction efficiency should be in the range of 90 – 110%.

- The slope of the standard curve should be between -3.1and -3.6.
- R2 ≥ 0.99.

### 3.5.9 Final dilution of DNA libraries

The final dilution is done to equalize all the concentrations of DNA libraries in order to ensure a similar read distribution for all the samples and a sufficient read depth.

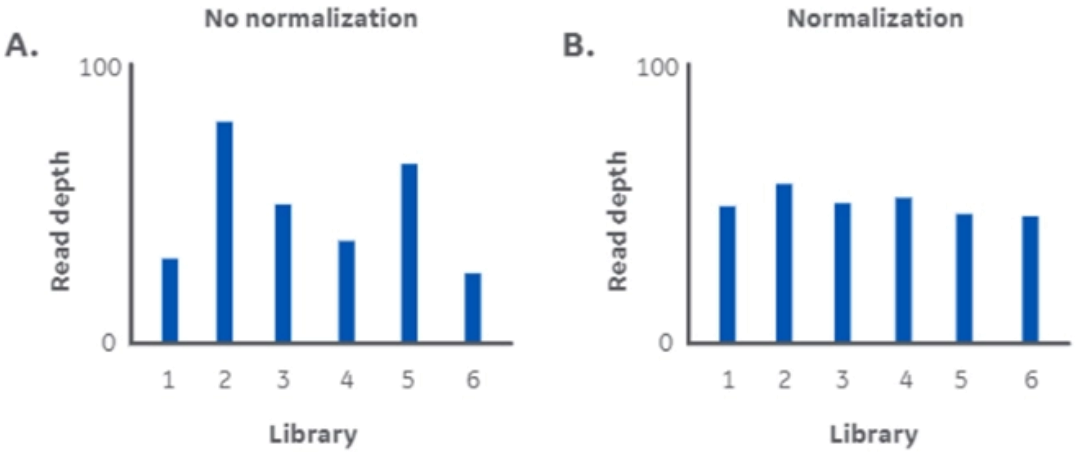



**Figure 3.5.9-1: DNA library normalization**

After performing qPCR, the concentrations for each dilution of every library were calculated. All libraries were diluted to 30 pM, then a volumetric pooling was done; 1 µl of each normalized library was added into a microcentrifuge tube. The content was mixed up and down gently.

## 3.6 Template preparation

The main purpose of template preparation is the creation of multiple copies of the same amplicon in order to have a robust detectable signal during sequencing.

### 3.6.1 Clonal Amplification and Enrichment

Template amplification and enrichment was performed using the Ion OneTouch™ 2 System (Thermo Fisher Scientific, USA), that includes Ion OneTouch™ 2 Instrument and Ion OneTouch™ ES Instrument (Thermo Fisher Scientific, USA).

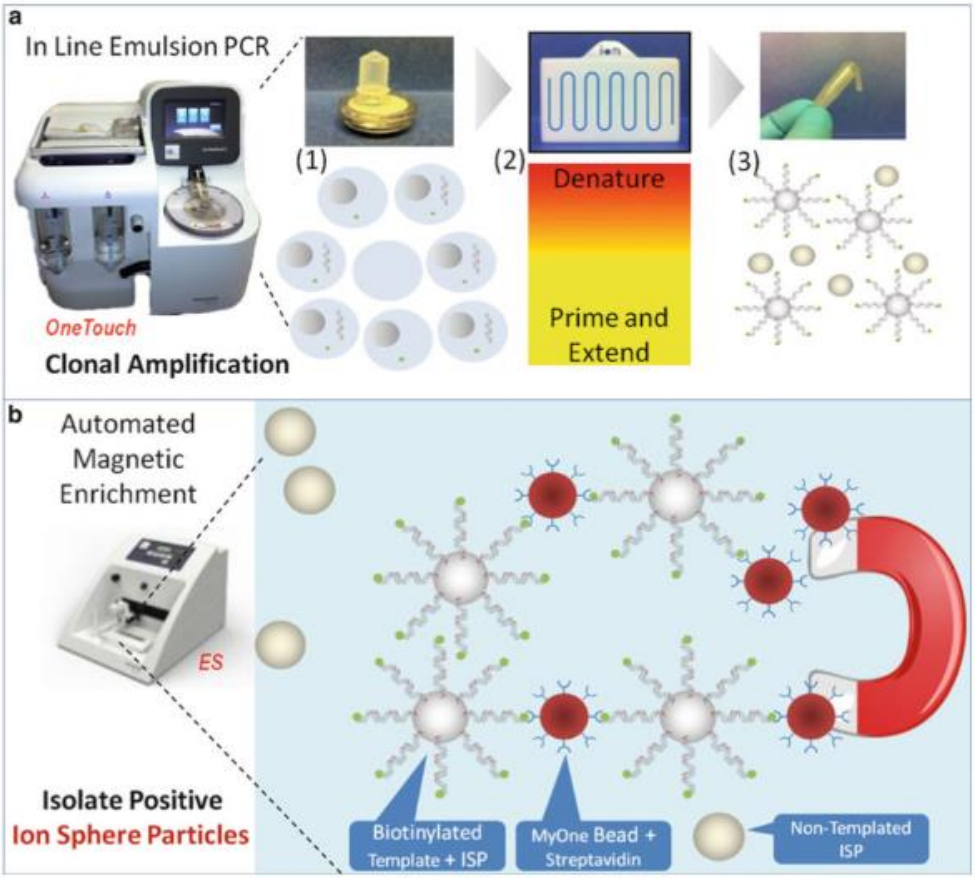



**Figure 3.6.1-1: Clonal amplification and enrichment using OneTouch™ instrumentation. (a) The Ion OneTouch™ 2 Instrument (b) The Ion OneTouch™ ES Instrument (Kohn *et al.*, 2013)**

This procedure uses an oil emulsion to attach DNA sequences to Ion Sphere™ particles (ISPs). Every droplet should include a unique DNA template bonded to an ISP to create a clonal colony (Kohn *et al.,* 2013).

### 3.6.2 Template-Positive Particles preparation

The preparation of templated Ion Sphere™ particles starts by the generation of microreactors by a reaction filter. PCR amplification plate allows the thermal cycling of the microreactors where clonal amplification occurs. Finally, the templated Ion Sphere™ particles are recovered after centrifuge (Kohn *et al.,* 2013).

The detailed description of the procedure is described below:

1. Ion OneTouch™ 2 Instrument (Thermo Fisher Scientific, USA) set up:
    - Dispense 150 µl of the Ion OneTouch™ Breaking Solution into each of the two Ion OneTouch™ Recovery Tubes.
    - Install the two Ion OneTouch™ Recovery Tubes and the Ion One Touch™ Recovery Router in the centrifuge.
    - Install the Ion OneTouch™ amplification plate.
    - Install the Ion OneTouch™ Oil on the left front port.
    - Install the Ion OneTouch™ Recovery Solution on the right front port.

2. Prepare the amplification solution:
   To a 2-mL tube containing 800 µl of Ion PGM™ Hi-Q™ View Reagent Mix, add the following components in the designated order resulting in volume of 1000 µl.

| **Ion PGM™ Hi-Q™ View Reagent** | **Volume** |
|---|---|
| Nuclease-free Water | 25 µl |
| Ion PGM™ Hi-Q™ View Enzyme Mix | 50 µl |
| Diluted library (26pM) | 25 µl |
| Ion PGM™ Hi-Q™ View ISPs | 100 µl |

3. Mix this amplification solution, then pipet the entire volume to the Ion OneTouch™ Reaction filter.
4. Pipet 850 µl of the Ion OneTouch™ Reaction Oil over the amplification solution.
5. Change the tip and pipet an additional 850 µl of the Ion OneTouch™ Reaction Oil.
6. Invert and install the filled Ion OneTouch™ Reaction Filter into the three holes on the top stage of the Ion OneTouch™ 2 Instrument.

7. Run the Ion OneTouch™ Instrument.

### 3.6.3 Template-positive ISPs recovery

In this step, template-positive ISPs are prepared for enrichment.

1. At the end of the run centrifuge the sample.
2. Remove both Recovery Tubes from the instrument, then remove all but ~100 µ1 of Ion OneTouch™ Recovery Solution from each Ion OneTouch™ Recovery Tube.
3. Add 500 µ1 of the Ion OneTouch™ Wash Solution to each Recovery Tube.
4. Pipet up and down to disperse the ISPs, then combine the suspension from each Recovery Tube into one new 1.5 ml Eppendorf tube.
5. Centrifuge the ISPs for 2.5 min at 15,500 × g and then remove all but 100 µL of supernatant and vortex the pellet for 10 s for complete resuspension.
6. Sample is ready for enrichment.

### 3.6.4 Enrichment of the Template-Positive ISPs

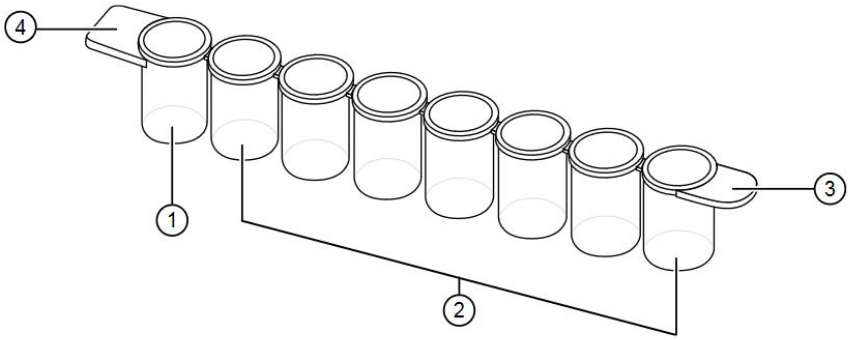
The non-templated ISPs are eliminated from the templated ISPs after clonal amplification. The Ion OneTouch™ ES utilize magnetic beads to separate template-positive Ion Sphere™ particles (Kohn *et al.,* 2013).

1. Prepare fresh Melt-Off Solution:

| **Reagent** | **Volume** |
|---|---|
| Tween$^{TM}$ Solution | 280 µl |
| 1M NaOH | 40 µl |
| Total volume | 320 µl |

2. Add 13 µL of Dynabeads™ MyOne™ Streptavidin C1 Beads to a new 1.5-mL Eppendorf Tube.
3. Place the tube on a magnet for 2 minutes, then carefully remove and discard the supernatant without disturbing the pellet.

4. Add 130 µL of My One™ Beads Wash Solution to the Dynabeads™ MyOne™ Streptavidin C1 Beads and vortex to mix.
5. Take an 8-well strip from the Ion OneTouch™ kit. Confirm that the square-shaped tab is on the left.
6. Transfer the template positive ISPs (100.0 µl) to Well 1.
7. Add the resuspended MyOne™ Beads (130.0 µl) to Well 2.
8. Add Ion OneTouch™ Wash Solution (300 µl) to Wells 3, 4 and 5.
9. Add the fresh melt-off solution (300 µl) to Well
10. Leave Wells 6 and 8 empty.




① Well 1
② Wells 2–8
③ Rounded tab
④ Square-shaped tab

**Figure 3.6.4-1: Flat Bottom 8-Well Strips**

### 3.6.5 Ion OneTouch™ ES Instrument preparation

1. Place a new tip on the Tip Arm.
2. Place a new PCR tube in the base of the Tip Loader and add 10 µl of Neutralization Solution.
3. Place the eight-well strip in right side of slot.
4. Perform the run.

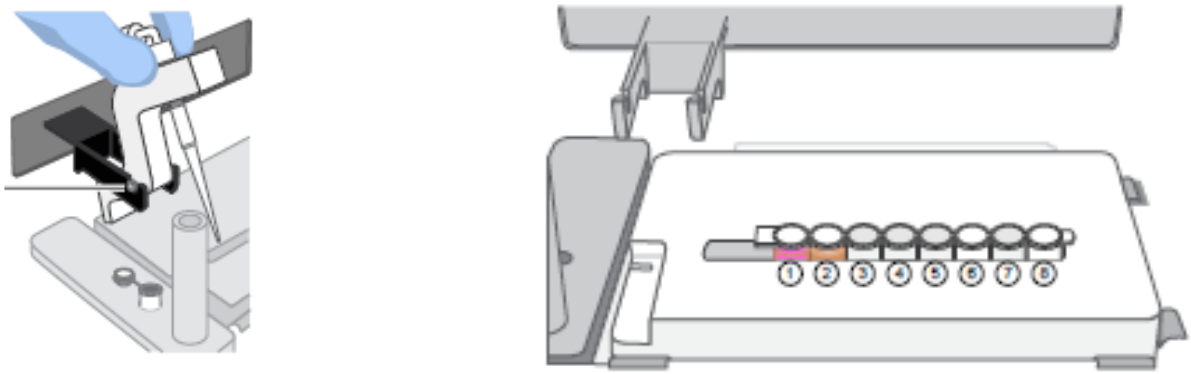


**Figure 3.6.5-1: Instrument installation**

## 3.7 Template sequencing

The sequencing was done with the Ion torrent PGM™ Sequencer (Thermo Fisher Scientific), the Ion 316™ Chip v2 BC was used.

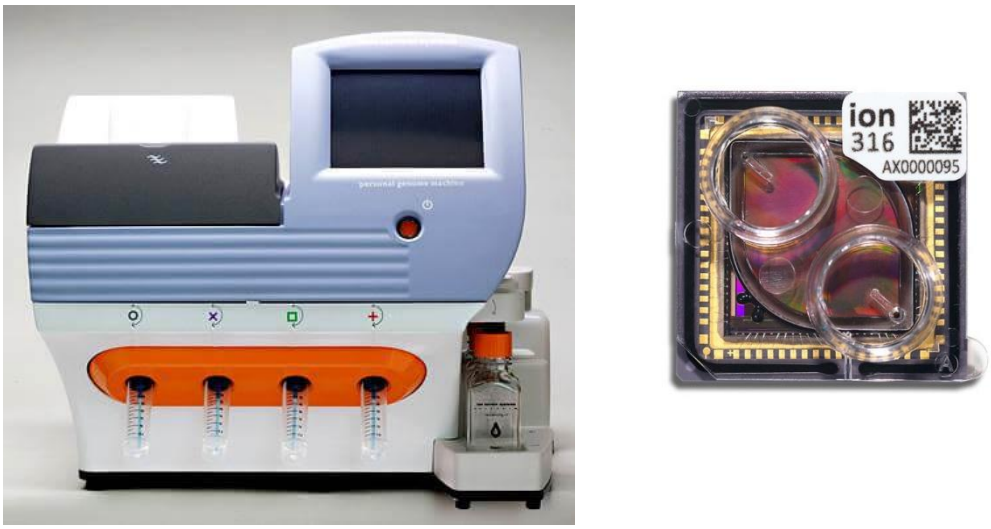



**Figure 3.7-1: Ion torrent PGM™ Sequencer and Ion 316™ Chip v2 BC**

### 3.7.1 Ion PGM™ System preparation

1. Clean the Ion Torrent as recommended by following on-screen prompts.
2. Wash all bottles with18 MΩ water three times.

3. Prepare Wash 1 bottle: Add 350 µl 100 mM NaOH.
4. Prepare Wash 3 bottle: Add 50 ml of W3 solution.
5. Prepare Wash 2 bottle: Add 2 liters of 18 MΩ water, add the entire bottle of W2 solution and finally add 70 µl of NaOH.
6. Add 20 µl of each dNTP to conical tubes.
7. Initialize the Ion Torrent and follow on-screen prompts.

### 3.7.2 Sample preparation

1. Add 5 µl of Control ISPs directly to the template-positive ISPs and mix by pipetting up and down.
2. Centrifuge for 2 minutes at 15,500 × *g*.
3. Remove the supernatant. Leave~15 µl in the tube and add 12 µl of Sequencing Primer to the ISPs.
4. Pipet the mixture up and down to disrupt the pellet.
5. Place the tube in the thermal cycler: 95°C for 2 minutes and then 37°C for 2 minutes.

### 3.7.3 Chip check and preparation

1. On the main menu of the Ion PGM™ Sequencer touchscreen, press Run.
2. Select the instrument used to prepare the template-positive ISPs: Ion OneTouch™ 2 System (OT2).
3. Replace the old chip with the new one. Close the chip clamp.
4. Use the scanner to scan the barcode located on the new chip, or enter the barcode manually.
5. Press Chip Check and look for leaks.

### 3.7.4 Chip loading

1. Remove the template-positive ISPs from the thermal cycler and add 3 µl of Ion PGM™ Hi-Q™ View Sequencing Polymerase.
2. Mix and incubate at room temperature for 5 minutes.

3. Remove residual liquid from the chip by tilting the chip at 45 °C, then inserting the pipette tip into the loading port. Discard the liquid.
4. Centrifuge the chip upside-down with tab pointing in for 5 seconds
5. Following polymerase incubation, load the entire sample (~30 µl) into the loading port by dialing down the pipette to gently and slowly deposit the ISPs at a rate of ~1 µl per second.
6. Remove any displaced liquid from the other port of the chip.
7. Centrifuge the chip with tab pointing in for 30 seconds.
8. Centrifuge the chip with tab pointing in for 30 seconds.
9. Set the pipette volume to 25 µl.
10. Tilt the chip at a 45° angle so that the loading port is the lower port.
11. Insert the pipette tip into the loading port and slowly pipette the sample in and out three times. Avoid creating small bubbles.
12. Slowly remove as much liquid as possible from the loading port by dialing the pipette. Discard the liquid.
13. Centrifuge the chip upside-down for 5 seconds to remove excess liquid.
14. Perform Run.
15. When the run is complete, proceed with a cleaning step.

### 3.7.5 Sequencing run assessment

The quality of a run depends on several factors:

1. The loading of the chip: when the loading density is higher, the result is better (figure 3.7.5-1).
2. Quality and quantity of the templated library, including a high percentage of final library and a low percentage of polyclonal (figure 3.7.5-2).
3. Size distribution, including an appropriate range of the sequences and a tight distribution with a low population of short fragments (figure 3.7.5-3).

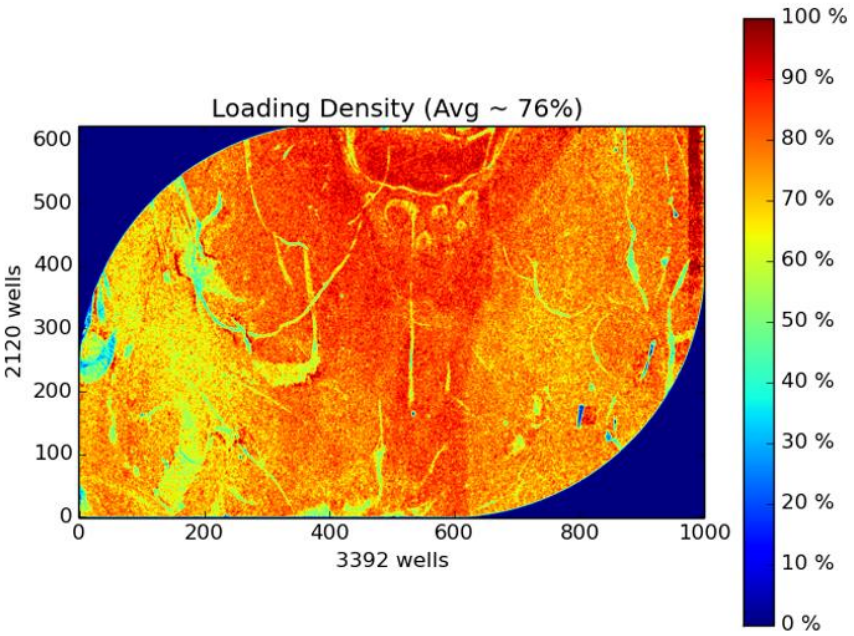



**Figure 3.7.5-1: Loading density on the Ion 316™ Chip**

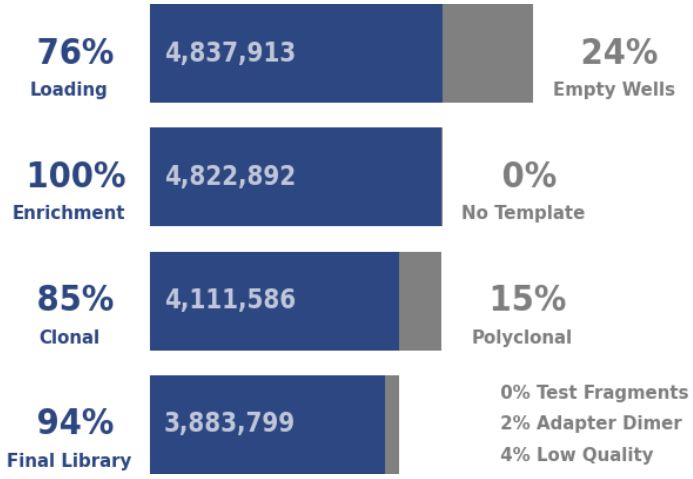



**Figure 3.7.5-2: Quality and quantity of the templated library**

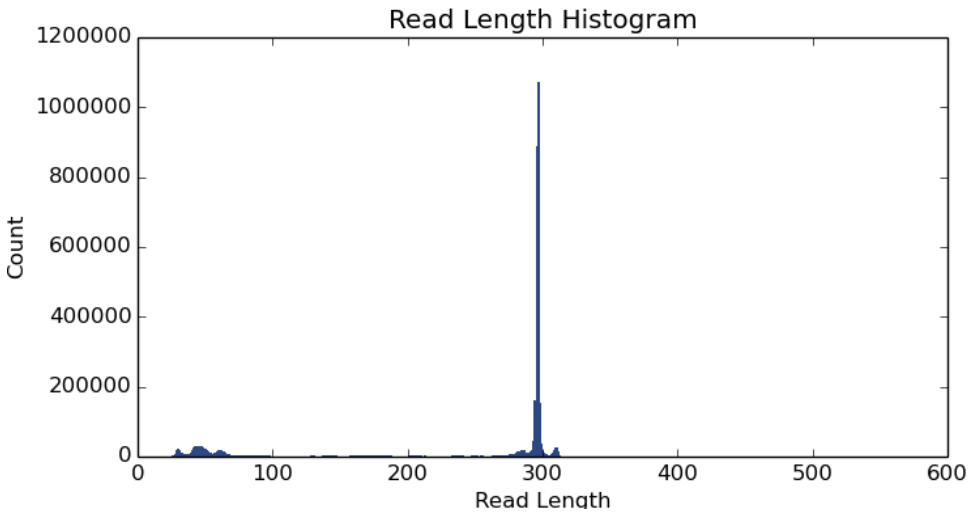



**Figure 3.7.5-3: Read length histogram**

## 3.8 Bioinformatic Analysis

The raw sequencing reads were filtered by the Ion Torrent Software to remove low quality and polyclonal sequences. Bacterial 16S rRNA gene sequences were retrieved in FASTQ format and analyzed using the QIIME2 platform, which is particularly developed for microbiome analysis (Bolyen *et al.,* 2019). Sequences were quality filtered, trimmed, denoised using DADA2 and chimeras were removed (Callahan *et al.,* 2016). VSEARCH was utilized, for the clustering and the taxonomical classification of the sequences, using the Greengenes database version 13_8 (Rognes *et al.,* 2016). The analysis of bacterial diversity was evaluated through alpha diversity (Chao1, Observed Species, Faith Phylogenetic Diversity, Pielou Evenness and Shannon index) and beta diversity (Bray Curtis distance matrix, Jaccard distance matrix and both weighted and unweighted UniFrac distance matrix). EMPeror tool was used for the visualization of principal coordinates analysis (PCoA) (Vázquez-Baeza *et al.,* 2013). Linear discriminant analysis (LDA) with an effect size (LefSe) algorithm (Segata *et al.,* 2011) was performed in the Galaxy Web module (http://huttenhower.sph.harvard.edu/galaxy/ (accessed on 20 October 2021) for biomarker identification.

## 3.9 Statistical Analysis

Alpha diversities were compared by non-parametric test using Kruskal–Wallis H test. Beta diversities were analyzed using permutational multivariate analysis of variance PERMANOVA with 999 permutations. Additionally, the PERMDISP test was done to test the homogeneity of dispersions between the groups of animals. The detection of taxa with significant differential abundance between cows and goats was done using the factorial Kruskal–Wallis and pairwise Wilcoxon tests. The alpha value was 0.05, with a threshold value of 2.0 for the logarithmic LDA scores of discriminative features.

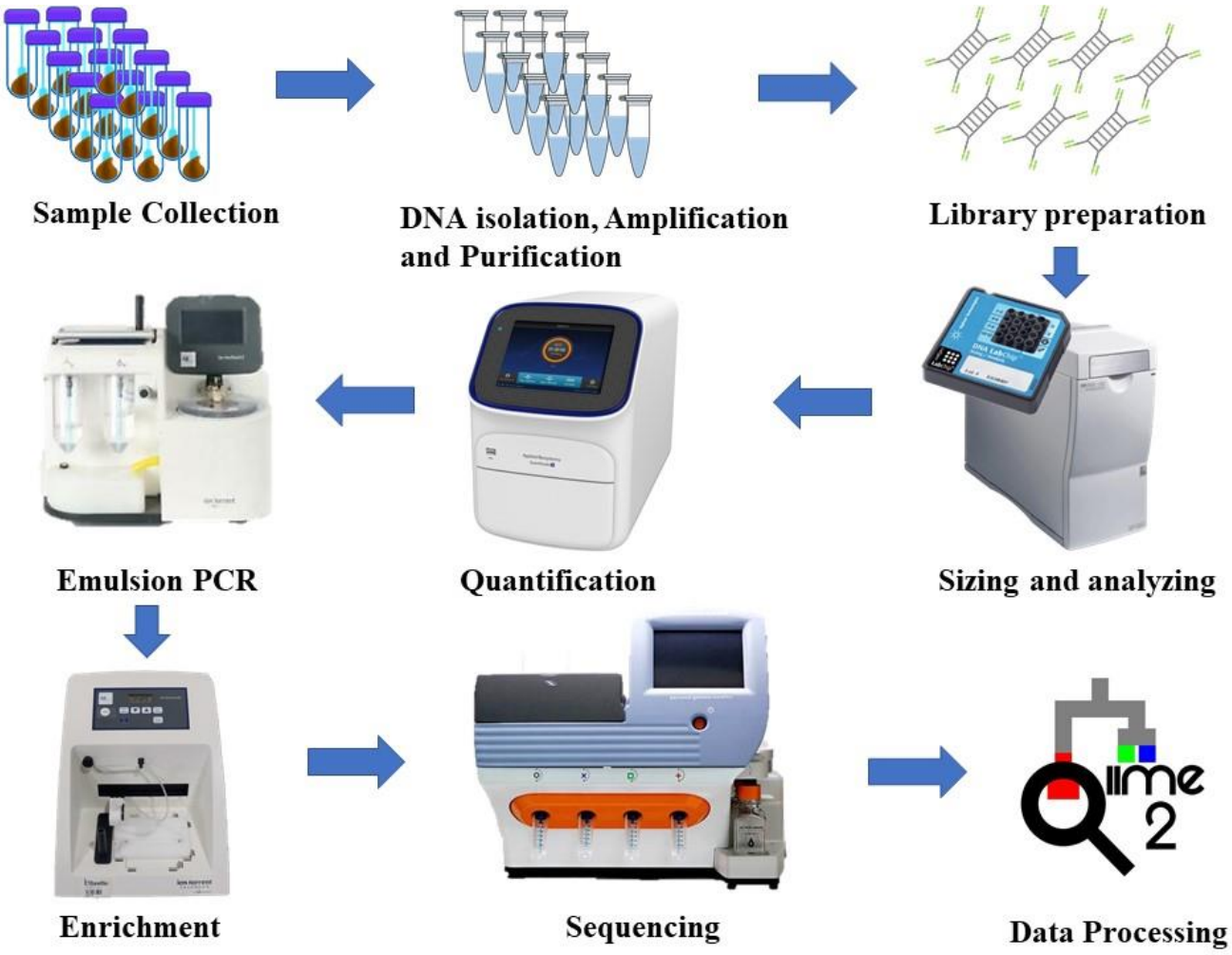



**Figure 3-1: Summary of the experimental technique for DNA sequencing using the Ion Torrent PGM™ Sequencer**

# 4. RESULTS

## 4.1 Sequencing Analysis

A total of 33 samples were subjected to 16S rRNA gene V4-V5 region sequence analysis. Bacterial sequencing generated a total of 6,442,853 reads of high-throughput sequencing. After passing through quality filtering (HTS), 2,324,963 reads were obtained. The sampling depth was rarefied to 2271 reads per sample. The rarefactions curves reached a plateau, showing that the sequencing depth was sufficient to establish the fecal core bacterial community for both cows and goats (Figure 4.1-1). The sequencing length of all amplicons was 290 bp. The total number of detected amplicon sequence variants (ASVs) was 11 802.

The sequencing analysis details are summarized in the table 4.1-1.

**Table 4.1-1: Sequencing analysis summary**

| | |
|---|---|
| Number of samples | 33 |
| Number of reads | 6,442,853 |
| Number of reads passed filtering | 2,324,963 |
| Rarefaction depth | 2271 |
| Number of ASVs | 11,802 |
| Total frequency | 2,205,492 |
| Sequencing length (bp) | 290 |

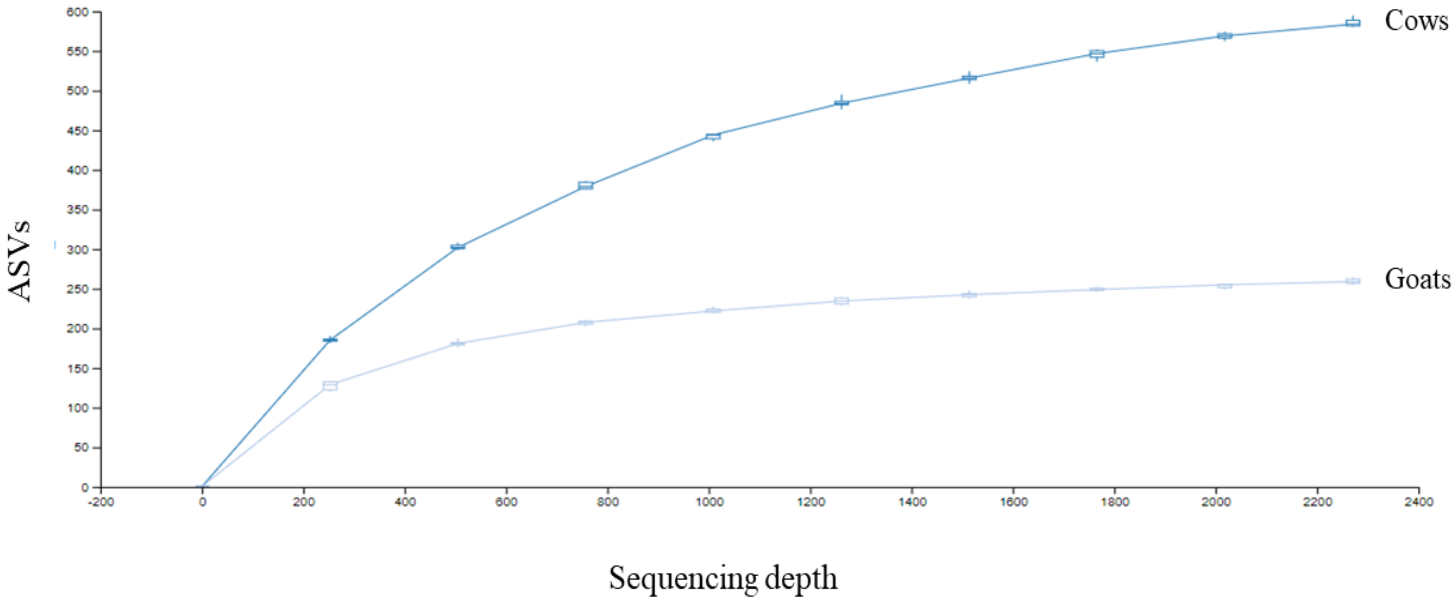



**Figure 4.1-1: Rarefaction curves representing the sequencing depth (number of reads) and the number of ASVs (amplicon sequence variants) found in feces of goats and cows.**

## 4.2 Alpha diversity

Alpha diversity metrics summarize the diversity of the species within a particular ecosystem. Several indices of diversity are used to assess the species richness and evenness (Thukral, 2017).

In our study, the bacterial population diversity of goats and cows' feces was determined using the Chao1 index, Observed Species, Faith Phylogenetic Diversity, Pielou Evenness index and Shannon entropy index.

Chao1 index is an estimator of the total richness based on the abundance of species (Chao and Chiu, 2016). Pielou Evenness index is an indicator of the equity in species abundance in each sample, measuring if there is some species dominating the samples or all species have relatively similar abundances (Pielou, 1966). Shannon index is an estimator of both richness and evenness, based on species abundance (Shannon, 1948). Faith Phylogenetic Diversity is an estimator if the feature diversity, based on the phylogenetic tree for species (Faith, 1992).

Alpha diversity metrics were calculated on individual samples and mean values are listed in table 4.2-1. The analysis revealed that diversity indices, Chao1, Shannon index and Faith Phylogenetic Diversity were slightly higher in cows compared to goats, but the difference was not significant ($p > 0.05$). This indicates that the richness in species between the two groups of animals is similar. However, Pielou Evenness index was significantly higher in cows compared to goats ($p < 0.05$), revealing a higher species abundance equity in cows.

Alpha diversity boxplots showing the different indices are illustrated in the figures 4.2-1, 4.2-2, 4.2-3, 4.2-4 and 4.2-5.

**Table 4.2-1: Summary of alpha diversity indices of bacterial community in cows and goats.**

| Index | Cows | Goats | p-value |
|---|---|---|---|
| **Chao1** | 877.19 | 688.54 | 0.16 |
| **Pielou Evenness Index** | 0.934 | 0.918 | 0.017* |
| **Shannon Entropy Index** | 8.286 | 7.498 | 0.11 |
| **Faith pd** | 60.82 | 56.16 | 0.13 |
| **ASV[1]** | 604 | 422 | 0.13 |

ASV[1], Amplicon sequence variant.

* Significant difference ($p < 0.05$)

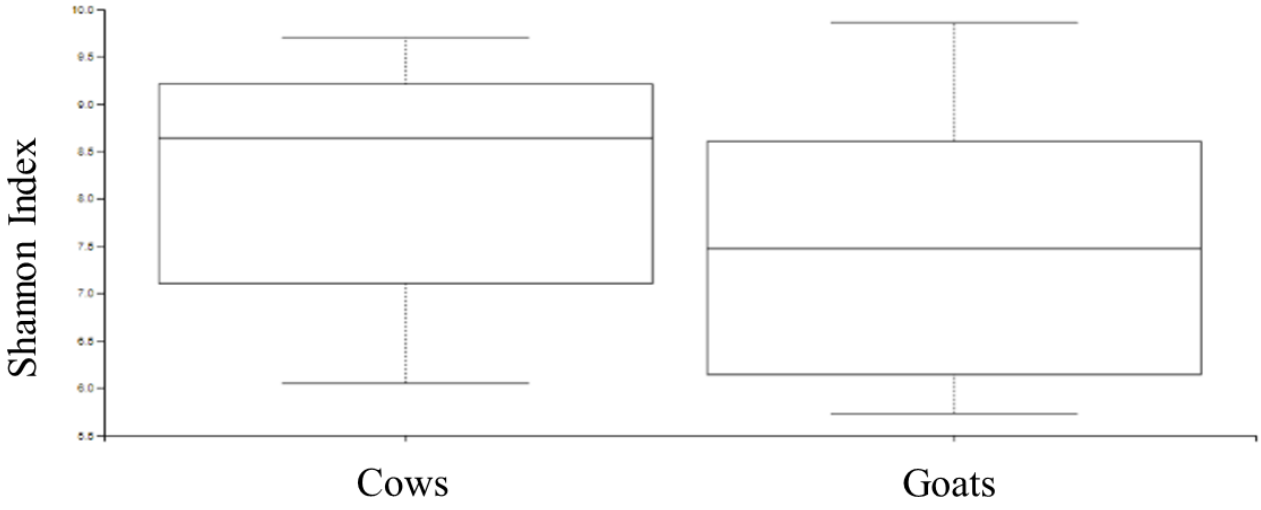



**Figure 4.2-1: Comparison of alpha-diversity indices in cows and goats. Shannon entropy index ($p > 0.05$).**

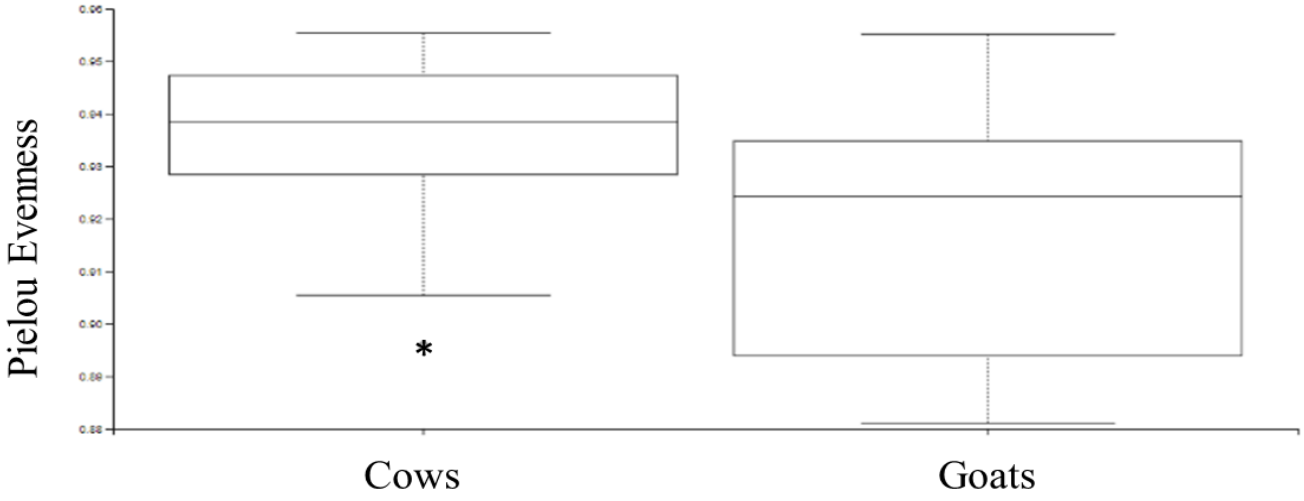



**Figure 4.2-2: Comparison of alpha-diversity indices in cows and goats. Pielou Evenness index ($p < 0.05$). Boxes with a star symbol show significant difference in bacterial alpha-diversity ($p < 0.05$).**

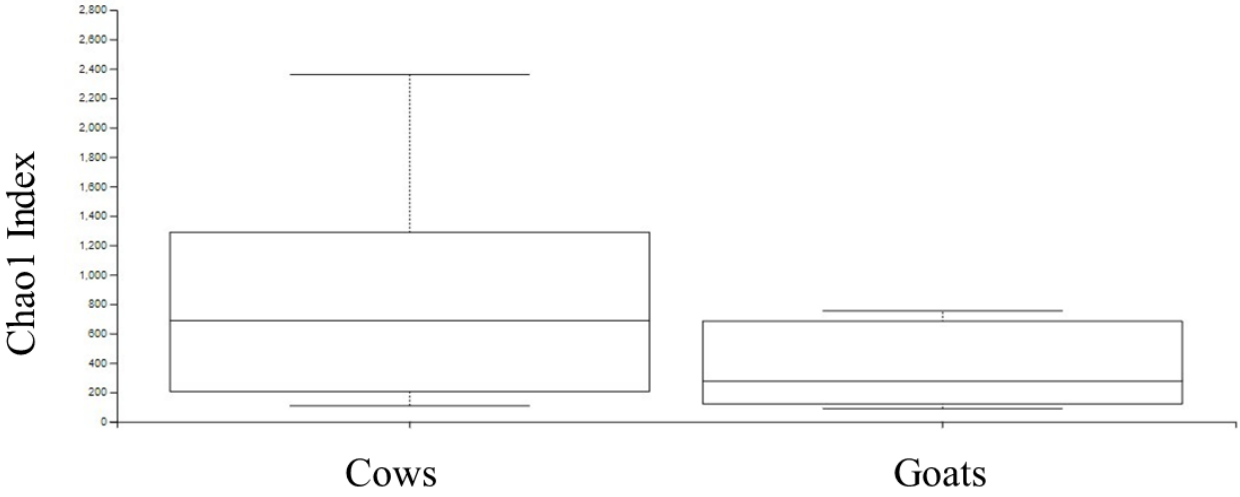



**Figure 4.2-3: Comparison of alpha-diversity indices in cows and goats. Chao1 index ($p > 0.05$).**

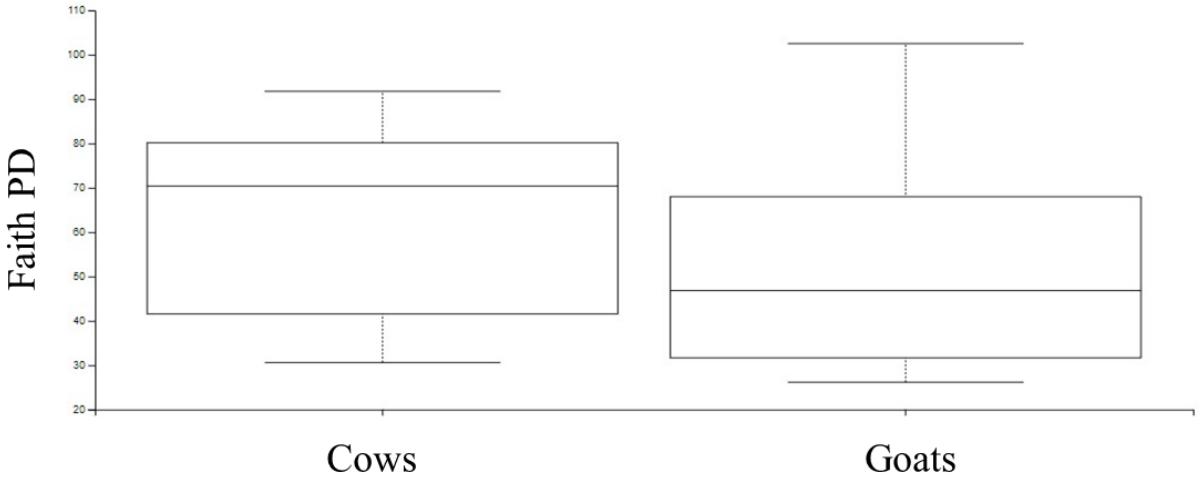



**Figure 4.2-4: Comparison of alpha-diversity indices in cows and goats. Faith Phylogenetic Diversity ($p > 0.05$).**

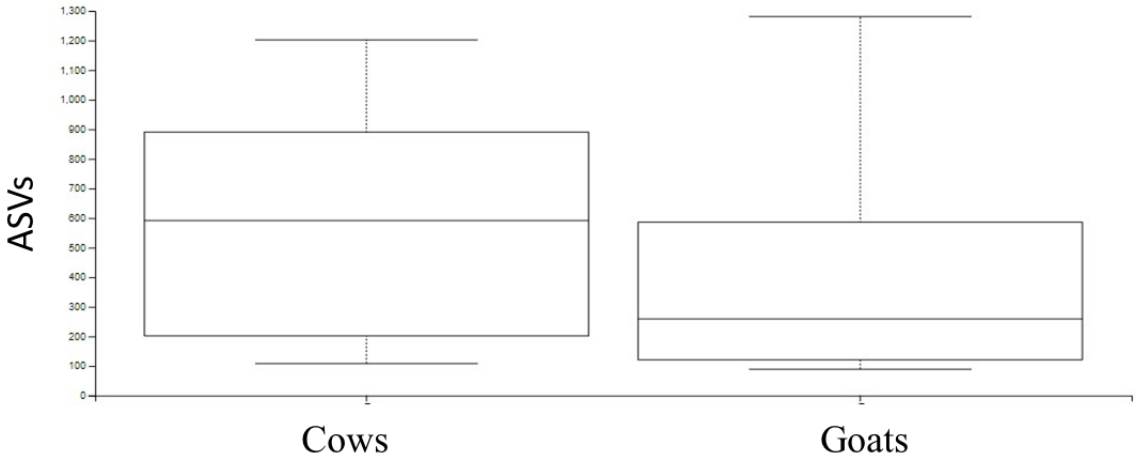



**Figure 4.2-5: Comparison of alpha-diversity indices in cows and goats. Observed ASVs ($p > 0.05$).**

## 4.3 Beta Diversity

Beta diversity evaluates the similarities and diversities among communities. The most used measures in microbiome research include Bray-Curtis index, Jaccard index and both weighted and unweighted UniFrac distances.

Bray-Curtis index quantifies the compositional dissimilarity between two populations. Jaccard index compares microorganisms for two communities to see which ones are shared and which are distinct. UniFrac distances assess the dissimilarities between populations while taking into consideration the phylogenetic tree information.

In our results, Beta diversity was assessed by several algorithms including Bray Curtis distance matrix, Jaccard distance matrix and both weighted and unweighted UniFrac distance matrix. Results are illustrated by PCoA plots. Permutational multivariate analysis of variance (PERMANOVA) based on the same similarity matrix was done to verify whether there is a significant difference in the distance between the groups of animals. In addition, permutational analysis of multivariate dispersions (PERMDISP) was done to test the homogeneity of dispersions between the groups and p-values are listed in the table 4.3-1. PCoA plots based on Bray Curtis distance matrix (figure 4.3-1), Jaccard distance matrix (figure 4.3-2) and both weighted and unweighted UniFrac distance matrix (figure 4.3-3 and 4.3-4, respectively) showed a distinct clustering of bacterial composition between goats and cows and PERMANOVA revealed statistically significant dissimilarities between the two groups of animals ($p < 0.05$). However, the results can be partially influenced by high intergroup variability (PERMDISP $p < 0.05$). Group significance plots based on Bray Curtis distance matrix, Jaccard distance matrix, weighted and unweighted UniFrac distance matrix are illustrated in the figures 4.3-5, 4.3-6, 4.3-7 and 4.3-8 respectively

**Table 4.3-1: Permutational multivariate analysis of variance (PERMANOVA) and dispersions (PERMDISP) showing significant differences in beta diversity between cows and goats ($p < 0.05$).**

| | PERMANOVA<br><br>p-value (*p < 0.05) | PERMDISP<br><br>p-value (*p < 0.05) |
|---|---|---|
| Bray Curtis distance | 0.001* | 0.01* |
| Jaccard distance | 0.001* | 0.002* |
| Weighted unifrac distance | 0.001* | 0.02* |
| Unweighted unifrac distance | 0.046* | 0.04* |

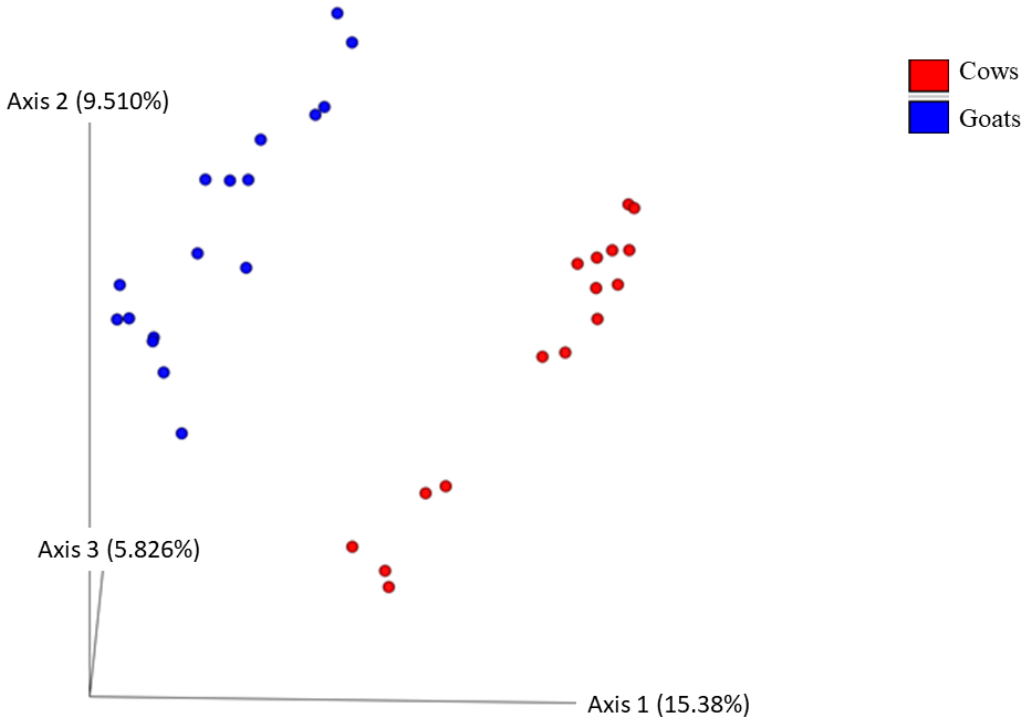



**Figure 4.3-1: Principal Coordinate Analysis (PCoA) of bacterial communities in goats and cows. The PCoA plot was constructed using Bray Curtis method.**

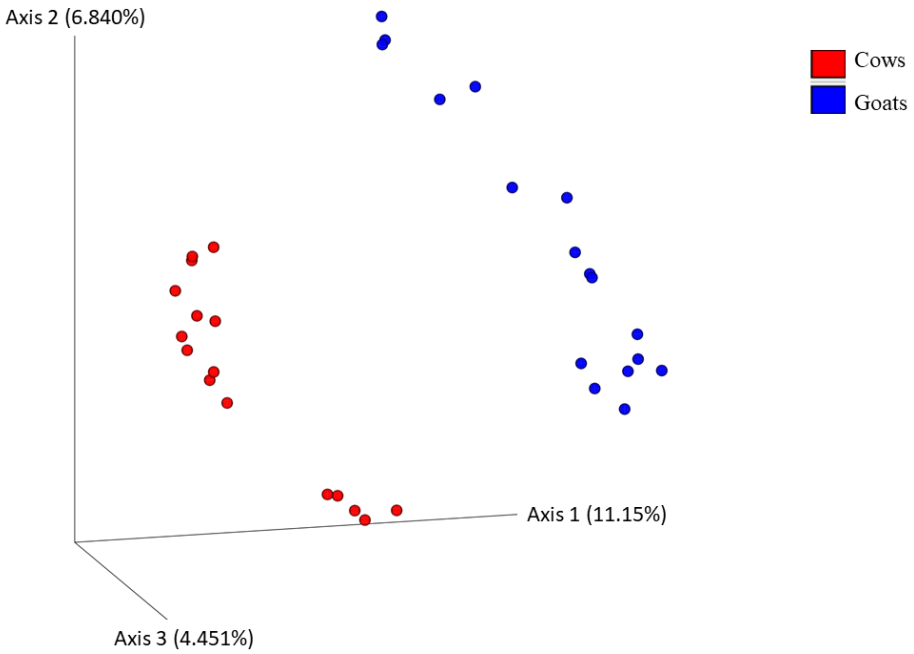



**Figure 4.3-2: Principal Coordinate Analysis (PCoA) of bacterial communities in goats and cows. The PCoA plot was constructed using Jaccard distance method.**

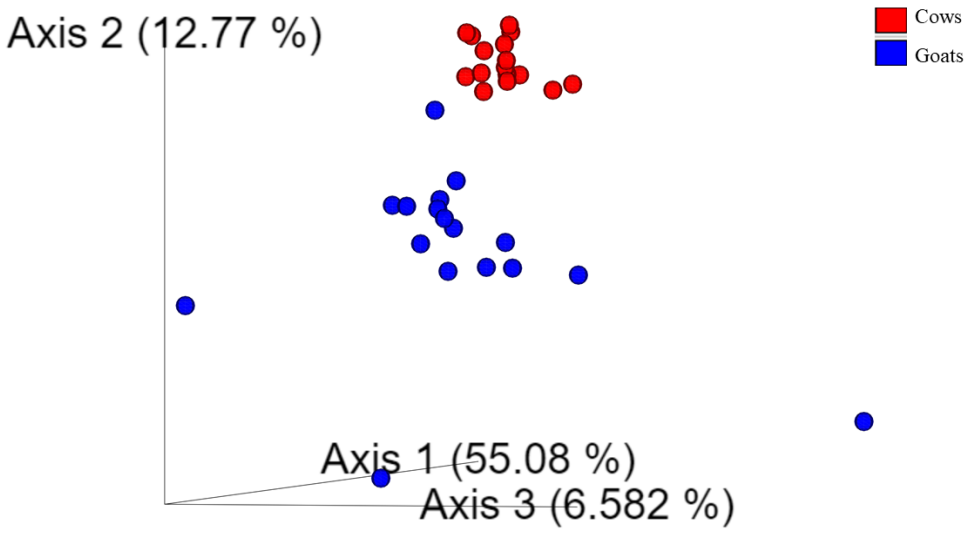



**Figure 4.3-3: Principal Coordinate Analysis (PCoA) of bacterial communities in goats and cows. The PCoA plot was constructed using weighted UniFrac distance matrix.**

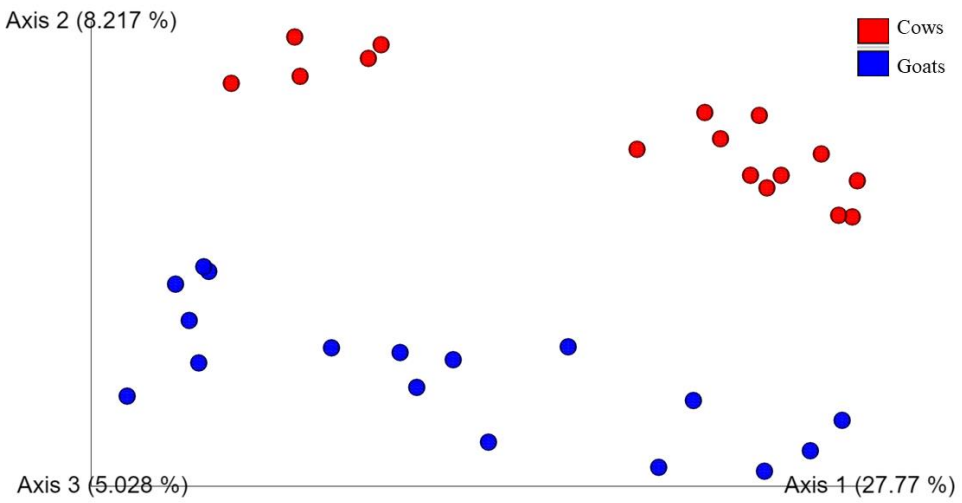



**Figure 4.3-4: Principal Coordinate Analysis (PCoA) of bacterial communities in goats and cows. The PCoA plot was constructed using unweighted UniFrac distance matrix.**

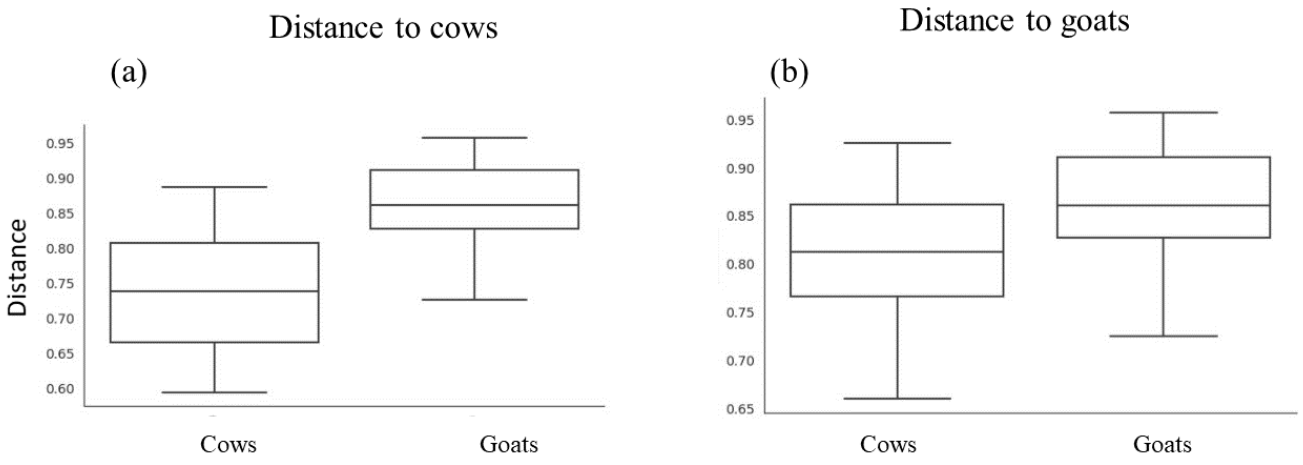



**Figure 4.3-5: Box plots based on Bray Curtis matrix showing significant difference in beta diversity between goats and cows ($p < 0.05$). (a) Distance to cows. (b) Distance to goats.**

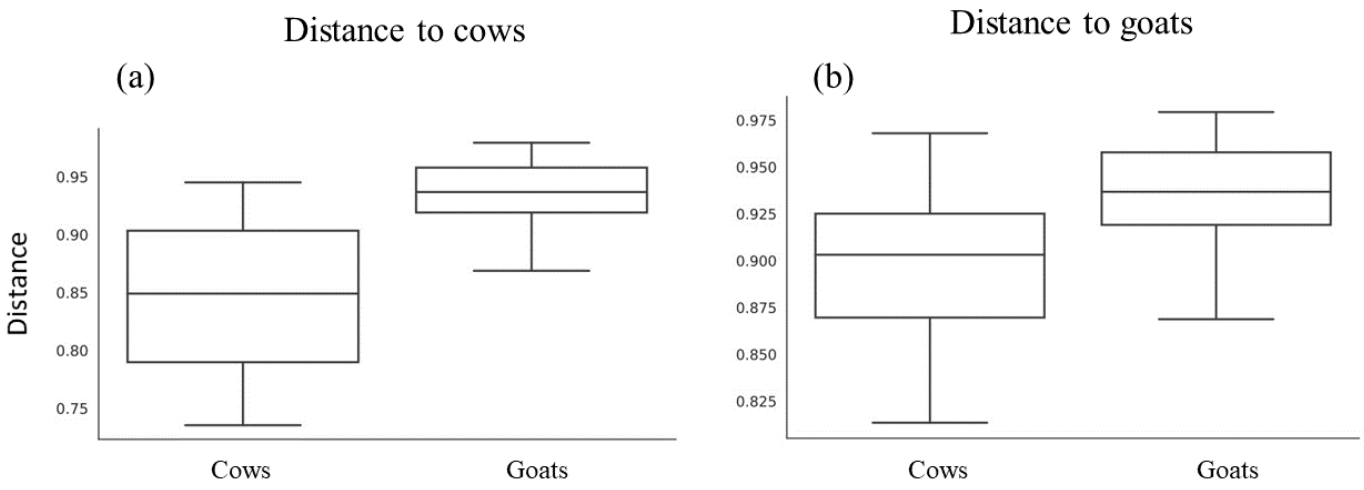



**Figure 4.3-6: Box plots based on Jaccard distance matrix showing significant difference in beta diversity between goats and cows ($p < 0.05$). (a) Distance to cows. (b) Distance to goats.**

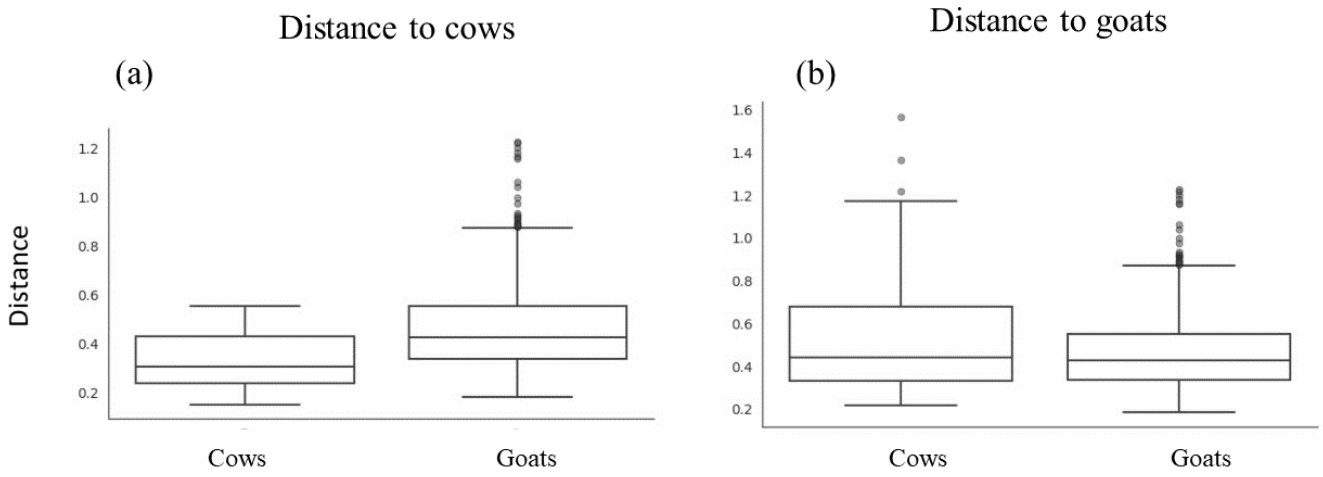



**Figure 4.3-7: Box plots based on weighted UniFrac distance matrix showing significant difference in beta diversity between goats and cows ($p < 0.05$). (a) Distance to cows. (b) Distance to goats.**

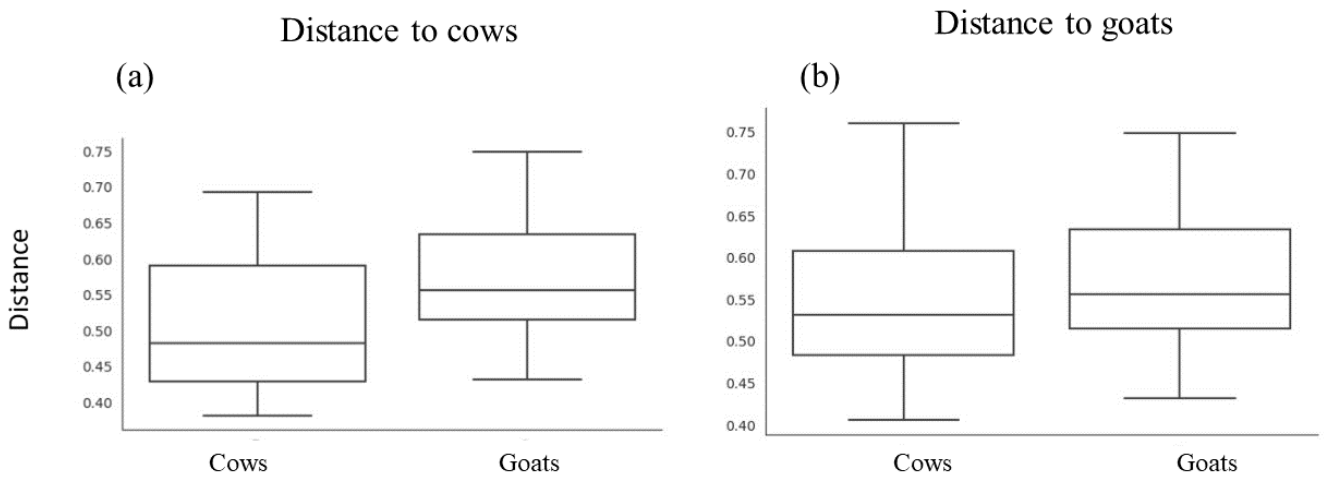



**Figure 4.3-8: Box plots based on unweighted UniFrac distance matrix showing significant difference in beta diversity between goats and cows ($p < 0.05$). (a) Distance to cows. (b) Distance to goats.**

## 4.4 Taxonomical composition

A total of 15 phyla, 26 classes, 40 orders, 92 families and 156 genera were detected in the samples. Some of the sequences were unassigned, they accounted for 17.5% ± 5% (mean ± standard deviation) in goats and 16.4% ± 3.7% in cows. On phylum level, Firmicutes (75.9% ± 3.7% and 75.8% ± 2% in goats and cows, respectively) and Bacteroidetes (17.2% ± 3.1% and 19.2% ± 1.8% in goats and cows, respectively) were the dominant phyla, followed by Proteobacteria (2.5% ± 1% and 2.1% ± 0.7% in goats and cows, respectively) and Spirochaetes (1.5% ± 0.6% and 0.3% ± 0.2% in goats and cows, respectively), as shown in figure 4.4-1. Rare phyla with relative abundance lower than 1% were Actinobacteria, Tenericutes, TM7, Planctomycetes, Lentisphaerae, Elusimicrobia, LD1, Verrucomicrobia, Cyanobacteria, Fibrobacteres and Fusobacteria and on the figure 4.4-1 are summarized as "others". The Firmicutes phylum was mainly dominated by the class Clostridia (75.8% ± 3.7% and 72.4% ± 2% in goats and cows, respectively) and a small abundance of Bacilli (0.2% ± 0.2% and 3.4% ± 2.3% in goats and cows, respectively). The Bacteroidetes phylum was mainly dominated by the class Bacteroidia (17.2% ± 3.2% and 19.2% ± 1.9% in goats and cows, respectively) In phylum Spirochaetes, the class Spirochaetes predominated (1.6% ± 0.6% and 0.3% ± 0.2% in goats and cows respectively), as shown in figure 4.4-2.

On order level, Clostridia was mainly dominated by the order Clostridiales (75.7% ± 3.6% and 72.4% ± 2% in goats and cows, respectively) and Bacilli were represented by the order Bacillales (0.06% ± 0.03% and 3% ± 2.5% in goats and cows, respectively). The Bacteroidia class was mainly dominated by the order Bacteroidales (17.2% ± 3.2% and 19.2% ± 1.9% in goats and cows, respectively). In Spirochaetes, the order Spirochaetales predominated (1.6% ± 0.6% and 0.3% ± 0.2% in goats and cows respectively), as shown in the figure 4.4-3.

On the family level, Clostridiales were mostly represented by *Ruminococcaceae* (37.2% ± 2.4% and 38.3% ± 3% in goats and cows, respectively), unidentified group of Clostridiales (17.2% ± 2.9% and 12.6% ± 1.6% in goats and cows respectively), *Lachnospiraceae* (6.6% ± 2.2% and 6.4% ± 1.6% in goats and cows, respectively), *Peptostreptococcaceae* (2.9% ± 2.4% and 5.6% ± 2% in goats and cows, respectively), *Mogibacteriaceae* (4.1% ± 1.2% and 3% ± 0.7% in goats and cows, respectively), unidentified group of Clostridiales (2.9% ±

1.9% and 2.7% ± 0.6% in goats and cows respectively), *Clostridiaceae* (1.6% ± 1.2% and 2.3% ± 0.5% in goats and cows, respectively) and *Christensenellaceae* (1.7% ± 0.7% and 0.5% ± 0.2% in goats and cows, respectively). Bacteroidales were mostly represented by an unidentified group of Bacteroidales (5.8% ± 1.2% and 2.6% ± 0.4% in goats and cows, respectively), *Bacteroidaceae* (3.4% ± 1.7% and 4.6% ± 0.7% in goats and cows, respectively), *Rikenellaceae* (3.5% ± 1.4% and 4% ± 0.8% in goats and cows, respectively), *RF16* (1.6% ± 1% and 3% ± 0.5% in goats and cows, respectively), *Paraprevotellaceae* (0.9% ± 0.6% and 1.9% ± 0.8% in goats and cows, respectively) and *p-2534-18B5* (0.5% ± 0.7% and 1.4% ± 0.5% in goats and cows, respectively). *Spirochaetaceae* (1.6% ± 0.6% and 0.3% ± 0.2% in goats and cows, respectively) predominated the order *Spirochaetales*, while *Planococcaceae* (0.03% ± 0.01% and 2.5% ± 2.3% in goats and cows, respectively) predominated the order Bacillales (figure 4.4-4).

At genus level, most of the sequences were not classified and were assigned as unclassified genus belonging to respective order or family. In both groups of animals, the most abundant genera were unclassified genus within the family *Ruminococcaceae* (33.5% ± 3% and 34.8% ± 3.2% in goats and cows, respectively) and unclassified genus within the order Clostridiales (17.2% ± 2.9% and 12.5% ± 1.6% in goats and cows, respectively). These two unknown genera represented 50.7% ± 5.9% and 47.3% ± 4.8% sequences in goats and cows, respectively. Each of other unclassified genera within the order Bacteroidales, Clostridiales and the families *Peptostreptococcaceae*, *Rikenellaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Mogibacteriaceae*, *RF16*, *Clostridiaceae*, *Christensenellaceae*, *p-2534-18B5* and *Planococcaceae* exhibited much lower relative abundance, but together formed 28.4% ± 15.1% and 32.5% ± 9.3% sequences in goats and cows, respectively. Only four group of sequences were identified as belonging to cultured genera of *Oscillospira* (1.6% ± 0.9% and 2.1% ± 0.5% in goats and cows, respectively), *Dorea* (1.5% ± 0.8% and 1.1% ± 0.6% in goats and cows, respectively), *Treponema* (1.6% ± 0.6% and 0.3% ± 0.2% in goats and cows, respectively) and *Ruminococcus* (1.5% ± 1.4% and 0.9% ± 0.4% in goats and cows, respectively) and uncultured genus *CF231* (0.7% ± 0.6% and 1.3% ± 0.5% in goats and cows, respectively), as shown in figure 4.4-5.

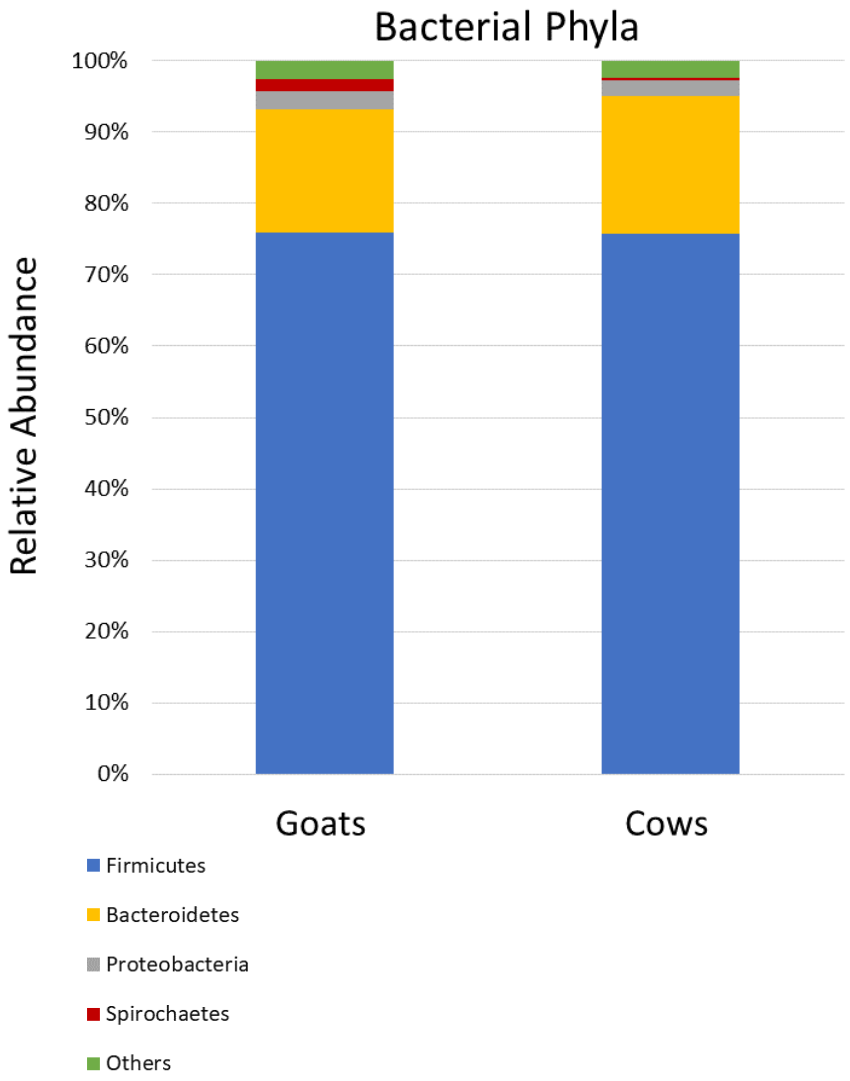



**Figure 4.4-1: Taxonomical level comparison of the fecal bacteria of goats and cows. The relative abundance is illustrated at phylum level. The taxa with a relative abundance lower than 1% are summarized as "Others".**

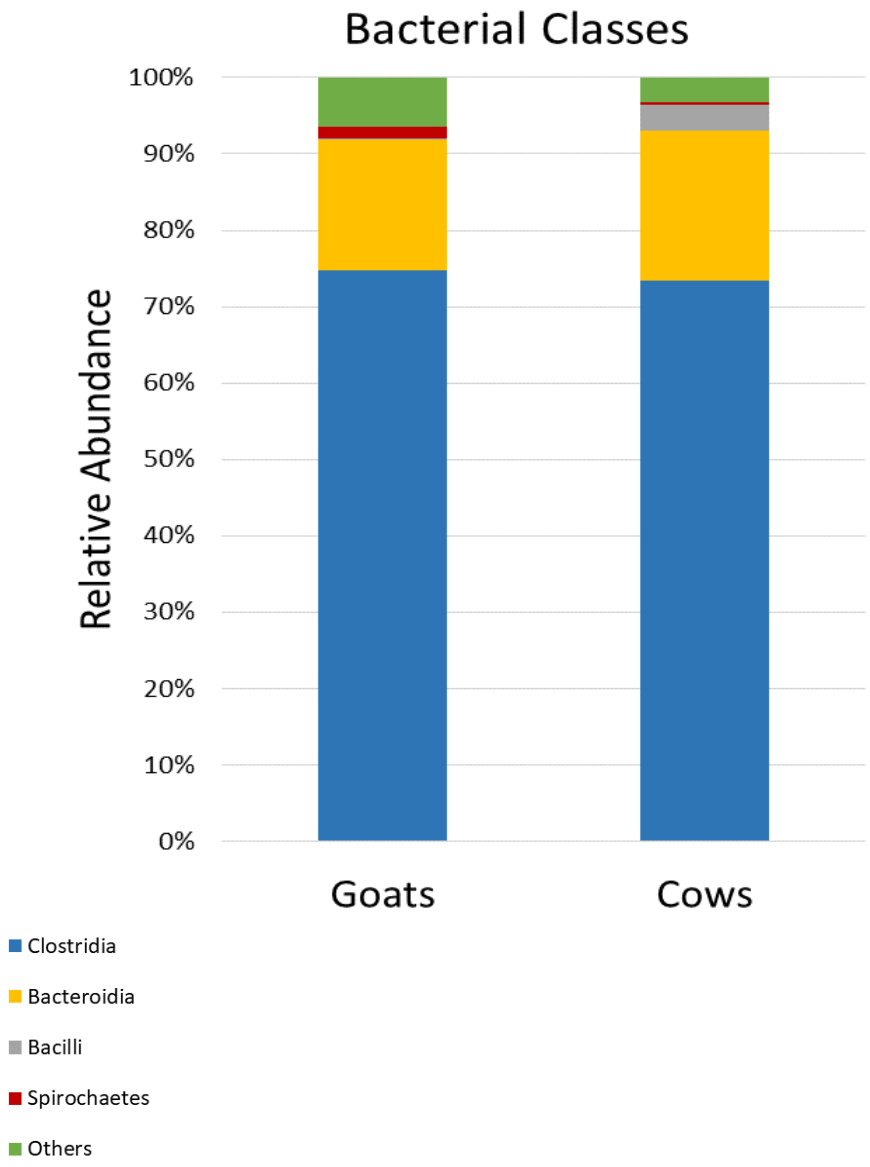



**Figure 4.4-2: Taxonomical level comparison of the fecal bacteria of goats and cows. The relative abundance is illustrated at class level. The taxa with a relative abundance lower than 1% are summarized as "Others".**

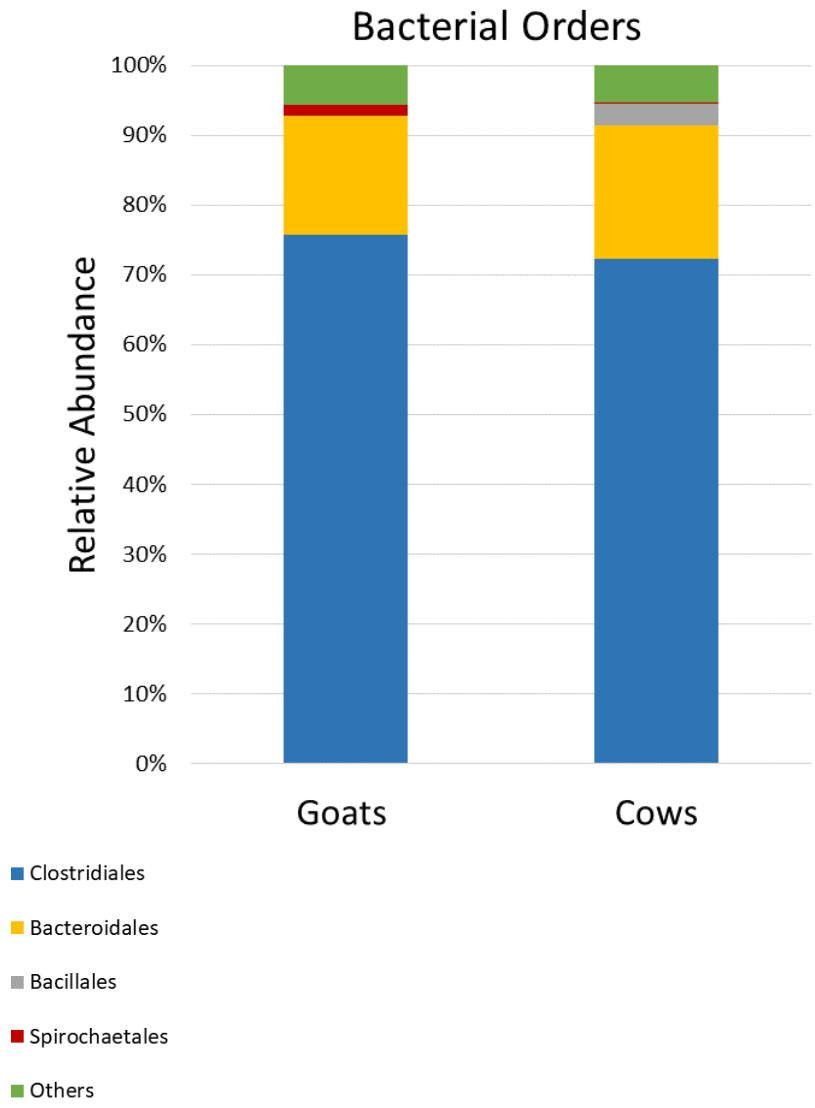



**Figure 4.4-3: Taxonomical level comparison of the fecal bacteria of goats and cows. The relative abundance is illustrated at class level. The taxa with a relative abundance lower than 1% are summarized as "Others".**

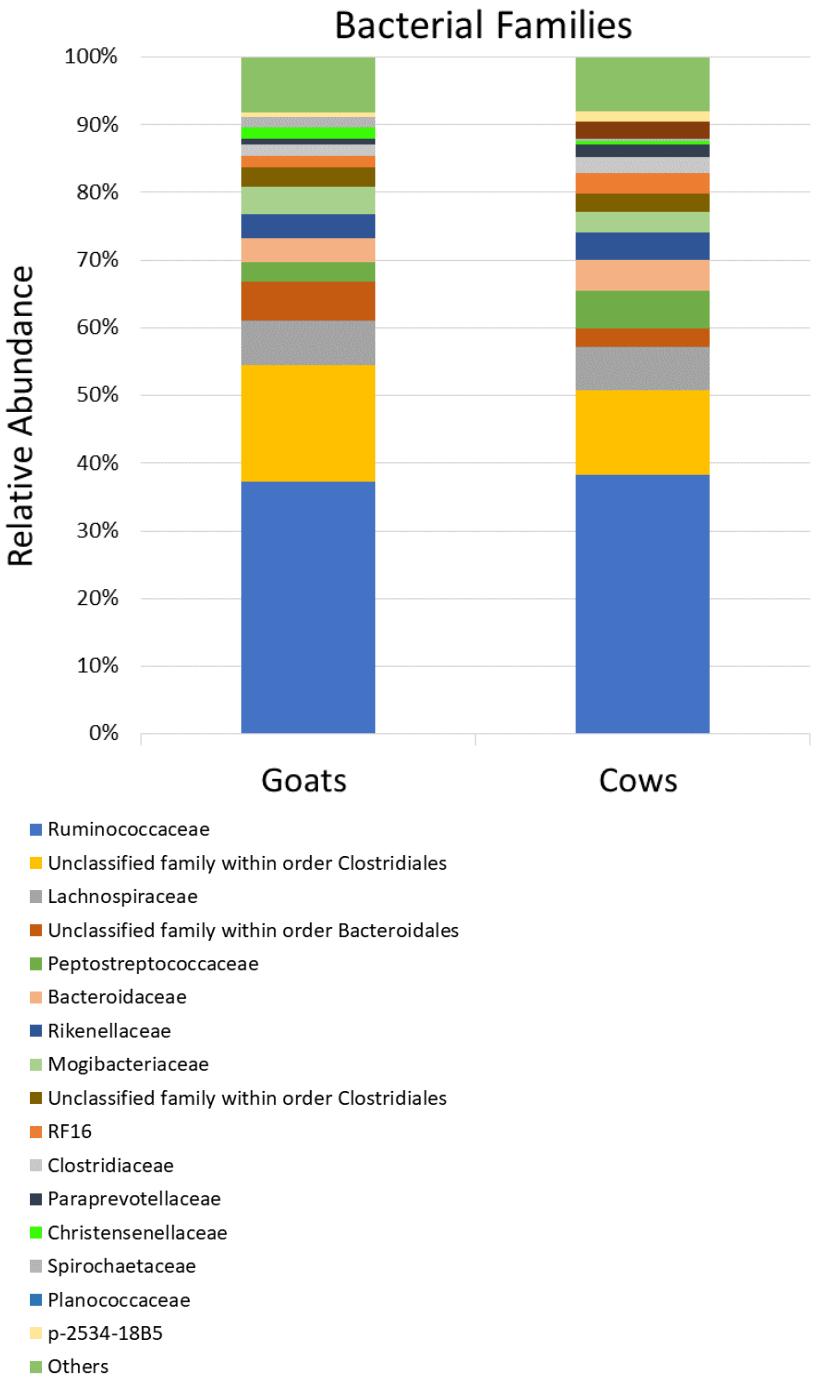



**Figure 4.4-4: Taxonomical level comparison of the fecal bacteria of goats and cows. The relative abundance is illustrated at family level. The taxa with a relative abundance lower than 1% are summarized as "Others".**

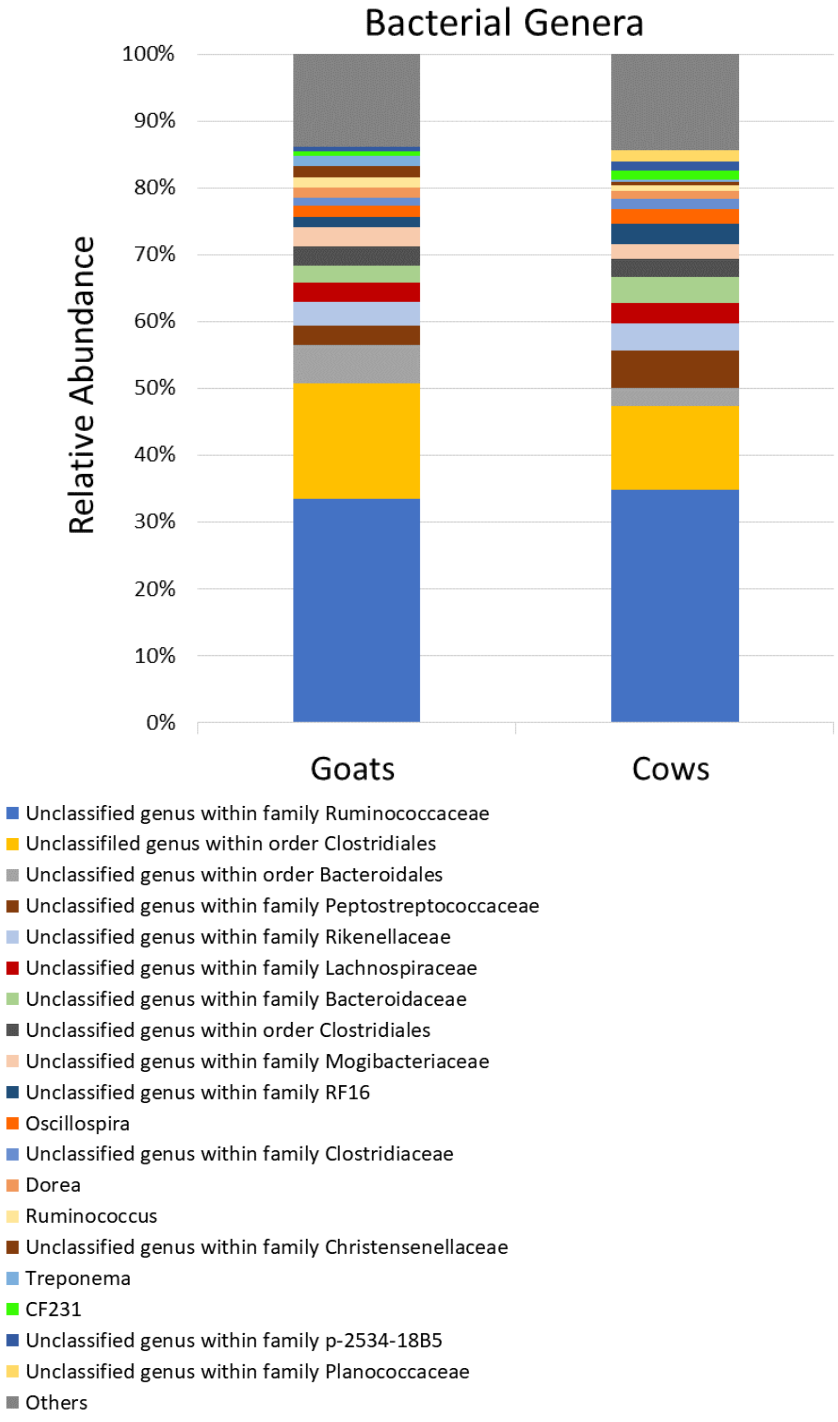



**Figure 4.4-5: Taxonomical level comparison of the fecal bacteria of goats and cows. The relative abundance is illustrated at genus level. The taxa with a relative abundance lower than 1% are summarized as "Others".**

To elucidate differences between microbiome composition of cows and goats, the Linear discriminant analysis effect size (LEfSe) was performed to determine the bacterial taxa with significantly different levels of abundance (figure 4.4-6). A total of 63 significantly different abundant taxa were identified in the two groups of animals (linear discriminant analysis score > 2). Twenty-three taxa had significantly higher relative abundance in group of goats (green bars) and 40 taxa had significantly higher relative abundance in group of cows (red bars).

On the order level, the figure 4.4-7 showed a higher relative abundance of Spirochaetales in the samples of goats compared to the samples of cows. On the contrary, the relative abundance of Bacillales was higher in the samples of cows compared to the samples of goats. On the family level, *Paraprevotellaceae* and *p-2534-18B5* (order Bacteroidale), *Peptostreptococcaceae* (order Clostridiales) and *Planococcaceae* (order Bacillales) were significantly more abundant in cows compared to goats. In contrary, *Christensenellaceae* (order Clostridiales) and *Spirochaetaceae* (order Spirochaetales) were significantly more abundant in goats compared to cows. At the genus level, *Treponema* (family *Spirochaetaceae*) and *Ruminococcus* (family *Ruminococcaceae*) were higher in relative abundance in group of goats, while *CF231* (family *Paraprevotellaceae*) was higher in cows.

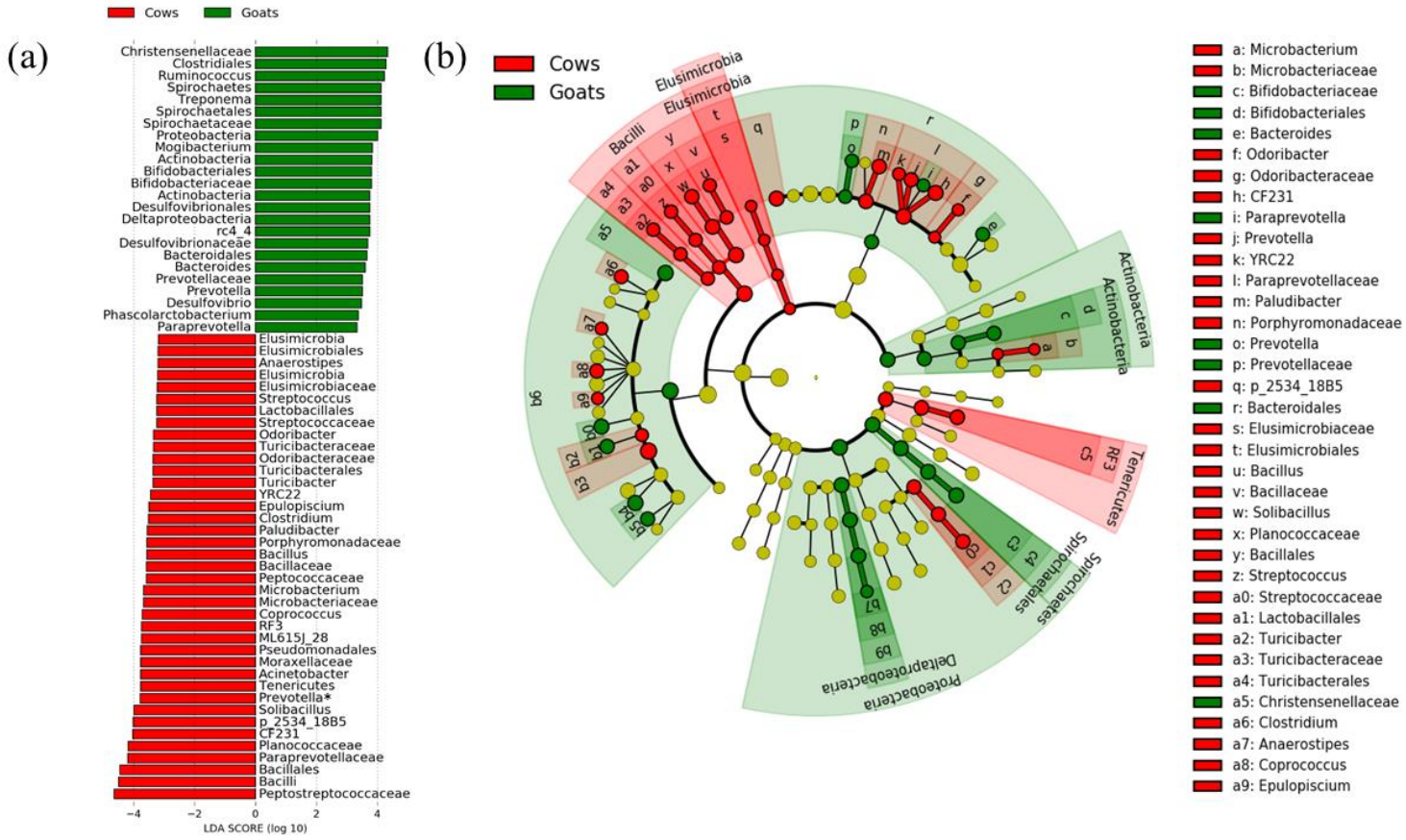



**Figure 4.4-6: Linear discriminant analysis (LDA) scores. (a) The bar plots represent the significantly differential taxa between cows (red) and goats (green). (b) Cladogram showed the differences in enriched taxa in cows (red) and goats (green). * This genus *Prevotella* belongs to the family *Paraprevotellaceae*.**

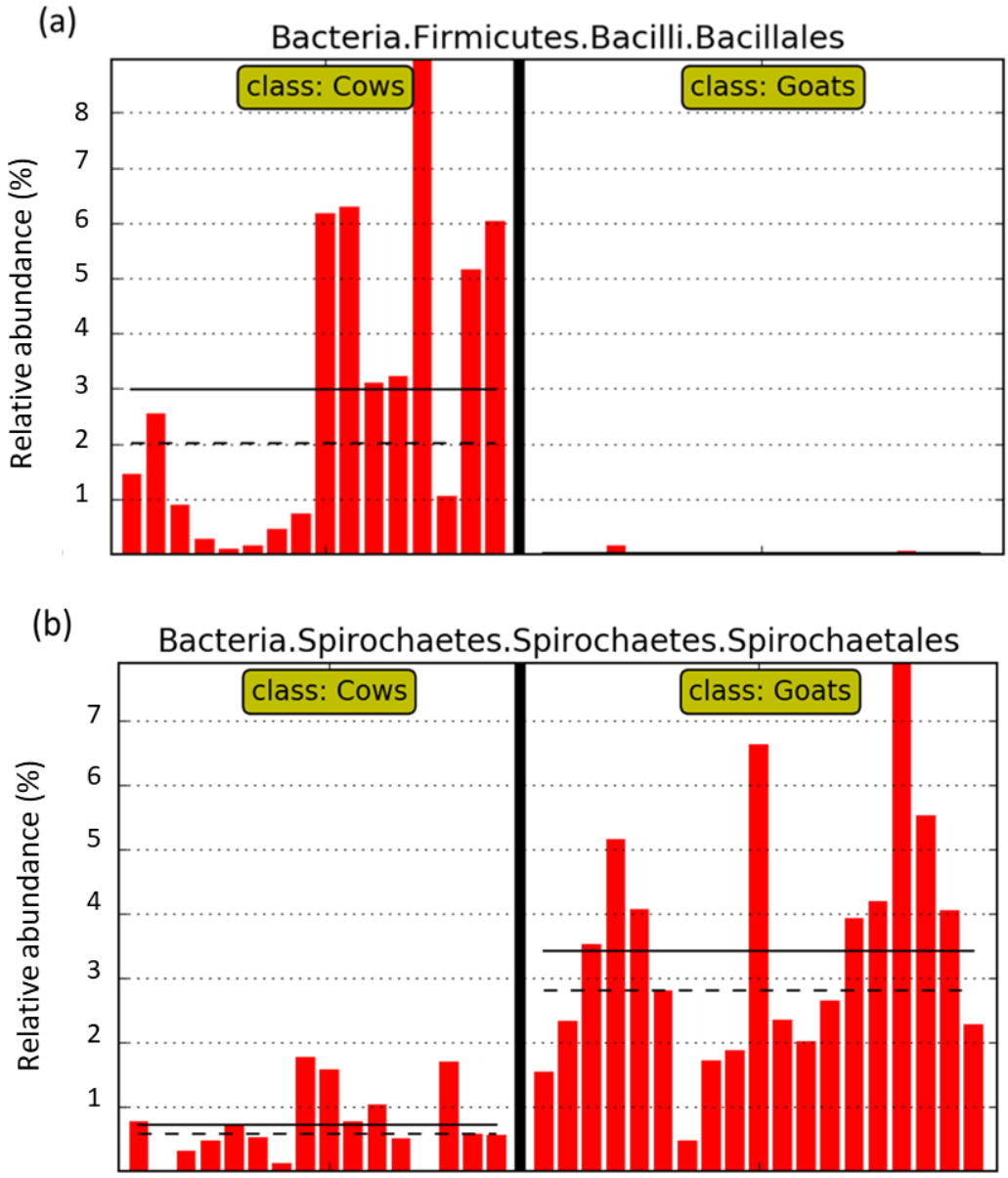



**Figure 4.4-7: Histogram showing the differences in bacterial abundance of Spirochaetales and Bacillales in each sample of goats and cows. (a) The order Bacillales was higher in cows ranging from 0.02% till 9% (mean 3% in cows). (b) The order Spirochaetales was higher in goats ranging from 0.5% till 8% (mean 1.6% in goats). The horizontal straight lines indicate the group means and the dotted lines indicate the group medians.**

# 5. DISCUSSION

Microbial community plays an important role in the overall nutritional and health status of the animals. Bacteria are the most prevalent microorganisms inhabiting the digestive tract of herbivores. For this reason, the study of bacterial diversity and composition in the gastro-intestinal tract of ruminants is important for understanding the microbial ecosystem and improving the digestive processes.

Recently, many studies were performed to determine the factors influencing microbial population, including host's diet (Henderson *et al.,* 2015; Fliegerova *et al.,* 2021; Hua *et al.,* 2017; Zhang *et al.,* 2019; Plaizier *et al.,* 2017; Grilli et al., 2016; Mao *et al.,* 2016), age (Zhang *et al.,* 2019; Fonty, 1987; Dias *et al.,* 2017; Liu *et al.,* 2017) and genetics (Wallace *et al.,* 2019; Difford *et al.,* 2018; Li *et al.,* 2019). Few of them explored the effect of host species on the rumen ecosystem (Henderson *et al.,* 2015; Ferreira *et al.,* 2017; Qian *et al.,* 2017; Zhang *et al.,* 2019), however, the knowledge about this domain remains still limited.

In this study, we examined and compared the diversity and composition of bacterial community of cows and goats' feces using advanced molecular biology technique. The two groups of animals were fed the same high fiber diet (pasture and hay) and kept in same location (Ceto, Italy). Both breeds were kept together, which can indicate the high probability of inter-animal transfer of rumen microbiota. Thus these circumstances of the animal husbandry provide ideal conditions for elucidating the extent to which the microbiome of digestive tract is affected by the host animal.

There are many differences between cows and goats due to the body size, rumen size and passage rate of rumen content, feeding behavior, intake, digestive function, utilization of nutrients, water economy and turnover rate and digestive efficiency (Hume, 2005; Silanikove, 2000; Giger-Reverdin *et al.,* 2019). Some of these differences are inherent, but others result from adaptation and interaction with various environmental factors. All these factors certainly affect the rumen ecosystem and bacterial diversity. Many studies however described a diet as a major determinant of bacterial community structure (Henderson *et al.,* 2015; Smith *et al.,*

2020), which implicates that the significant differences in the composition of the microbiome between cows and goats fed the same diet could not be supposed.

Indeed, in our study, the indices of alpha diversity describing mainly number of different species, which is richness and species diversity, i.e. the uniformity of individual distribution in the community, did not show significant differences between cows and goats. Also the bacterial phylogenetic diversity was not significantly different between the studied groups of animals. On the other hand, the animal species had the influence on the count of individual bacterial species. The Pielou Evenness index, which is a measure of biodiversity which quantifies how equal the community is numerically, revealed a significant difference, showing a higher species evenness in cows compared to goats. The algorithms calculating the distance between samples (i.e. beta diversity) also revealed the separation of goats and cows. Each group of animals form its own cluster and both clusters were well separated and community difference was statistically significant. However, the high variability within each group has to be taken into consideration. The individual bacterial composition in feces resulted in the spreading of samples in the Principal coordinates analysis (PCoA) and was the reason of the difference in dispersion between groups and therefore the PERMDISP analysis indicated significant differences between groups. If the groups of animals were compact in their fecal bacterial composition, then the PERMDISP would be insignificant. All used algorithms (Bray-Curtis, Jaccard, weighted/unweighted UniFrac) resulted in the same outcomes indicating significant differences between cows and goats regardless of whether qualitative or quantitative measure of community differences were taken into account and regardless including/excluding of phylogenetic relationships between features. However, the difference between the two groups of host animals was increased with the weighted UniFrac distance (55.1% on axis 1) compared with the unweighted UniFrac distance (27.8% on axis 1), because the weighted analysis takes into consideration not only presence/absence of taxa, but also the relative abundance of certain bacteria, while unweighted analysis evaluates only presence/absence of bacterial taxa. This indicates that relative abundance of certain bacteria contributed to the bacterial community difference between cows and goats.

Our results are in agreement with the work of Qian et al. (2017) comparing bacterial composition of rumen contents of cattle, red deer and sheep fed the same diet. Their study did not find any significant difference in the bacterial richness (Chao1) and diversity (Shannon) indices, but revealed the significant distances in the bacterial community structure across all three hosts using weighted UniFrac distance algorithm, which considers quantitative measure of community differences, while unweighted UniFrac distance algorithm, which considers qualitative measure of community differences, resulted in much worse separation. This indicates the connection between relative abundance of bacteria and host species, similarly to our results. Ferreira et al. (2017) compared cows, sheep and goats grazing together in the same area and described a clear effect of animal species on rumen bacterial structure, even if the method (T-RFLP) used was different. Zhang et al. (2019) also described significant differences in the rumen bacterial composition among dairy goats and different breeds of cattle (high-yield dairy cows, low-yield dairy cows, Luxi cattle and Bohai Black cattle), but the study did not specify, if the animals were fed the same diet or not.

Despite the significant differences found in bacterial diversity and composition between goats and cows, the dominant bacteria in feces were similar for cows and goats. The most abundant phyla Firmicutes and Bacteroidetes are known to be dominant in all ruminants, even if they differ in their prevalence. The prevalence of Firmicutes has been found in many studies of ruminal bacterial composition in cattle and goats (Noel *et al.,* 2017; Cunha *et al.,* 2011; Tajima *et al.,* 1999; Kim *et al.,* 2011; Li et al., 2014) and the majority of the studies done on fecal bacterial community of cows and goats found Firmicutes as dominant phylum regardless of the type of diet (Plaizier *et al.,* 2017; Hagey *et al.,* 2019; Shanks *et al.,* 2011; Mao *et al.,* 2012; Huang *et al.,* 2020; De Jesus-Laboy *et al.,* 2012). On the other hand, several studies have found Bacteroidetes to be the most abundant phylum in the rumen (Smith *et al.,* 2020; Qian *et al.,* 2017; Zhang *et al.,* 2019; Jewell *et al.,* 2015). Regarding goats, dominance of Firmicutes was observed in rumen fluid of goats fed high fiber diet, while Bacteroidetes was dominant phylum in goats fed high grain diet (Fliegerova *et al.,* 2021; Hua *et al.,* 2017). The similar trend was described for cows as well (Deusch *et al.,* 2017). Another study including a large cohort (n=334) of dairy cows fed high concentrate diet (forage-to-

concentrate ratio of 45:55) described the similar abundance of Firmicutes and Bacteroidetes (Xue *et al.,* 2018).

The fecal bacteriome of both cows and goats analyzed in this work is characterized by very high numbers of unclassified taxa. This is in well agreement with study of Henderson et al. (2015), who analyzed bacteria from more than 700 samples from ruminants and camelids and identified unclassified *Lachnospiraceae*, *Ruminococcaceae*, Bacteroidales and Clostridiales as the most abundant groups. In our study *Ruminococcaceae*, *Lachnospiraceae*, unclassified Bacteroidales and Clostridiales formed together 66.8% in goats and 59.9% in cows. On the genera level, majority of 79.1% bacteria in goats and 79.6% in cows were unclassified and 4 classified genera (*Oscillospira*, *Dorea*, *Treponema*, *Ruminococcus*) formed 6.2% in goats and 4.4% in cows.

Surprisingly, we have not detected *Prevotella* genus in our samples, which was identified by Henderson et al. (2015) as the most abundant genus in cattle (15%) and mainly in goats (30%). Absence of this bacterium in our samples is interesting finding, because *Prevotella* is known to be versatile genus, which exhibits substantial metabolic diversity and is present in the rumen of different animals across a variety of diets (Henderson *et al.,* 2015; Grilli *et al.,* 2016).

Our results indicate that the distance between cows and goats was caused by quantitative differences of some bacterial taxa. Linear discriminant analysis effect size (LEfSe) showed a total of 63 bacterial taxa with significantly different relative abundance in goats and cows. Mainly, the relative abundance of Spirochaetales, *Christensenellaceae*, *Spirochaetaceae*, *Treponema* and *Ruminococcus* was higher in goats compared to cows. In contrary, the relative abundance of Bacillales, *Paraprevotellaceae*, *p-2534-18B5*, *Peptostreptococcaceae*, *Planococcaceae* and *CF231* was higher in cows compared to goats. This is in line with the study of Ferreira et al. (2017), describing a high relative abundance of the *Ruminococcaceae* family in goats that was not detected in sheep and cattle. However, our findings are in disagreement with the study of Zhang et al. (2019), who found that the abundance of *Ruminococcus* was significantly higher in the rumen of high-yield dairy cows compared to those in the rumen of dairy goats. Also the abundance of unclassified genus *CF231* was

significantly lower in the rumen of high-yielding dairy cows compared to the rumen of dairy goats. These contradictive results may be related to the diet as well as difference between the breeds of animals, because several studies have found significant differences in the ruminal microbial population among several breeds of cattle (Sadan *et al.,* 2020; Zhu *et al.,* 2021).

The positive outcome of our study is, that we have not detected any potentially pathogenic or opportunistic microbes such as *Campylobacter*, *Salmonella*, *Bergeriella* or taxa of *Neisseriaceae*, which have been found by some researchers in cows (Abu Aboud *et al.,* 2016; Hagey *et al.,* 2019) and goat (Wetzels *et al.,* 2015, Li *et al.,* 2014). The incidence of opportunistic bacteria in mentioned studies can be associated with high-grain diet. Increased portion of grains in diet is used to saturate the nutritional demands of animals. The high-grain diet can satisfy the ruminant's energy and proteins needs, in order to achieve a better performance, but, on the other hand, it can cause alterations in the rumen microbiome (Mao *et al.,* 2013; Nagata *et al.,* 2018), leading even to metabolic disorders (Khiaosa-ard and Zebeli, 2018; González *et al.,* 2012). The influence of high-grain diet on rumen microbial communities is well studied in economically important ruminants (Fliegerova *et al.,* 2021; Hua *et al.,* 2017; Zhang *et al.,* 2019; Plaizier *et al.,* 2017; Grilli *et al.,* 2016; Mao *et al.,* 2016; Petri *et al.,* 2013), but the microbiome of grazing animals is less studied.

In terms of feed efficiency, differences in the efficiency of fermentation processes between goats and cows cannot be simply inferred from differences in their bacterial community, even though a relationship between grazing behavior and bacterial composition in the rumen is known (Ferreira *et al.,* 2017). In general, it is known that cattle are typical grazers (Hodgson *et al.,* 1991), while goats are known to be browsers and mixed feeders (Clark *et al.,* 1982). They have different abilities to utilize plant resources, resulting in different productive responses. Therefore, efficient utilization of forage varies by species. Cattle have a higher intake and digestive ability than small ruminants because they have a larger intestinal capacity (Ferreira *et al.,* 2017). On the other hand, goats seem to have more specialized bacterial populations in their rumen allowing them to digest more fibrous feeds. Indeed, our results suggest an increase in fibrolytic bacteria, specifically *Ruminococcus*, which was found to have a higher relative abundance in goats compared to cows. However, feed efficiency

depends on many factors. On high-fiber, low-quality forages, goats have better digestive efficiency than other ruminants and one of the main reasons for this is the longer mean retention time of digesta in the rumen. Goats can also detoxify tannins and polyphenols (Giger-Reverdin *et al.,* 2019) and they are highly resistant to environmental factors (Huston, 1978).

The study of the microbiome of pasture-raised animals is very important with the respect to Europen Community organic farming support. Action Plan aims to reach the target of 25% of agricultural land under organic farming at the EU level by 2030 (2021/2239(INI)). Moreover, the requirements for organically grown animal-derived products are rising constantly due to increasing demands for consumer products having minimal inputs of chemicals. Organic products are becoming more and more popular commodities, which make claims to a desirable characteristic and features of organic farming systems (Sutherland *et al.,* 2013). This means not only a lack of synthetic chemicals used in production processes, but consumers purchasing organic food also expect higher welfare standards for animals in comparison with non-organic production systems (Hughner *et al.,* 2007; Rosati and Aumaitre 2004, Jones *et al.,* 2016). Pastured ruminants are also healthier, showing lower levels of lameness, hoof pathologies, hock lesions, mastitis, uterine disease and mortality compared to animals on continuously housed systems (Arnott *et al.,* 2017). However, several studies have reported an increase in milk somatic cell count and bacterial load, leading to an increased risk of mastitis when ruminants are on pasture during summer (Regi *et al.,* 1987; Lamarche *et al.,* 2000; Busato *et al.,* 2000; Baroni *et al.,* 2006).

In addition, the ruminant grazing management has positive effect on landscape ecology contributing to grassland biodiversity maintenance (Rook and Tallowin, 2003; Claps *et al.,* 2020). Products from pasture-raised animals are known to be healthier and more nutritious compared to those in stable-raised animals without the possibility of moving and grazing outside. Organic meat and dairy products are lower in calories and total fat having higher levels of vitamins and a more beneficial balance of omega-3 and omega-6 fats and level conjugated linoleic acid (CLA) than conventional products (Jensen, 2000; Prandini *et al.,* 2007; Claps *et al.,* 2020).

Given the expected increase in the number of pasture-raised animals, it is very important to link this type of animal husbandry with a scientific approach. Microbes occupying the digestive tract of ruminants are crucial for providing the host with energy from the breakdown of plant cell wall carbohydrates. A better understanding of this complex microbiome, the dietary factors that influence it, the host's impact on the rumen microbiome and the impact of rumen fermentation on the host is the key to developing approaches to improve the conversion of animal fibrous diet into edible food for human.

# 6. CONCLUSIONS

In the present study we determined the diversity and the composition of the bacterial population in the feces of cattle and goats using high-throughput sequencing. The subsequent conclusions were derived from the results.

A relatively high diversity of bacteria was found in goats and cattle's feces.

Alpha diversity showed that the richness in species between the two groups of animals was similar, however a higher species abundance equity in cows compared to goats was revealed.

Beta diversity showed distinct clustering of bacterial composition between goats and cows and PERMANOVA revealed statistically significant dissimilarities between the two groups of animals ($p < 0.05$).

Concerning the taxonomical composition, the dominant bacteria at phylum level was Firmicutes, followed by Bacteroidetes, Proteobacteria and Spirochaetes for both goats and cows. The fecal microbiome at genus level for both animals was composed by *Oscillospira*, *Treponema*, *Ruminococcus*, *CF231* as well as Unclassified *Ruminococcaceae*, *Peptostreptococcaceae*, *Rikenellaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Mogibacteriaceae*, *RF16*, *Clostridiaceae*, *Christensenellaceae*, *p-2534-18B5*, *Planococcaceae*, Clostridiales and Bacteroidales.

A total of 63 significantly different taxa were found between goats and cows. A higher relative abundance of Spirochaetales, *Christensenellaceae*, *Spirochaetaceae*, *Treponema* and *Ruminococcus* was found in goats compared to cows. In contrary, a higher relative abundance of Bacillales, *Paraprevotellaceae*, *p-2534-18B5*, *Peptostreptococcaceae*, *Planococcaceae* and *CF231* was found in cows compared to goats.

Our results suggest that ruminants' species have a significant influence on the bacterial structure composition in feces. Comparing cows and goats, the impact is caused mainly by the different relative abundance of several bacterial species, while the bacterial species richness and diversity were similar for both groups of animals.

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