# Correlations Between Concentration of Vitamin B12 in Milk and the Composition of the Bovine Microbiota

**Julian Lopez**
Department of Food Science and Agricultural Chemistry

**Faculty of Agricultural and Environmental Sciences**
**McGill University, Montreal**

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**This thesis is dedicated to my family**

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## Preface

The preparation of this thesis followed the graduate and postdoctoral studies thesis guidelines of McGill University. This thesis is written and organized in the traditional monograph-style, containing six chapters; introduction, literature review, methods, results, discussion, and conclusion.

### Contribution of Authors

I am the primary author of the six chapters of this thesis. I conducted the research work, participated in the extraction and sequencing of the bacterial DNA from milk, rumen and fecal samples, analyzed the data and wrote this thesis under the supervision of Professor Jennifer Ronholm. An Bui and Coralie Raymond participated in the extraction of bacterial DNA in rumen and fecal samples, respectively. Melissa Duplessis and William Poisson collected the samples and measured vitamin B12 concentrations in each sample. Daniel Rico designed the rumen sampling strategy. Roger Cue provided statistical analysis of the relationships between the vitamin B12 concentrations at each body site.

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## List of Abbreviations

| | |
|---|---|
| ADF | Acid detergent fiber |
| AdoCbl | Adenosylcobalamin |
| Cbl | Cobalamin |
| CFU | Colony-forming unit |
| CNCbl | Cyanocobalamin |
| CP | Crude protein |
| DC | Dendritic cells |
| DIM | Days in milk |
| DMBI | Dimethylbenzimidazole |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| FDA | Food and Drug Administration |
| FDR | False discovery rate |
| GRAS | Generally recognized as safe |
| HMECs | Human mammary epithelial cells |
| IF | Intrinsic factor |
| LDA | Linear Discriminant Analysis |
| LEfSe | Linear discriminant analysis effect size |
| MCM | Methylmalonyl CoA mutase |
| MDP | Marker Data Profiling |
| MeCbl | Methylcobalamin |
| mL | Milliliter |

| | |
|---|---|
| mm | Millimetre |
| mRNA | Messenger RNA |
| NDF | Neutral Detergent Fiber |
| NFC | Non-Fiber carbohydrate |
| ng | Nanogram |
| NMDS | Non-metric Multidimensional Scaling |
| OHCbl | Hydroxocobalamin |
| OTU | Operational Taxonomic Units |
| PCoA | Principal Coordinate Analysis |
| pg | Picogram |
| RBS | Ribosome-binding site |
| RDA | Recommended daily intake |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| SCC | Somatic cell count |
| SCFA | Short-chain fatty acids |
| UCG | Uncultured Genus |
| VFA | Volatile fatty acids |

## Abstract

Vitamin B12 is an essential nutrient that only bacteria and archaea can synthesize. Humans must acquire vitamin B12 through diet, and most Canadians obtain their recommended daily intake (RDI) of vitamin B12 through the consumption of milk or other dairy products. However, the vitamin B12 concentrations found in a 250 mL glass of milk are highly variable and can provide between 23-56% of the RDI. This variability can result in vitamin B12 deficiency in vulnerable groups despite the regular consumption of dairy products. The goal of this project was to identify bacterial populations that inhabit the rumen, lower intestine, and mammary gland that are associated with either very high- or very low-vitamin B12 concentrations in milk. Vitamin B12 is produced by bacteria and archaea in the rumen and absorbed into the cow's bloodstream in the intestine, while during circulation, the vitamin B12 can be used by the cow for her own metabolic needs and a proportion of what remains is secreted into the milk. The microbes present in the lower intestine also require vitamin B12 for survival and may metabolize it before it is absorbed into the bloodstream, but this microbial population can also synthesize the vitamin. It is also possible that this vitamin is consumed by the microbial population residing in the mammary gland of the cow, and that this population also affects the final concentration of vitamin B12 in milk. In this study, we collected rumen, blood, fecal, and milk samples from 47 dairy cows at two time points. We measured the vitamin B12 present in each sample, and characterized the microbial population in the rumen, fecal, and milk samples using 16S rRNA targeted amplicon sequencing. We found that the vitamin B12 concentration in milk is not statistically correlated to the concentration of vitamin B12 in the rumen. However, there is a strong correlation between the presence of *Prevotella, Succinivibrio,* and *Shuttleworthia* in the rumen with high concentrations of vitamin B12 in the rumen. Specific bacterial taxa correlated with high levels of vitamin B12 in the intestine and

mammary gland could not be clearly identified. This study has provided potential targets for modifying the rumen microbiome for increased vitamin B12 production.

**Résumé**

La vitamine B12 est un nutriment essentiel que seules les bactéries et les archées peuvent synthétiser. Les humains doivent acquérir de la vitamine B12 dans leur régime alimentaire et la plupart des Canadiens obtiennent leur apport journalier recommandé (RDI) en vitamine B12 en consommant du lait ou d'autres produits laitiers. Cependant, les concentrations de vitamine B12 trouvées dans un verre de lait de 250 mL sont très variables et peuvent fournir entre 23 et 56% du RDI. Cette variabilité peut entraîner une carence en vitamine B12 chez les groupes vulnérables malgré la consommation régulière de produits laitiers. Ce projet visait à identifier les populations bactériennes présentes dans le rumen, le bas intestin et les glandes mammaires et associées à des concentrations très élevées ou très faibles en vitamine B12 dans le lait. La vitamine B12 est produite par les bactéries et les archées dans le rumen et est absorbée par le sang de la vache dans le gros intestin. Pendant la circulation, elle est utilisée par la vache pour ses propres besoins métaboliques et une partie de ce qui reste est sécrétée dans le lait. Les microbes présents dans le bas de l'intestin peuvent également utiliser la vitamine B12 avant son absorption dans la circulation sanguine, mais cette population microbienne peut également synthétiser la vitamine. Il est également possible que cette vitamine soit consommée par la population microbienne résidant dans la glande mammaire de la vache et que cette population affecte également la concentration finale de vitamine B12 dans le lait. Dans cette étude, nous avons prélevé des échantillons, à deux reprises, de rumen, de sang, de selles et de lait chez 47 vaches laitières. Nous avons mesuré la vitamine B12 présente dans chaque échantillon et caractérisé la population microbienne dans les échantillons de rumen, de selles et de lait en utilisant le séquençage d'amplicon ciblés par l'ARNr 16S. Il est important de noter que nous avons constaté que la concentration de vitamine B12 dans le lait n’est pas corrélée statistiquement à la concentration de vitamine B12 dans le rumen. Cependant, il existe

une forte corrélation entre les genres *Prevotella*, *Succinivibrio* et *Shuttleworthia* et des concentrations élevées de vitamine B12, ce qui suggère que ces bactéries pourraient être des productrices de vitamine B12. En revanche, les taxons bactériens en corrélation avec la consommation bactérienne de vitamine B12 dans l'intestin et la glande mammaire n'ont pas pu être clairement identifiés. Cette étude a fourni des cibles potentielles pour la modification du microbiome bovin afin de fournir des taux élevés et constants de vitamine B12 naturelle dans le lait.

# Chapter 1. Introduction

## 1.1 General Introduction

Vitamin B12 is an essential nutrient that can only be synthesized by bacteria and archaea (Roth *et al*. 1996). Although this is an essential vitamin for health, humans cannot synthesize it and must acquire it through diet. Vitamin B12 deficiency can cause pernicious anemia, cognitive disabilities, neuropathy, and sustained spinal cord degeneration (Hoffbrand, 2015). Canadians obtain most dietary vitamin B12 through the consumption of dairy products, milk is an ideal source of this vitamin since it can withstand pasteurization and is not destroyed by daylight or prolonged refrigerator storage (Duplessis *et al*. 2016). Drinking one 250 mL glass of milk per day is advertised to provide about 46% of the recommended vitamin B12 requirements; however, in practice, concentrations are highly variable. For example, when analyzed for a research study a 250 mL glass of milk was found to contain anywhere from 23-56% of the daily recommended vitamin B12 intake (Duplessis *et al*. 2016).

Bacteria and archaea synthesize vitamin B12 in the rumen of dairy cows, some vitamin B12 is then absorbed into the bloodstream in the small intestine, while unabsorbed vitamin B12 is excreted in the feces (O'Leary & Samman, 2010). The cow uses some of the circulating vitamin B12 for her own nutritional requirements, and a proportion of the remaining vitamin B12 is secreted in her milk. It is known that some of the commensal bacteria in the rumen and lower digestive tract, and possibly the mammary gland, also consume vitamin B12 (Girard *et al.* 2009*)*. Therefore, the final concentration of vitamin B12 in milk is the difference between the amount produced and the amount utilized by both the cow and her microbiome. However, the detailed knowledge of the specific members of the bacterial communities that both produce and consume vitamin B12 is not known. Detailed knowledge of the specific bacterial taxa involved in vitamin

B12 production and consumption in the rumen, lower intestine, and mammary gland could contribute to targeted attempts to manipulate these populations and increase vitamin B12 levels and consistency in bovine dairy products – thus improving human nutrition.

### 1.2 Rationale

There is potential to optimize the use of the microbiota of dairy cattle to increase the nutrients in milk – this is particularly true for vitamin B12 which is only synthesized by members of the microbiota. It is known that both diet and bovine genetics play a role in determining the vitamin B12 content of milk. However, neither of these variables fully explain the observed variance, and it is likely that the exact composition of the microbiota plays a major role. Therefore, this project was undertaken to elucidate which particular bacterial taxa are correlated with the level of vitamin B12 ultimately found in bovine milk. If clear correlations are identified, future work aimed at modifying the microbiota for optimal vitamin B12 production is feasible.

### 1.3 Research Hypothesis

Specific bacterial taxa in the bovine rumen, lower intestine, and mammary gland can be correlated with the concentration of vitamin B12 in milk.

### 1.4 Specific Thesis Research Objectives

To answer the research hypothesis and meet the general objective of this thesis, four specific objectives were defined:

1) Simultaneously collect milk, rumen, fecal, and blood samples from 47 dairy cattle at two different time points (June 2018 and August 2018).

2) Use a 16S rRNA targeted amplicon sequencing strategy to characterize the bacterial community present in each rumen, fecal, and milk sample.

3) Measure the vitamin B12 concentration of each rumen, fecal, blood, and milk sample.

4) Integrate the 16S rRNA targeted amplicon sequencing datasets with the vitamin B12 concentration metadata to identify correlations between particular microbial taxa and the concentration of vitamin B12.

# Chapter 2. Background and Literature Review

## 2.1 Vitamin B12

### 2.1.1 Corrinoid Chemistry

Vitamin B12 is an essential component of human nutrition that is exclusively synthesized by certain bacteria and archaea (Roth *et al*. 1996). The important role of vitamin B12 in human health was discovered by Minot and Murphy (1926) who identified a correlation between a diet rich in cattle liver (which is high in vitamin B12 content) and the improvement of pernicious anemia. However, the discovery of the actual molecule took place in 1948 when Abeles and Dolphin (1976) isolated it for the first time. The vitamin B12 molecule is composed of three parts: a central corrin ring, an upper (Co-β) axial ligand, and a lower (Co-α) ligand containing a nucleotide loop (Martens *et al*. 2002) (Figure 1). Molecules, such as vitamin B12 that contain central corrin rings, are classified as corrinoids. Vitamin B12 contains a cobalt atom at the center of its corrinoid ring, and therefore can also be known as cobalamin (Martens *et al*. 2002).

Vitamin B12 is part of a group of structurally related vitamers that include over a dozen corrinoids, eight of which have been found in the human gut in varying proportions (Allen & Stabler, 2008). These related corrinoids include adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin (OHCbl), and cyanocobalamin (CNCbl) (Martens *et al*. 2002; Takano *et al*. 2015) (Table 1). AdoCbl and MeCbl have vitamin B12 activity, but other forms are considered pseudovitamin B12 and do not have enzymatic activity in vitamin B12 dependent enzymes (Biedendieck *et al*. 2010; Guo & Chen 2018). Pseudovitamin B12 can be produced by some bacteria including *Streptomyces griseus*, *Escherichia coli*, and *Propionibacterium shermanii* (Ellenbogen & Cooper, 1991). The dynamic interaction between vitamin B12 and other corrinoids has been demonstrated by analyzing the bacteria of the human intestine (Allen & Stabler, 2008).

Certain bacteria can use vitamin B12 and convert it into a series of several corrinoids in a process known as corrinoid remodeling. In this process, the lower ligand of the corrinoid is removed, and another one is added (Gray & Escalante-Semerena, 2009). The process of corrinoid remodeling provides bacteria with the ability to obtain the specific corrinoids that they require for their metabolism from a larger pool of related molecules (Gray & Escalante-Semerena, 2007). Pseudovitamin B12, as well as other corrinoids, can also be used by some bacteria to produce vitamin B12 through a series of biosynthetic steps called the salvage pathway (Takahashi *et al.* 2012).

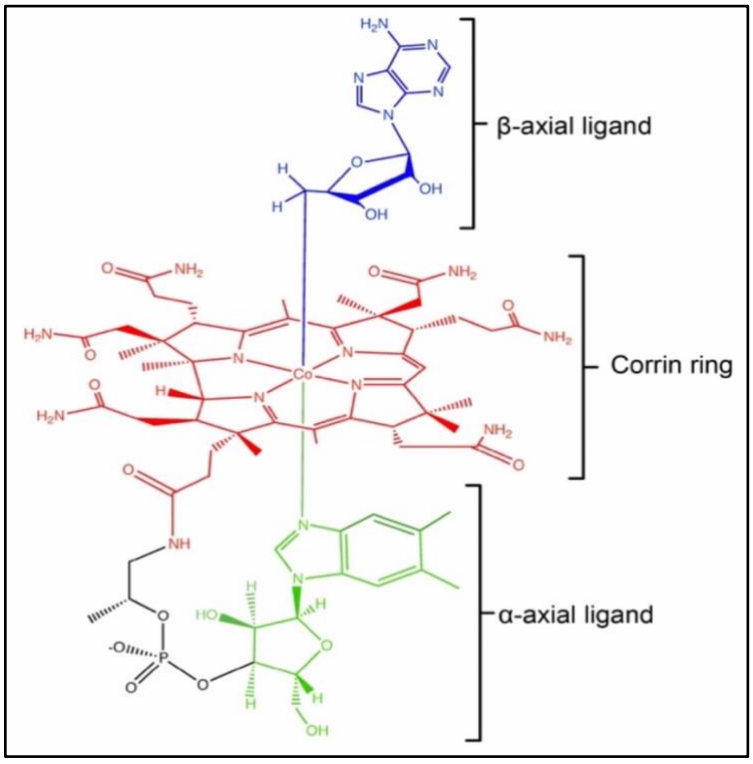


**Figure 1. Chemical Structure of Vitamin B12**. Vitamin B12 is a member of a family of vitamers that each contain a cobalt positioned in the center of a corrin ring. Each of the vitamers in this family differ based on the composition of the β-axial ligand.

**Table 1. Cobalamin molecule β-axial ligand and corresponding cobalamin name (**Krautler, 2005)

| β-axial ligand | Name |
|---|---|
| R´=CH3;R=CN: | Vitamin B12 |
| R´=CH3;R=CH3: | Methylcobalamin |
| R´=CH3;R=5´-deoxy-5´adenosyl | Coenzyme B12 |
| R´=CH3;R=e-: | Cob(II)alamin (B12r) |
| R´=CH3;R=CN: | Pseudovitamin B12 |
| R´=H; R=CN: | Pseudovitamin B12 |

### 2.1.2 Physiological Functions of Vitamin B12

Vitamin B12 is a co-factor for several enzymes involved in critical metabolic pathways in humans and other mammals. Functions of vitamin B12 include: i) purine and pyrimidine synthesis; ii) transfer of methyl groups; iii) formation of proteins from amino acids; and iv) carbohydrate and fat metabolism (McDowell, 2000). The two main vitamin B12 dependent-enzymes in mammals are methionine synthase and methylmalonyl CoA mutase (MCM) (Takahashi *et al.* 2012). Methionine synthase catalyzes the methylation of homocysteine to methionine, using folate and vitamin B12 as co-factors (Moretti & Caruso, 2019). Since vitamin B12 is critical to the proper metabolism of folic acid, vitamin B12 deficiency also leads to folic acid deficiency – even when folic acid is in adequate supply, which results in anemia (McDowell, 2000). In addition, since vitamin B12 is required for the proper metabolism of homocysteine, homocysteine increases in the plasma of vitamin B12 deficient individuals triggering potential health problems such as cognitive and cardiovascular diseases (McCaddon *et al*. 2002; Miller, 2003). MCM catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondrion, which is involved in the digestion of branched amino acids and odd-chained fatty acids to produce succinyl-CoA, an intermediate product of the Krebs cycle (the main energy-yielding metabolic pathway in cells) (Takahashi *et al*. 2012).

### 2.1.3 Dietary Sources of Vitamin B12

Vitamin B12 production is limited to prokaryotic biosynthesis by a subset of bacteria and archaea. However, the majority of eukaryotic organisms (excluding plants and fungi) have enzymes that require vitamin B12 as a cofactor (Roth *et al*. 1996). For this reason, mammals must rely on diet or bacterial symbiotic relationships to obtain their daily vitamin B12 requirements (Fang *et al*. 2017; Sych *et al*. 2016). In ruminants, the rumen microbiota provides a reliable source of vitamin B12, since the microbial community responsible for synthesis (rumen) is upstream of the site of absorption (small intestine) (Girard *et al.* 2009). However, in humans, the microbial population responsible for vitamin B12 synthesis resides in the large intestine, which is downstream from the small intestine, which has the receptors for vitamin B12 absorption (Degnan *et al*. 2014b). Since, humans do not engage in coprophagy, they must acquire vitamin B12 through the consumption of animal-based food products, or artificially fortified foods. For Canadians, the primary food source for acquiring vitamin B12 is through the consumption of dairy products (Girard *et al.* 2009).

Other common sources of vitamin B12 in diet are animal-based products such as red-meat, fish, and eggs (Gille & Schmid 2015). High levels of vitamin B12 in animal-based products are associated with the microbial synthesis in the animals' digestive system as well as the physiological construction of their digestive system (Watanabe, 2007). For example, in ruminants, vitamin B12 is produced in the rumen and then transported to the intestine where it is effectively absorbed into the bloodstream (Girard *et al*. 2009; Degnan *et al*. 2014b). Carnivores are capable of bioaccumulating vitamin B12 in their livers, allowing them to maintain reserves of the vitamin (Engelking, 2010).

Vitamin B12 concentrations in plant-based products are low (Watanabe *et al*. 2014; Watanabe *et al.* 2002), and the trace levels vitamin B12 sometimes measured in plants are typically associated with either environmental contamination or fermentation processes (Denter & Bisping, 1994; Watanabe *et al*. 2014). Edible algae are an exception to this rule and can provide a substantial amount of vitamin B12 (Watanabe *et al*. 2002). The two most widely consumed edible algae are dried green algae (*Enteromorpha spp*.), and edible purple laver also called nori (*Porphyra spp.*) (Watanabe *et al*. 1999). Edible algae contain concentrations of vitamin B12 ranging from 32 to 78 µg/100 g dry weight (Watanabe *et al*. 2002). Studies which examined vitamin B12 deficiency in rats showed that hepatic levels of vitamin B12 increased in these mammals after feeding them purple laver (Takenaka *et al*. 2001). These results suggest that algae could be a source of vitamin B12 and an alternative for a strict vegetarian or vegan population. However, the bioavailability of edible algae vitamin B12 in humans is unknown. Initial studies have indicated that the vitamin B12 present in algae has no effect on the blood levels of vitamin B12 in deficient children, and this suggests that algae vitamin B12 may not be as bioavailable to humans as it is to rats (Dagnelie *et al*. 1991). Therefore, vegetarians and vegans are encouraged to maintain a controlled intake of vitamin B12 through supplements or fortified foods to prevent deficiency (Baik & Russell, 1999; Allen *et al*. 2010; Watanabe *et al*. 2014).

#### 2.1.3.1 Eukaryotic Vitamin B12 Absorption

All vertebrates have a metabolic requirement for vitamin B12, and this requirement can be met directly if the host mammal is able to absorb the vitamin B12 produced by the gut microbiota, or indirectly if the host mammal cannot absorb the vitamin B12 produced by its microbiota, but instead must consume food items rich in vitamin B12 (Degnan *et al.* 2014b). In vertebrates whose

small intestine precedes their large intestine the vitamin B12 produced in the large intestine is not absorbed. However, in animals that practice coprophagy, such as mice and beavers, the vitamin B12 produced in the large intestine is consumed along with fecal material by litter mates, and therefore these animals also receive a direct source of vitamin B12 (Mickelsen, 1956). Since, humans must acquire vitamin B12 through diet, elaborate mechanisms exist in the human intestine that allow vitamin B12 absorption while blocking the absorption of other corrinoids. This specificity of absorption likely exists to protect vitamin B12-dependent enzymes from exposure to corrinoids that cannot function effectively as co-factors (Nielsen *et al*. 2012). The human vitamin B12 intestinal absorption system is saturated around 1.5–2.0 µg per meal; therefore, to have adequate vitamin B12 nutrition the vitamin must be consumed regularly, instead of just in high-amounts at a single time point (Watanabe, 2007). If ultra-high levels (e.g. 1000-fold higher than recommended daily allowance) of vitamin B12 are consumed in a single feeding through supplementation, there will be higher levels of vitamin B12 and all other corrinoids in the feces, but not in the plasma, and these changes are transient with all corrinoid levels returning to normal in about 10 days (Allen & Stabler, 2008). The corrinoids increase in the feces is associated with the bacterial remodeling of vitamin B12 in the intestinal tract, where the lower ligand of this molecule is changed (Gray & Escalante-Semerena, 2009).

Vitamin B12 absorption in the human small intestine is a process that requires three proteins: haptocorrin, intrinsic factor (IF), and transcobalamin (Nielsen *et al*. 2012). In the stomach, vitamin B12 is released from food components through digestion and is bound by haptocorrin. If the vitamin B12 remains bound to proteins, and not in its free form, it may not bind haptocorrin and may not be readily absorbed. Haptocorrin will remain attached to vitamin B12 until it reaches the duodenum. In the duodenum, haptocorrin is degraded by intestinal enzymes,

and free vitamin B12 binds IF. In the small intestine, IF-B12 complex is endocytosed by a receptor complex called cubam, which is expressed on enterocytes in the ileum and is responsible for the absorption of most vitamins (Nielsen *et al.* 2012). Inside the enterocyte, IF is degraded and vitamin B12 is released into the plasma from the basolateral side (Festen, 1991). The level of IF in the intestine can be depleted due to auto-immune gastritis resulting in vitamin B12 deficiency and the development of pernicious anemia (Lahner & Annibale, 2009), or neurological complications (O'Leary & Samman, 2010). Vitamin B12 absorption can also be affected by medications or infections (Ankar & Bhimji, 2018). In the serum, transcobalamin binds vitamin B12, and approximately 50% is delivered to the liver, where it is stored, but it can also be re-introduced to the intestinal tract via the biliary system, and the remainder will be distributed to other tissues (Nielsen *et al* 2012).

The vitamin B12-transcobalamin complex is normally used to transit vitamin B12 to a variety of cell types (Morkbak *et al*. 2007). Mammary epithelial cells are thought to acquire vitamin B12 in the same way other cells do, via an endocytic receptor that binds the vitamin B12-transcobalamin complex from plasma (Quadros *et al*. 2009). For example, human mammary epithelial cells (HMECs) have a low affinity for haptocorrin bound with vitamin B12, but a very high affinity for transcobalamin bound with vitamin B12 (Adkins & Lonnerdal, 2001). HMECs use a transmembrane receptor called CD320 protein to acquire vitamin B12 (Quadros *et al*. 2009). Upon endocytosis by HMECs, transcobalamin is degraded in the cell lysosome to release vitamin B12, and then vitamin B12 is stored, used, or expelled (Nielsen *et al*. 2012). The mechanism of secretion of vitamin B12 by mammary epithelial cells into milk is thought to be similar as the secretion system present from in body cells like gut epithelial cells: a protein transmembrane called ABCC1 can transport vitamin B12 in its free form to milk (Beedholm-Ebsen *et al*. 2010). Finally,

free vitamin B12 could create a complex with the haptocorrin present in milk to be ready to be metabolized by newborns (Nielsen *et al*. 2012). The same vitamin B12 transporter proteins have been found in cow's mammary epithelium, and consequently, a similar process is thought to happen in the bovine mammary gland, however, empirical evidence of this is still lacking (Rutten *et al*. 2013).

The anatomy of dairy cattle (*Bos taurus*) is significantly different from that of humans or mice where the small intestine precedes the colon. Dairy cattle are classified as ruminants which indicates that they have a four-chamber stomach including the rumen, reticulum, omasum, and abomasum (Colville & Bassert, 2015; Hasheider, 2007) (Figure 2). In general, ruminant digestion allows animals to survive in harsh environments with few nutrients, since stomach is designed to process and extract energy from large volumes of material that are low in nutrients (Dehority, 2003).

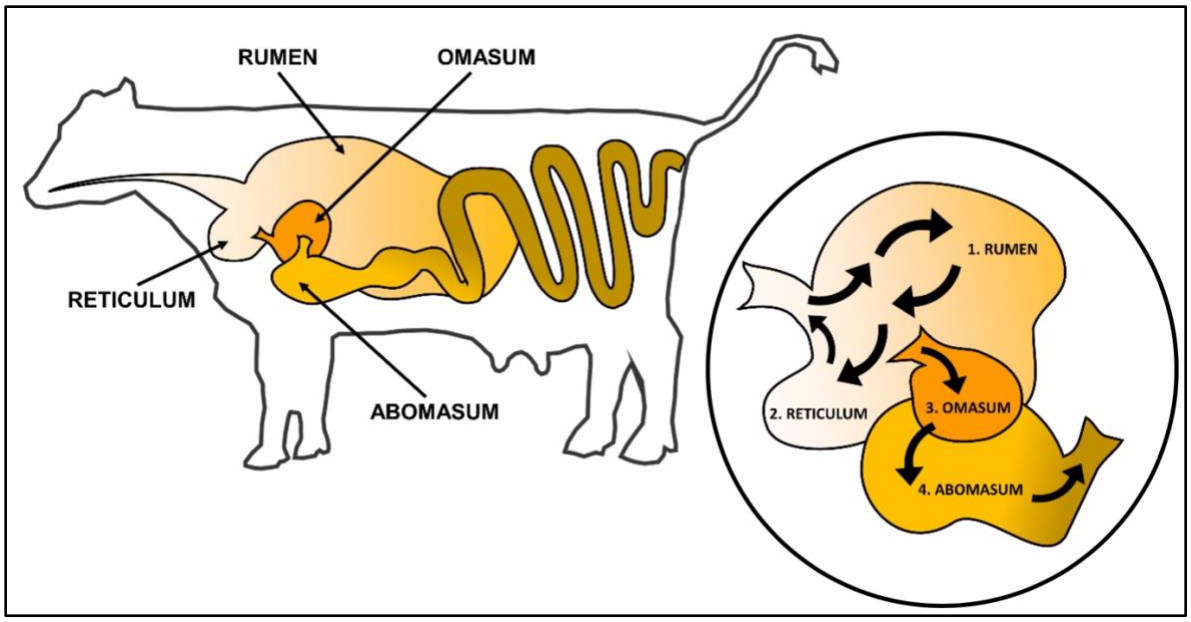


**Figure 2. The Ruminant Stomach Anatomy**. The ruminant stomach presents four separate chambers. Food moves first into the rumen (1), reticulum (2), omasum (3) and finally into the abomasum (4) before entering the intestine.

The rumen is the largest compartment of a cow's stomach and also contains the most dense and diverse microbial population in the bovine digestive tract (Mackie *et al.* 2000). The rumen microbiota is adapted to break-down most of the cows feed including soy, grass, silage, urea, and grains (Hall & Silver, 2001). Material flows from the rumen to the reticulum, omasum, abomasum, and then into the small intestine. Most nutrients including vitamin B12 are absorbed in the small intestine, before material flows into the large intestine which absorbs excess water. Finally, fecal material is transported and expelled via the anus (Hall & Silver, 2001). Unlike monogastric mammals, like human and mice, cows are able to absorb the vitamin B12 that their gastrointestinal bacteria produce since the site of production comes prior to the site of absorption and thus have no need to consume it through diet (Torres *et al*. 2016).

The intestinal absorption process in ruminants has been found to be similar to the one in humans, where the binding of the complex vitamin B12-IF with specific receptors at the ileum level is a crucial step in the absorption process (Girard *et al*. 2009). However, some studies indicate that ruminal absorption is performed in a biphasic pattern. Girard *et al*. (2001) reported that 50% of vitamin B12 absorbed was released to the portal vein during the first 4-10 hours post ingestion and 35% 20-24 hours, after the intake of vitamin B12, the net flux of vitamin B12 to the portal vein being still positive. The existence of two different transport mechanisms could explain the biphasic pattern of absorption of vitamin B12 (Girard *et al.* 2001). Passive absorption, which is a simple diffusion through ileum, could explain the increased and low efficiency of absorption to the portal vein during the first 4 hours post ingestion. The second mechanism, which requires IF and receptor proteins on the epithelial cells, has a saturable transport process and could be responsible for the net flux of vitamin B12 to the portal vein after 24 hours (Le Grusse & Watier, 1993; Combs, 1998; Quadros *et al*. 1999). According to previous studies, around 1.4% of 500 mg

of a vitamin B12 dose is absorbed to the portal vein; similar absorption rates have been found in sheep with a 1-3 % of the dose absorbed under same vitamin dose conditions (Smith & Marston, 1970; Girard *et al*. 2001).

### 2.2 Prokaryotic Synthesis of Vitamin B12

Vitamin B12 synthesis is complex and has ancient evolutionary roots (Raux *et al.* 1999). Due to the elaborate structure and high metabolic cost of synthesizing vitamin B12, only a few diverse bacteria and archaea are known to produce it (Doxey *et al*. 2015) (Figure 3). It is unclear why some prokaryotes maintain vitamin B12 synthesis for potential community benefit when it is so costly to produce (Giovannoni, 2012). Vitamin B12 can be synthesized by either of two pathways: the *de novo* pathway or the salvage pathway. Both pathways can be performed in either anaerobic or aerobic environments (Figure 4) (Yin and Bauer, 2013).

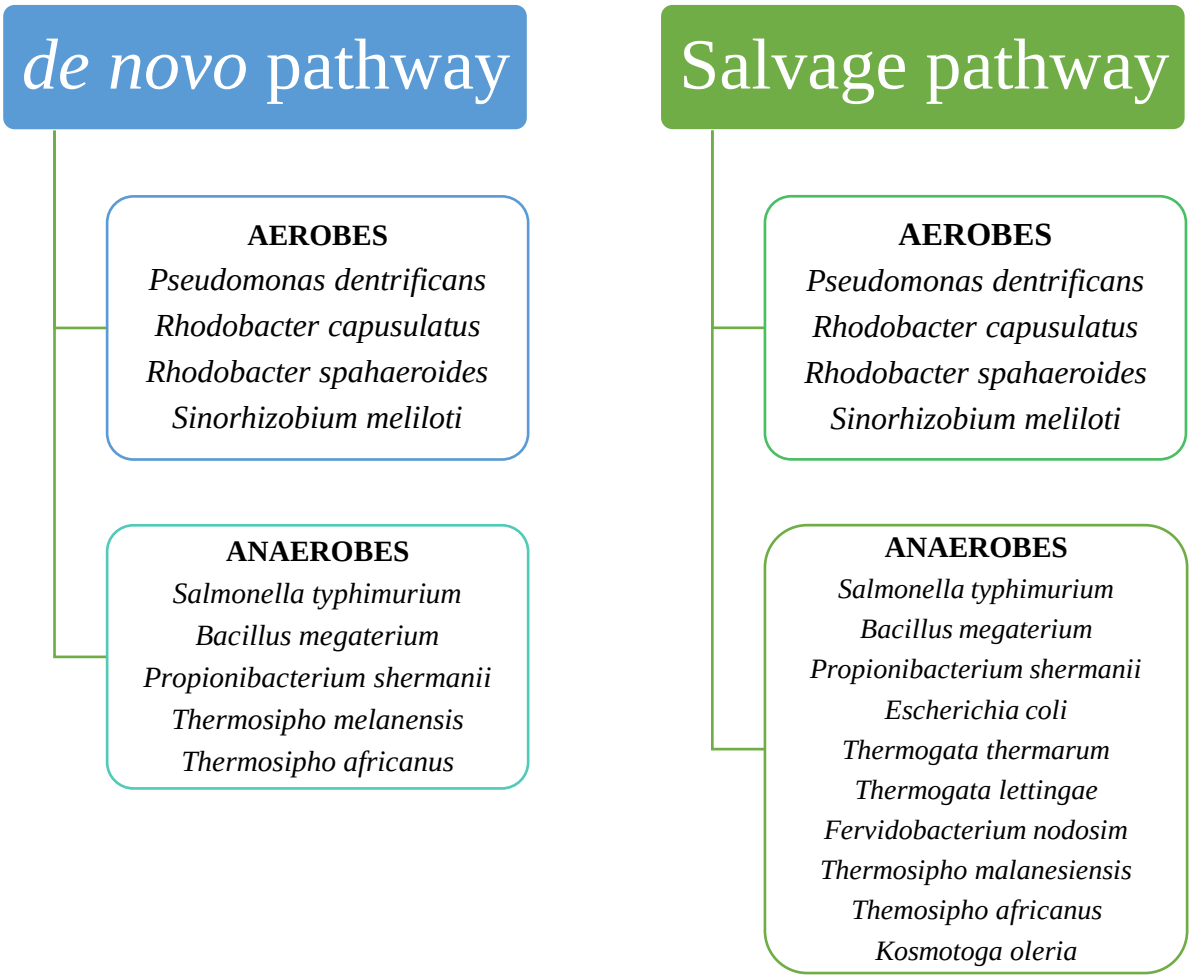


**Figure 3. Primary Vitamin B12 Producing Bacteria Involved in Both Biosynthetic Pathways** (Martens *et al*. 2002; Fang *et al.* 2017). Vitamin B12 can be synthesized by the de novo pathway, where the vitamin is synthesized from glutamate and a cobalt atom, or the salvage pathway, which requires the presence of corrinoid precursors. Both pathways can be performed in either aerobic or anaerobic environments.

The *de novo* synthesis pathway involves more than 30 genes and starts with glutamate to synthesize vitamin B12 (Martens *et al*. 2002). The primary difference between the aerobic and anaerobic versions of this pathway is the timing of addition of the central cobalt atom, which occurs earlier in the anaerobic pathway. The aerobic pathway also requires oxygen to promote ring-contraction, while the anaerobic pathway does not have this requirement (Martens *et al*. 2002; Fang *et al*. 2017). The aerobic and anaerobic pathways follow the same order of peripheral

methylation (Escalante-Semerena & Warren, 2008). However, the reactions diverge at the substrate precorrin-2 until the production of adenosyl cobyric acid (Fang *et al*. 2017). Many of the methyltransferase enzymes involved in these reactions show high degrees of sequence similarity (Gray & Escalante-Semerena, 2007).

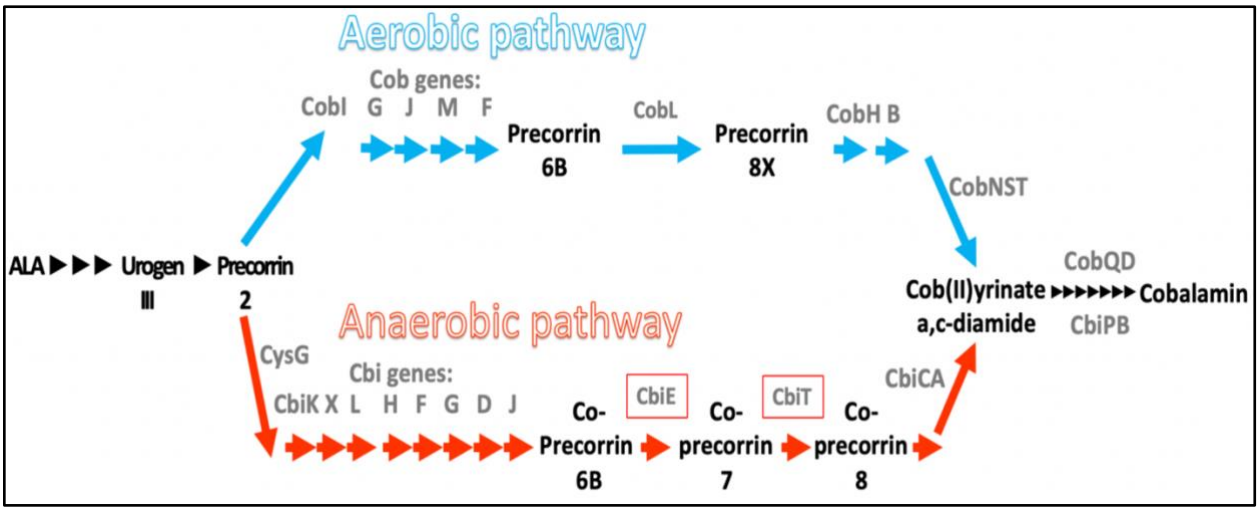


**Figure 4. Vitamin B12 Biosynthetic Pathways**. Diagram representation of vitamin B12 biosynthetic pathways and genes associated with the synthesis of cobalamin from its precursor ALA. Cobalamin precursors are shown in black while in gray all the genes involved in the vitamin B12 biosynthetic process. Cob genes (CobI, CobG, CobJ, CobM, CobF, CobL, CobHB, CobNST, and CobQD) are involved in the aerobic pathway converting precorrin 2 into Cob (II)yrinate a, c-diamide in presence of oxygen. Similarly, Cbi genes (CbiK, CbiX, CbiL, CbiH, CbiF, CbiG, CbiD, CbiJ, CbiE, CbiT, CbiCA, and CbiPB) are part of anaerobic pathway converting percorrin 2 into Cob (II)yrinate a,c-diamide in absence of oxygen. Cob (II)yrinate a,c-diamide is finally transformed to cobalamin (vitamin B12) by CobQD (aerobic pathway) or CbIPB (anaerobic pathway).

The salvage pathway is a biosynthetic pathway that requires energy through ATP hydrolysis to convert corrinoids into vitamin B12 (Gray & Escalante-Semerena, 2007). In Gram-negative bacteria, exogenous cobalamins are transported into the bacteria via an ATP-binding cassette (ABC) transport system (membrane permease-ATPase) that consists of BtuC, BtuD, and BtuF (periplasmic-binding protein components) to be transformed into vitamin B12 (Fang *et al.* 2017; Escalante-Semerena, 2007) (Figure 5). The ABC transport system for corrinoid uptake has also been found in archaea (Woodson *et al*. 2005).

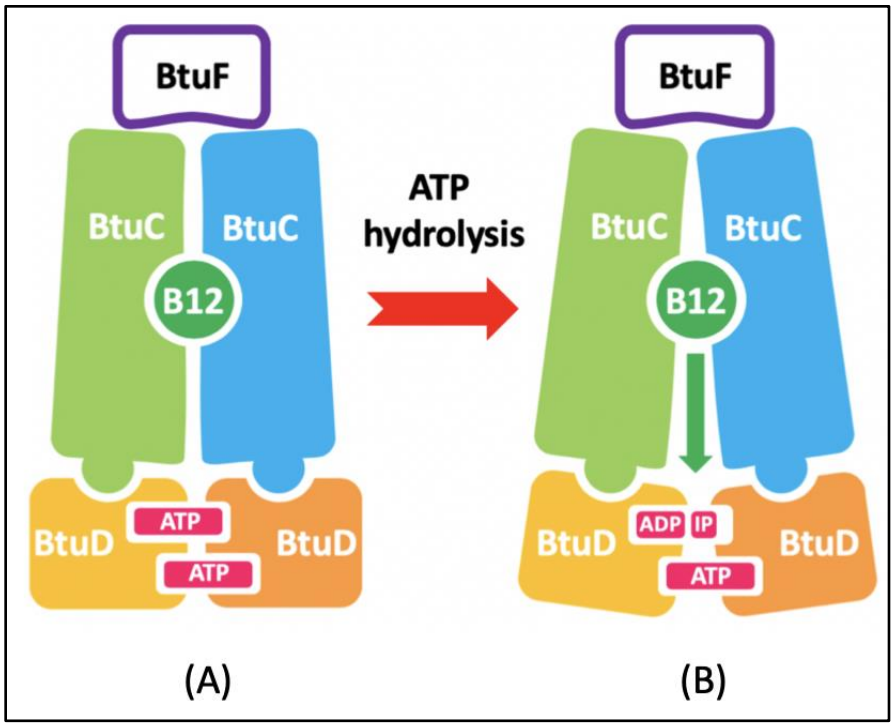


**Figure 5. ATP-binding Cassette (ABC) Transport System, BtuCD Transporter.** Schematic diagram of the conformational changes triggered by hydrolysis of one ATP at the active site and transport of vitamin B12 into cell. Upon BtuF and vitamin B12 binding, the transporter turns into a closed state and contains vitamin B12 in the middle of the transporter system (Figure 5A). After hydrolysis of one of the binding ATPs, an active site is disrupted between BtuDs proteins leading to the opening of the transportation system and vitamin B12 translocation into the cell (Figure 5B).

At the molecular level, vitamin B12 riboswitches (small RNAs) work regulating the synthesis of vitamin B12, vitamin B12 transport proteins, and the expression of other vitamin B12 -dependent enzymes (Vitreschak *et al*. 2003). Vitamin B12 riboswitches regulate the expression of btuB gene which encodes a vitamin B12 transporter protein, and the cob operon that regulates vitamin B12 synthesis in *Salmonella enterica* serovar Typhimurium (Vitreschak *et al*. 2003). The expression of vitamin B12 riboswitches is negatively regulated by the presence of vitamin B12 (Lundrigan *et al*. 1991; Richter-Dahlfors & Andersson 1992). For example, in *Escherichia coli*, the btuB leader sequence can bind to vitamin B12 (AdoCBL) and provoke conformational changes in the RNA (Vitreschak *et al*. 2003). This conformation change creates two alternative RNA

structures, repressing and anti-repressing vitamin B12 translation (Nahvi *et al*. 2002). Another translational regulation mechanism of these genes is the inhibition of the initial translation by a conserved RNA hairpin that blocks the ribosome-binding site (RBS) (Richter-Dahlfors *et al*. 1994; Nou & Kadner, 1998). High vitamin B12 concentration can also sequester RBS and interrupt the translation initiation. Alternatively, a low vitamin B12 concentration promotes the formation of an anti-terminator hairpin, enabling RNA polymerase to complete transcription of the downstream gene (Mandal & Breaker, 2004; Serganov & Nudler, 2013). Finally, once the bacteria produce a high concentration of vitamin B12, an alternative Rho-Independent termination hairpin or Rho binding induces the premature transcriptional termination (Mandal & Breaker, 2004).

### 2.3 Synthetic Vitamin B12 Production

The first completely synthetic production of vitamin B12 occurred in 1973, and the process included 70 synthesis steps. The completely chemical synthesis of vitamin B12 is difficult and expensive (Woodward, 1973). Thus, the primary industrial method for producing synthetic vitamin B12 occurs via microbial fermentation (Vandamme, 1992; Martens *et al*. 2002). *Pseudomonas denitrificans*, *Propionibacterium shermanii*, and *Sinorhizobium meliloti* are the bacterial taxa most commonly used in this process due to their high vitamin B12 productivity and rapid growth rates (Martens *et al*. 2002; Fang *et al*. 2017) (Table 2). The genus *Propionibacterium* is generally recognized as safe (GRAS) and is most commonly used for the synthetic production of vitamin B12 in the supplement industry (Piwowarek *et al*. 2017).

**Table 2. Species of microbial producers and microbiological processes recommended for producing vitamin B12** (Martens *et al*. 2002).

| Species of microorganism | Main component of culture medium | Conditions of fermentation | Vitamin B12 production (mg/l) |
|---|---|---|---|
| *Propinobacteium freudenreichii* | Glucose | Anaerobiosis | 206.0 |
| *Rhodopseudomonas promaticus* | Glucose | Anaerobiosis | 135.0 |
| *Propionibacterium shermanii* | Glucose | Anaerobiosis | 60.0 |
| *Pseudomonas denitrificans* | Sucrose | Aerobiosis | 60.0 |
| *Nocardia rugose* | Glucose | Aerobiosis | 18.0 |
| *Rhizobium cobalaminogenum* | Sucrose | Aerobiosis | 16.5 |
| *Micromonospora sp.* | Glucose | Anaerobiosis | 11.5 |
| *Streptomyces olivaceus* | Glucose | Anaerobiosis | 6.0 |
| *Nocardia gardneri* | Hexadecane | Aerobiosis | 4.5 |
| *Butyribacterium nethylotrophicum* | Methanol | Anaerobiosis | 3.6 |
| *Pseudomonas sp.* | Methanol | Anaerobiosis | 3.2 |
| *Arthrobacter hyalinus* | Isopropanol | Anaerobiosis | 1.1 |

The fundamental difference between *Propionibacterium* and *Pseudomonas* is the fermentation process required during vitamin B12 production. *Propionibacterium* species are good producers of vitamin B12; however, they have specific growth requirements for vitamin B12 production such as low oxygen concentrations, long fermentation cycles, and complex and expensive media requirements (Martens *et al*. 2002). Vitamin B12 production using *Propionibacterium* requires two stages. The first stage includes three days of fermentation where the bacteria grow in an anaerobic environment and produce the precursor of vitamin B12, in the second stage the intermediate molecule is linked to cobamide to complete the formation of vitamin B12 and this latter step also takes from one to three days (Eggersdorfer, 1996).

*P. denitrificans* can produce vitamin B12 under aerobic conditions in just three days (Scott, 1998). The mechanisms that *P. denitrificans* uses to synthesize vitamin B12 have been studied in detail, and several strains of *P. denitrificans* have been improved by mutagenesis (Martens *et al*. 2002). *P. denitrificans* produces vitamin B12 via the aerobic pathway, and each of the genes

associated with this process have been studied in detail (Table 3) (Martens *et al*. 2002). Genomic studies performed in *S. enterica* serovar Typhimurium (Roth *et al*. 1996) and *Bacillus megaterium* (Raux *et al*. 1997) have also helped to identify the biosynthetic genes required in the anaerobic synthesis of vitamin B12 (Table 3).

Several attempts have been made to increase the production of vitamin B12 in the organisms used in synthesis fermentations. The most common strategy is to use random mutagenesis to create high producing strains. Mutagenesis is achieved by the treatment of microorganisms with mutagenic agents such as UV light, followed by the selection of strains with practical advantages such as: increased productivity, improved growth rates, and genetic stability (Martens *et al.* 2002).

**Table 3. Pathway products and genes required for the transformation of ALA into adenosylcobalamin** (Martens *et al*. 2002).

| **Aerobic pathway products** ***(P. denitrificans)*** | **Gene** | **Anaerobic pathway products** ***(S.typhymurium, P.shermani)*** | **Gene** |
|---|---|---|---|
| Aminolaevulinc acid | HemB | Aminolaevulinc acid | HemB |
| Prophobilinogen | HemC | Prophobilinogen | HemC |
| Hydroxymethylbilane | HemD | Hydroxymethylbilane | HemD |
| Uroporphyrinogen III | CobA | Uroporphyrinogen III | CysG (cobA) |
| Precorrin-2 | CobI | Precorrin-2 | CysG (cbiX-cysGB) |
| Precorrin-3 | CobG | Cobalt- Precorrin-2 | CbiL |
| Precorrin-3 hydroxylactone | CobJ | Cobalt- Precorrin-3 | CbiH |
| Precorrin-4 | CobM | Cobalt- Precorrin-4 | CbiF |
| Precorrin-5 | CobF | Cobalt- Precorrin-5 | |
| Precorrin-6 | CobK | Cobalt- Precorrin-6 | CbiJ |
| Dihydro Precorrin-6 | CobL | Cobalt- Dihydro Precorrin-6 | CbiE |
| Precorrin-8 | CobH | Cobalt- Percorrin 8 | CbiT |
| Hydrigenobyrinic acid | CobB | Cobronic acid | CbiC |
| Hydrigenobyrinic acid a,c-diamide | CobNST | Cob(II)yrinic acid a,c-diamide | |
| Cob(II)yrinic acid a,c-diamide | | Cob(I)yrinic acid a,c-diamide | CobA |
| Cob(I)yrinic acid a,c-diamide | CobO | ado-Cob(I)yrinic acid a,c-diamide | CobP |
| ado-Cob(I)yrinic acid a,c-diamide | CobQ | ado-Cobyric acid | Cob |
| ado-Cobyric acid | CobC,D | ado-Cobinamide | CbiU |
| ado-Cobinamide | CobP | ado-GDP-Cobinamide | CobS |
| ado-GDP-Cobinamide | CobV | Ado-Cobalamin | |

## 2.4 Prokaryotic Consumption of Vitamin B12

Several prokaryotes lack the ability to synthesize vitamin B12 although they encode vitamin B12-dependent enzymes, meaning that they also must acquire this vitamin from other prokaryotes in their community (Table 4) (Garsin, 2010). Approximately 80% of sequenced human intestinal bacteria are estimated to require exogenous vitamin B12, while less than 25% of the human microbiome can produce vitamin B12 through *de novo* synthesis (Degnan *et al*. 2014a).

The consumption of vitamin B12 by gastrointestinal bacteria results in direct competition for vitamin B12 with the host and has the potential to result in vitamin B12 deficiency in spite of adequate consumption (Degnan *et al.* 2014a). It has been found that people with a high bacterial concentration in their small intestines tend to have low serum vitamin B12 levels, and this may be due to the bacterial consumption of vitamin B12 (Albert *et al.* 1980).

**Table 4. Corrinoid-dependent enzymes in humans and their gut microbiota** (Degnan *et al.* 2014b)

| Ortholog | Humans | Gut microbiomes | Percentage of gut microbiomes |
|---|---|---|---|
| MetH | + | + | 67 % |
| MCM/Spca/MutAB | + | + | 25 % |
| RNR II/NrdJ | - | + | 35 % |
| GImEs | - | + | 8 % |
| Mgm | - | + | 1 % |
| IcmAB/Icm/Hcm | - | + | 4 % |
| EutBC | - | + | 17 % |
| PduCDE | - | + | 11 % |
| KamED | - | + | 8 % |
| OraSE | - | + | 2 % |
| QueG | - | + | 33 % |
| PceA | - | + | 1 % |
| AcsECD | - | + | 4 % |
| Mta/Mtt | - | + | 30 % |
| HpnR/Hyp | - | + | 43 % |

Several cofactor transport systems are associated with bacterial vitamin B12 uptake. The BtuBFCD transporter is the unique route of vitamin B12 acquisition in *E. coli* and is limited to Gram-negative bacteria (Chimento *et al.* 2003). However, the BtuF and ABC transporter BtuCD are present in both Gram-positive and Gram-negative bacteria. The BtuB protein is one of the most common corrinoid transporters. Homologs of the gene encoding this protein have been identified in almost 70 % of the vitamin B12-dependent Gram-negative bacteria. Each homolog is positioned

downstream of a B12 riboswitch, and the majority of species have two, three or more copies of these transport proteins, indicating the importance of vitamin B12 acquisition to cellular survival (Degnan *et al*. 2014a). Some bacteria have multiple corrinoid transporters. For example, *Bacteroides thetaiotaomicron* has three vitamin B12 transport systems: btuB1, btuB2, and btuB3. Even though all three systems are capable of transporting vitamin B12; each one exhibits different levels of affinity towards other corrinoids (Goodman *et al*. 2009). Therefore, bacteria with multiple transporters could have a higher level of competitiveness in the community relative to other bacteria that are not capable of transporting corrinoids with modified ligands. In 2014, Degnan *et al*. found that 40% of the bacteria only contain the genes required for the last steps of attachment of the lower ligands to vitamin B12. These bacteria do not possess the extra 5-20 genes necessary to initiate the synthesis of vitamin B12, indicating that a large number of bacteria take advantage of the corrinoids produced by other bacteria for their own synthesis of vitamin B12 (Degnan *et al*. 2014a).

### 2.5 Ruminant Vitamin B12 Production and Feed/ Management Practices

Dairy farms are interested in practices that improve any aspects of milk quality without using economically costly dietary supplements (Walker *et al*. 2004). Several characteristics of feed have been shown to have a correlation with the level of ruminal synthesis of vitamin B12 (Girard *et al*. 2009). In general, cattle feed is classified into two groups: 1) concentrate foods: high in energy (corn, oats, barley) and high protein products (soybean, canola, peanuts), and 2) forage: high fiber products, including, hays, silage, and straw. Both types of foods are necessary to maintain health and rumen function. However, a high ratio of forage has been correlated to an increase of vitamin B12 (Huber *et al*. 1965; Salem *et al*. 1993). For example, Santschi *et al.* (2005)

reported an increase in the synthesis of active vitamin B12 in a diet with a 60:40 forage-to-concentrate ratio diet than in a 40:60 forage to concentrate ratio diet. Vitamin B12 production in the rumen is positively correlated with the concentration of neutral detergent fiber (NDF), acid detergent fiber (ADF), and high alfalfa maturity and negatively correlated with high concentrations of non-fiber carbohydrates (NFC) such as starch, simple sugars, soluble fiber, increasing orchard grass maturity, and crude protein (CP) (Schwab *et al*. 2006; Castagnino *et al*. 2017; Castagnino *et al*. 2016). Thus, an abundance of fiber in the rumen appears to favor ruminal vitamin B12 synthesis, while starch appears to have a negative effect. Apparent ruminal synthesis is about 3-fold greater for cows receiving a high-fiber diet, when compared with cows receiving a high-starch diet (Beaudet *et al*. 2016). Meanwhile, a cobalt supplementation – cobalt at the core of the vitamin B12 molecule, improves vitamin B12 concentrations in milk, but only during early lactation (Akins *et al*. 2013; Duplessis *et al*. 2014). Also, direct intramuscular injections of vitamin B12, into dairy cows increases the levels of vitamin B12 in liver, plasma, and milk (Girard & Matte, 2005).

## 2.6 The Bovine Microbiome

### 2.6.1 Rumen and Intestinal Microbiota

The bovine rumen presents a complex microbiota that is involved in converting indigestible plant material into nutrients and energy for the animal (Dias & Ametaj, 2017). Studies have proposed that a milliliter of ruminal fluid contains around $10^{10}$ bacterial cells that represent more than 200 different species (Mackie *et al*. 2000). Bovine microbiota has a synergic relationship with the host and is adapted to metabolizing substrates present in the cow's feed including cellulose, hemicellulose, protein, starch, urea, and pectin (Zhou *et al*. 2015; Jin *et al*. 2018; Kameshwar *et al*. 2019) (Figure 6). Also, the rumen microbiota is involved in the fermentation processes that

produce volatile fatty acids (VFA) which are the primary source of energy for cows (Castillo-Gonzales *et al*. 2014).

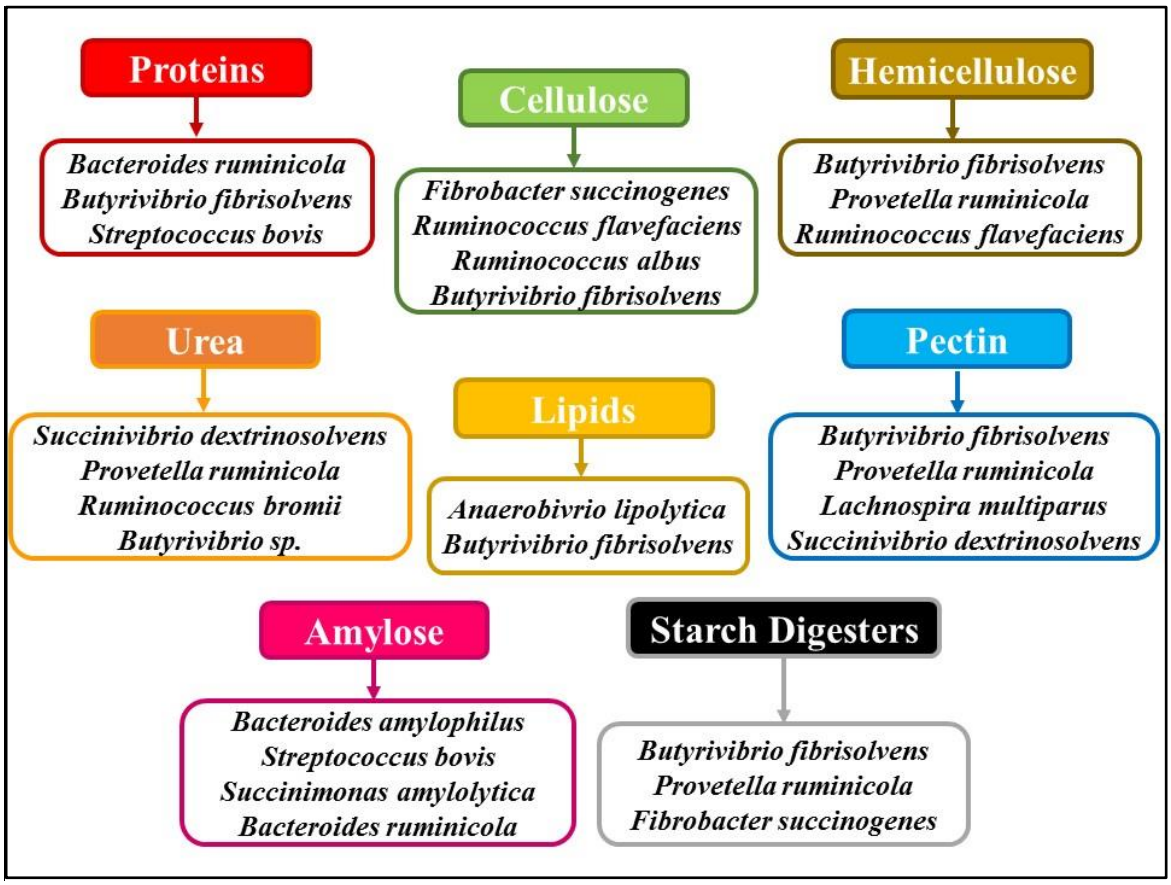


**Figure 6. Principal Microorganisms Associated with the Degradation of the Components of Cattle Feed.** This figure shows the principal nutrients and components in cattle diet (proteins, cellulose, hemicellulose, urea, lipids, pectin, amylose, starch) and bacteria that play a role in the degradation of these food components.

At the phyla level, the rumen microbiota is dominated by *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Jami & Mizrahi, 2012; Jami *et al*. 2014; Deusch *et al*. 2017). At the genus level, *Prevotella*, *Butyrivibrio* and *Ruminococcus* are the most dominant genera in the rumen. However, the exact microbiota composition is affected by the bovine health condition, geographical location, and by the diet (Li *et al*. 2018; Welkie *et al*. 2010; Jami & Mizrahi, 2012; Kittlelmann *et al*. 2013;

Pitta *et al*. 2016; Callaway *et al*. 2010; Henderson *et al*. 2015). In high forage diets, cellulose digesters are essential to degrade cellulose and hemicellulose (Figure 6) (Koike & Kobayashi, 2009). Also, if the cattle's diet is changed too quickly, an accumulation of VFA can occur, and the rumen's pH can decrease drastically, thus increasing the *Streptococcus bovis* and *Lactobacillus* species (Chen *et al*. 2012).

Rumen microbiota is the site of the synthesis of vitamins such as thiamine, riboflavin, niacin, vitamin B6, folates, and vitamin B12 (Seck *et al*. 2017). In the particular case of vitamin B12, just a few bacterial species are able to produce it (Dryden *et al*. 1962; Seck *et al*. 2017). *Selomonas spp., Butyribrio fibriosolvens and Propionibacterium shermanii* are thought to be the largest vitamin B12 synthesizers in the rumen (Stemme *et al*. 2008; Dryden *et al*. 1962; Duplessis *et al*. 2016), and although the production of vitamin B12 is directly related to the rumen microbiota, there are no studies which directly correlate rumen microbiota with vitamin B12 levels in the milk. However, some studies have found associations between microorganisms related to milk-fat yield. Jami *et al*. (2014) found associations between the ratio of *Firmicutes: Bacteroidetes* with the percentage of fat in milk. Specifically, that a high concentration of *Firmicutes* and a low abundance of *Bacteroidetes* increased milk-fat rates (Jami *et al*. 2014). This suggests that other physiological parameters in milk, like vitamin B12, could be positively correlated with specific populations in the rumen microbiota.

The microbiota of other compartments of the bovine gastrointestinal tract have received little research attention relative to the rumen, although the microbial community of the large intestine plays a vital role in animal health and can be used to monitor food quality (Shanks *et al*. 2011). The composition of the intestinal microbiota is strongly correlated to diet (Mao *et al*. 2015). For example, diets that are high in starch contribute to a higher abundance of *Bacteroidetes* and

decreased relative abundance of *Firmicutes* in large intestine samples (Shanks *et al*. 2011; Dias & Ametaj, 2017). The bovine large intestine microbiota is primarily composed of *Firmicutes* (55.3%) and *Bacteroidetes* (25.4%) with other phyla including *Proteobacteria* (2.5%), and *Tenericutes (*2.9%) (Shanks *et al*. 2011).

### 2.6.2 Milk microbiota

Milk is an excellent source of nutrients, and it contains high-quality proteins, fats, minerals, and vitamins (Pereira, 2014), and because of the high concentration of nutrients, milk is an excellent medium for microbial growth. Understanding the composition of the bovine milk microbiota is a newly emerging area of research. In the very recent past, the mammary gland was thought to be sterile, and any bacteria present in milk were assumed to be from external contamination or a chronic pathology (Addis *et al*. 2016). However, high-sensitivity next-generation sequencing techniques have revealed that diverse microbial communities reside in mammary gland and have some bacterial species in common with those in the bovine intestinal tract. A theory that states that mammary gland microbial communities that have originated in the bovine gut and were transported to mammary gland through the immune system exists and is called *the entero-mammary pathway* (Perez *et al*. 2007; Donnet-Hughes *et al*. 2010; Jost *et al*. 2014; Rodriguez, 2014; Young *et al*. 2015) (Figure 7).

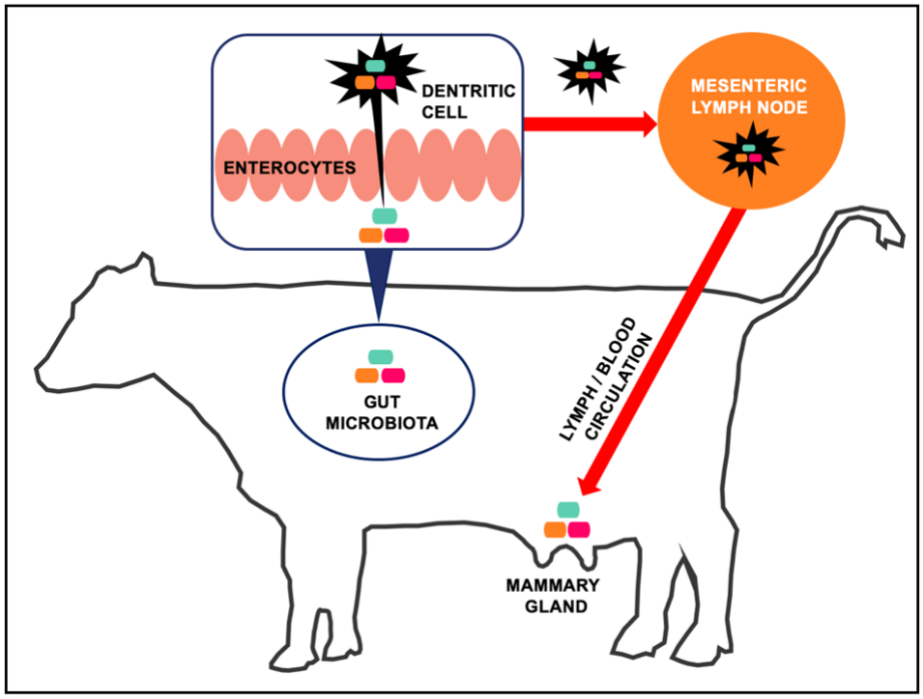


**Figure 7. Entero-Mammary Pathway in Ruminants.** The entero-mammary pathway theory suggests that intestinal bacteria can migrate to the bovine mammary gland via dendritic cells which transit these bacteria to the lymphatic nodes, blood circulation, and ultimately the mammary gland (Young *et al*. 2015).

The entero-mammary pathway is favored over the idea that the mammary gland becomes colonized by environmental contamination, since strictly anaerobic bacteria such as *Bifidobacterium* and *Ruminococcus* taxa have been isolated from raw milk, making environmental contamination less likely (Addis *et al*. 2016; Gueimonde *et al*. 2007; Young *et al*. 2015). The entero-mammary pathway proposes that some bacteria can move out from the gut lumen through dendritic cells (DC). The dendritic cells make their way between the enterocytes without damaging the epithelial barrier and travel through the mesenteric nodules to finally reach the mammary gland and be part of the milk composition (Figure 7) (Perez *et al*. 2007; Rodríguez, 2014; Jost *et al*. 2014; Donnet-Hughes *et al*. 2010).

Most work conducted to understand the mammary gland microbiota in dairy cows has focused on mastitis, and several studies have compared the bacterial populations of raw milk from cows with an active case of mastitis to healthy animals (Kuehn *et al*. 2013; Oikonomou *et al*. 2014; Zhang *et al*. 2015; Kim *et al*. 2017). However, very few studies have focused exclusively on the microbial milk population from healthy cows, although, those studies have observed that healthy bovine milk samples are mostly composed of the phyla: *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteroidetes* (Bhatt *et al*. 2012). However, environmental conditions are known to influence the composition of the mammary gland microbiota. Expansion of the *Actinobacteri*a population appears to be correlated with low temperatures, while a higher abundance of *Firmicutes* is related to high-temperatures (Li *et al*. 2018). *Bacteroidetes* populations are correlated with low humidity, while the *Proteobacteria* population is correlated with high humidity (Li *et al*. 2018). The milk microbiota has also been correlated with milk parameters like fat and protein content. A few bacteria are negatively correlated to the degradation of protein, such as: *Bacteroides*, *Streptococcus*, *Agrococcus*, *Ornithinimicrobium*, and *Atopobium* but the same bacteria have been shown to contribute to milk fat degradation (Li *et al*. 2018). Further studies are necessary to understand the complicated relationships between the milk microbiota and other nutritional raw milk parameters.

In this work, I aim to further understand the relationships between specific bacterial taxa in the bovine rumen, lower intestine, and mammary gland and the final concentration of vitamin B12 in milk.

# Chapter 3. Methods

## 3.1 Animal Care Approval

The experimental protocol was approved by the Institutional Committee for Animal Care of the Agriculture and Agri-Food Canada Research and Development Centre (Sherbrooke, Quebec, Canada). Care and use of cows followed the recommended code of practice of the National Farm Animal Care Council (2009).

## 3.2 Sample Collection Design

Forty-seven Holstein cows fitted with cannulas from the dairy herd at the Agriculture and Agri-Food Canada Research and Development Centre (Sherbrooke, Quebec, Canada) were involved in this study. All animals were co-housed, and the diet was controlled, consistent, and recorded. Milk, fecal, blood, and rumen samples were collected at the same time point for each cow, and most animals were sampled twice with about three months separating sampling events producing a total of 92 complete (milk, rumen, fecal, and blood) sample sets. Sampling was performed between May and August 2018. Cows were at different physiological stages of lactation, but each animal was free from mastitis with somatic cell count (SCC) values lower than 250,000 cells/mL during sampling. Sample collection was performed by trained and qualified members of the Agriculture and Agri-Food Canada Research and Development Centre Team (Sherbrooke, Quebec, Canada).

### 3.2.1 Milk Sampling

Milk samples were collected using a sterile technique. An initial check of the milk was performed by stripping the teat 4-5 times and collecting the milk onto a dark-bottomed container

to examine the milk for clumps or off-colouring. Then the teat was placed in an iodine pre-dip followed by a 30 seconds contact time. The teat was then dried with a clean and disposable wipe. Each teat was wiped with 70% ethanol and the milk was collected manually, using a gloved hand, into 50 mL falcon tubes which were immediately placed on ice. Samples were labeled and stored at –20°C until processing.

### 3.2.2 Rumen Sampling

Whole rumen digesta samples were collected approximately 8 hours after feeding according to Rico *et al*. 2015. Digesta samples were collected through a cannula sequentially from five different sections of the rumen: 1- cranial dorsal, 2- cranial ventral, 3- central, 4- caudal dorsal, and 5-caudal ventral), and mixed. The composite sample (approximately 50 mL) was lyophilized at -20° C (Ultra 35-XL; Virtis Company, Inc.) and ground in a Wiley mill grinder (A. H. Thomas Co., Philadelphia, PA) and passed through a 1 mm sieve. Each sample was labeled and stored at -20° C until processing.

### 3.2.3 Feces Sampling

Fifty milliliters of feces were collected by direct rectum sampling. The rectal wall was massaged to stimulate rectal evacuation and resulting feces were collected. Samples were lyophilized at -20° C (Ultra 35-XL; Virtis Company, Inc.) and ground in a Wiley mill grinder (A. H. Thomas Co., Philadelphia, PA) and passed through 1 mm sieve (Rico *et al*. 2014). Finally, all samples were labeled and stored at -20°C until processing.

### 3.2.4 Blood Sampling

Blood samples were taken by caudal venipuncture using a vacutainer system (Becton, Dickinson and Co., Franklin Lakes, NJ). Blood was collected in tubes with the anticoagulant

ethylenediaminetetraacetic acid (EDTA) for B12 analyses. Blood samples were centrifuged within one hour of collection for 15 minutes at 3,000 × g and 4°C. Plasma samples were labeled and stored at –20°C until analysis.

### 3.3 Vitamin B12 Quantification

#### 3.3.1 Rumen and Feces Vitamin B12 Quantification

This method was adapted from AOAC International (2005; 952.20). An extractive solution was prepared by dissolving 13 g of disodium hydrogen phosphate (Fisher Scientific), 12 g of citric acid (Sigma-Aldrich), and 10 g of sodium metabisulfite (Fisher Scientific) in 1 L of ultrapure water. A sample of 0.1 g of solid material (either rumen or fecal solids) was suspended in 20 mL of extractive solution, and 150 µL of 1.0 M sodium cyanide (Sigma-Aldrich) was added before autoclaving at 100°C for 10 minutes. The tubes were then cooled at room temperature and the pH was adjusted to between 6.2 to 6.5 with 3.3 M. The volume of the solution was made up to 30 mL by the addition of ultrapure water and samples were centrifuged (3,000 × g, 10 minutes, 4°C) to remove any additional solids. A volume of 200 µL of the supernatant was used to determine the concentration of the biologically active form of vitamin B12 using the SimulTRAC-SNB Vitamin B12/Folate RIA Kit for the quantitative determination (SimulTRAC-S Vitamin B12 [Co57]/ Folate [I125], MP Biomedicals). This kit uses a SimulTRAC-SNB Binder that contains purified porcine intrinsic factor and radioactive vitamin B12 (cyanocobalamin) as a tracer to compete against sample's vitamin B12. All samples were analyzed in duplicate. The interassay coefficients of variation were 3.84 for rumen and 3.32 for feces. Vitamin B12 quantification was performed by trained and qualified members of the Agriculture and Agri-Food Canada Research and Development Centre Team (Sherbrooke, Quebec, Canada).

### 3.3.2 Milk and Plasma vitamin B12 quantification

Vitamin B12 concentrations in milk were analyzed in duplicate by radioassay using the SimulTRAC-SNB Vitamin B12/Folate RIA Kit for the quantitative determination (SimulTRAC-S Vitamin B12 [Co57]/ Folate [I125], MP Biomedicals) described in the previous section. The interassay coefficients of variation were 3.70 for milk and 3.96 for plasma. Vitamin B12 quantification was performed by trained and qualified members of the Agriculture and Agri-Food Canada Research Centre Team (Sherbrooke, Quebec, Canada).

## 3.4 Bacterial DNA extraction

### 3.4.1 Bacterial DNA Isolation from Milk

Frozen milk samples were thawed overnight in a refrigerator at 4° C. Then samples were gently homogenized using a vortex mixer (Scientific Industries) for 5 seconds. Three milliliters of milk from each udder quarter of the same cow were combined in a 15 mL sterile conical tube to obtain a composite sample of the cow sample to analyze. Six milliliters of the composite milk sample were centrifuged at 17,900 x g for 5 minutes to pellet bacterial cells and remove fat from the milk sample. The pellet was used for bacterial DNA extraction using the Sox Soil DNA Extraction Kit (Metagenom Inc.) following the manufacturer's instructions for a 200 mg sample. After extraction, DNA samples were quantified using the Nanodrop™ 2000/2000c Spectrophotometer and stored at -20 °C until 16S rRNA PCR amplification.

### 3.4.2 Rumen and Feces DNA extraction

DNA from the rumen and feces samples were extracted according to the Sox Soil DNA Extraction Kit (Metagenom Inc.) following the manufacturer's instructions for a 200 mg sample.

DNA samples concentrations were quantified using a Nanodrop™ 2000/2000c Spectrophotometer and stored at -20 ℃.

### 3.5 16S rRNA Targeted Amplicon Sequencing

#### 3.5.1 Library Preparation

Illumina MiSeq paired-end sequencing was performed "in-house" to determine the bacterial community composition of each sample using the V4 region of the 16S rRNA gene as a proxy. The V4 region of the 16S rRNA gene was amplified from the mixed microbial DNA sample using a set of custom primers (F548 and R806) (Table 5). The set of custom primers consisted of the same forward and reverse primers labelled with different barcodes, such that there were 8 different forward primers and 12 different reverse primers. Each sample was amplified via a unique combination of forward and reverse primers so that up to 96 samples could be de-multiplexed after sequencing (Kozich *et al.* 2013). Each sample was amplified via PCR using HotStar Taq (Qiagen) with 25 cycles of 95 ℃ for 20 seconds, 55 ℃ for 15 seconds, and 72 ℃ for 5 minutes. The PCR amplicons were approximately 250 bp. The PCR products were purified using AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions then quantified using the Quant-iT™ dsDNA High-Sensitivity Assay Kit (ThermoFisher). Any amplicons that were found to have a concentration of less than 1.5 ng/ µL were re-amplified.

**Table 5. Custom primers for 16S rRNA Targeted Amplicon Sequencing**

| Primer Name | Sequence 5'-3' | size (bp) |
|---|---|---|
| F548-SA501 | AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA502 | AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA503 | AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA504 | AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA505 | AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA506 | AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA507 | AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| F548-SA508 | AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 68 |
| R806-SA701 | CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA702 | CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA703 | CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA704 | CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA705 | CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA706 | CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA707 | CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA708 | CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA709 | CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA710 | CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA711 | CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| R806-SA712 | CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 64 |
| V4-Read-1 | TATGGTAATTGTGTGCCAGCMGCCGCGGTAA | 31 |
| V4-Read-2 | AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT | 32 |
| V4-Index | ATTAGAWACCCBDGTAGTCCGGCTGACTGACT | 32 |

After quantification, the amplicons for each individual sample were independently diluted using DNA/RNA free water to a final concentration of 1.5 ng/uL. A sample library was prepared by combining 3 µl of each sample in a microcentrifuge tube.

### 3.5.2 Amplicon Sequencing

The 16S rRNA amplicons were sequenced using a 500-cycle V2 reagent kit (Illumina) that had been removed from storage at -20°C and incubated in the refrigerator overnight prior to sequencing. The MiSeq reagent cartridge was removed from the freezer and thawed overnight at 4° C and well foils 12, 13, 14, and 17 were broken using a 1000 pipette tip. Next, 3 µl of the Read 1 Sequencing Primer (100 µM) was loaded into well 12, Read 2 Sequencing Primer was loaded into well 14, and the Index Primer was loaded into well 13 (Table 5). To denature the double-stranded DNA, 5 µl of the sample library and 5 µl of fresh sodium hydroxide 0.2N (Sigma) were combined in a fresh 1.5 mL microcentrifuge tube. This was incubated at room temperature for 5 minutes. After 5 minutes, 990 µl of HT1 was added and the denatured library was stored on ice. In a separate microcentrifuge tube, 2 µl of PhiX (Illumina) and 3 µl DNA/RNA free water were combined with 5 µl of 0.2N sodium hydroxide. This was incubated at room temperature for 5 minutes, after which 990 µl of HT1 was added. This resulted in a 4pM sample library and a 4 pM PhiX solution. To create a 15 % PhiX library 850 µl of sample library was combined with 150 µl of PhiX and 600 µl of this solution was added to well 17 of the MiSeq reagent cartridge.

The flow cell 500-cycle V2 reagent kit was rinsed well with Milli-Q water and loaded into the Illumina MiSeq along with the prepared MiSeq reagent cartridge. The amplicons were sequenced for 251 sequence reads in the forward and 251 sequence reads in the reverse directions. All raw sequence read data were up-loaded to the SRA database and can be found under the accession number SUB5240395.

### 3.6 Data Analysis

#### 3.6.1 Vitamin B12 Concentration

A Pearson correlation coefficient was used to estimate the correlation between rumen-milk, feces-milk, and rumen-feces vitamin B12 concentrations. The following parameters were considered: Effect size among samples (≥0.5= large effect size, ≥0.3= medium effect size, ≤0.1=low effect size, 0=no correlation) and a significant p-value ≤0.05. Rumen and feces samples that did not present their corresponding milk sample (due to dry lactation periods) were excluded from the correlation analysis.

#### 3.6.2 16S rRNA Targeted Amplicon Sequencing

Sequence data was processed using Mothur version 1.39.5. 16S rRNA sequences were analyzed through a quality filtering, trimming and error-free read identification (Kozich *et al.* 2013). The operational taxonomic unit (OTU) selection was performed using Mothur picking and SILVA v.132 as a reference database. Sequences that did not occur in the dataset at least 20 times were removed from the analysis. A similarity of 97% was used as a cut-off to performed cluster process. Details about the exact pipeline, including each command used and each associated parameter can be found in Appendix 1. All sequences were analyzed together to do an overall bovine microbiome analysis, in addition three separate analyses were performed with each type of sample (milk, rumen or feces samples sequences).

### 3.7 Statistical Analysis

Alpha-diversity and beta-diversity analyses were calculated using Marker Data Profiling (MDP) analysis on MicrobiomeAnalyst web platform (Dhariwal *et al*. 2017). For the analysis, two

files generated by mothur were used: a consensus taxonomy file and a shared file. An additional metadata file was created using a tab-delimited text file. Metadata files for all analysis were designed to group the sequences according to the type of samples analyzed and their concentration of vitamin B12. For example, all milk samples were grouped in 3 groups in the metadata file according with their vitamin B12 levels (high, medium, and low) to further discriminatory analysis. The same criteria were applied to rumen and feces samples. Initially, a data integrity check and data filtering were performed to analyze read counts per sample and identify potential outliers due to under-sampling or sequencing errors. Samples with less than 10000 sequence read counts were removed and re-sequenced, and OTUs that were not present in at least 5% of samples were filtered out of the dataset. Data normalization was performed to rarefy data to the minimum library size. Because of the limitations of the resolution on taxonomical classification using 16S rRNA targeted amplicon sequencing, the analysis was restricted to the genus level and above. The Chao1 richness index was used as an alpha-diversity metric using ANOVA as a statistical method. The beta-diversity analysis and significance testing were performed at a genus-level using a Principal Coordinate Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS), a Bray-Curtis index and permutational MANOVA (PERMANOVA) statistical method. P-values < 0.05 were considered to be significant.

### 3.8 Metadata correlation analysis

Linear discriminant analysis effect size (LEfSe) was performed through the online software presented in MicrobiomeAnalyst web plataform (Dhariwal *et al*. 2017). LEfSe employed a Kruskal-Wallis rank sum test to detect features with significant differential abundance genus with regard to both groups (high and low vitamin B12 samples), followed by a Linear Discriminant

Analysis (LDA) to evaluate the effect size of differential abundance genus. The default settings of LEfSe in microbiomeanalyst.ca were used: adjusted p-value cut-off: 0.05, Log LDA score of 1.0 and a False Discovery Rate (FDR) of 0.05.

## 4.1 Vitamin B12 concentrations in rumen, fecal, blood, and milk samples

A total of ninety-two rumen, ninety-two feces samples, ninety-two plasma (Blood) samples, and seventy-four milk samples were collected and analyzed. Vitamin B12 results were analyzed and grouped according to their kind of sample and vitamin concentration (Figure 8).

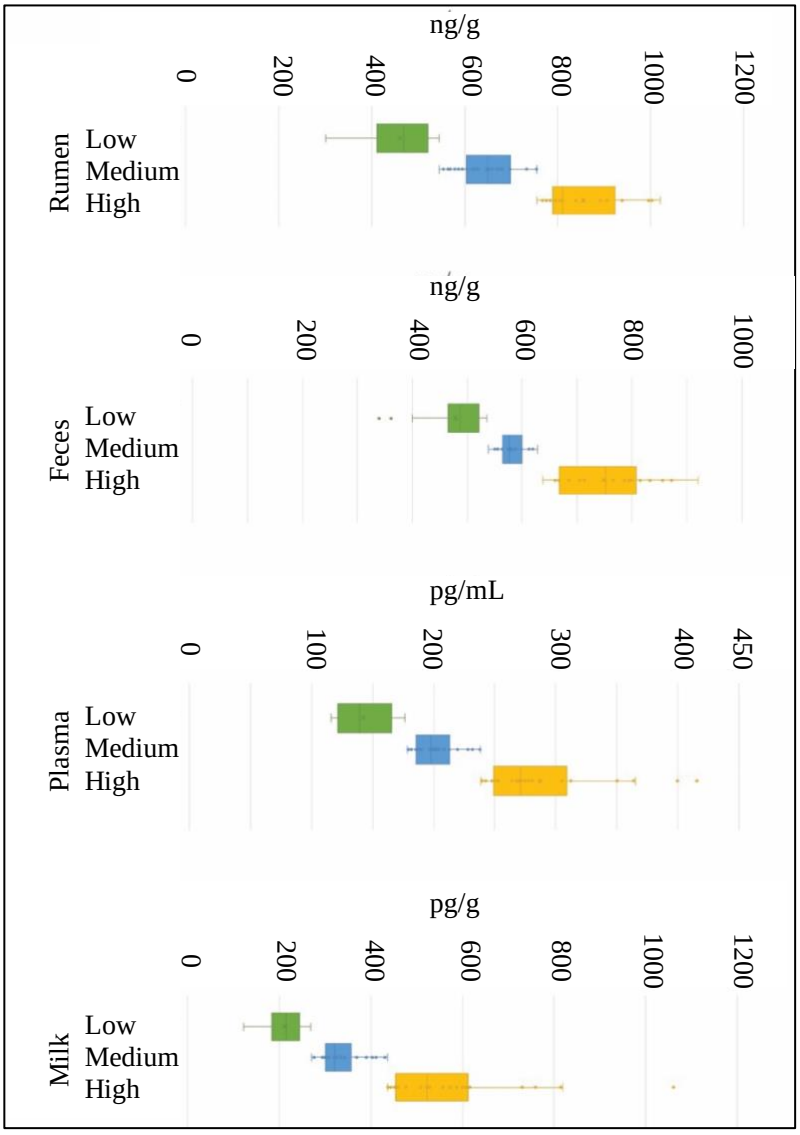


**Figure 8. Vitamin B12 Concentrations in Plasma, Milk, Rumen and Feces Samples.** Samples from each group were divided into three categories according to their own group vitamin B12 concentration values: low (green), medium (blue) and high (yellow) vitamin B12 concentration groups.

A Pearson correlation coefficient was performed between all vitamin B12 concentration results for the different types of samples from lactating cows (milk, rumen, feces & plasma) (Table 6). No significant correlations where found among vitamin B12 concentration of the different types of samples: plasma/milk (r=0.19 p-value=0.09), rumen/milk (r=0.008, p-value= 0.94), rumen/plasma (r=0.20, p-value= 0.08). However, a positive small effect size correlation was found between rumen/feces vitamin B12 concentration (r=0.25, p-value=0.02).

**Table 6. Pearson Correlation analysis of vitamin B12 concentrations between milk, rumen, plasma and feces.**

| **Pearson Correlation Coefficients<br>Prob > \|r\| under H0: Rho=0<br>Number of Observations** | | | | |
|---|---|---|---|---|
| | **Feces** | **Milk** | **Plasma** | **Rumen** |
| **Feces** | 1.00000 | -0.09046 | 0.16353 | 0.25396 |
| | 75 | 0.4434<br>74 | 0.1610<br>75 | 0.0279<br>75 |
| **Milk** | -0.09046 | 1.00000 | 0.19733 | 0.00805 |
| | 0.4434<br>74 | 74 | 0.0919<br>74 | 0.9457<br>74 |
| **Plasma** | 0.16353 | 0.19733 | 1.00000 | 0.20001 |
| | 0.1610<br>75 | 0.0919<br>74 | 75 | 0.0853<br>75 |
| **Rumen** | 0.25396 | 0.00805 | 0.20001 | 1.00000 |
| | 0.0279<br>75 | 0.9457<br>74 | 0.0853<br>75 | 75 |

### 4.2 Bovine microbiome

The similarities and differences between the bacterial populations present in the rumen, feces, and milk were evaluated by processing 16S rRNA targeted amplicon sequencing data from each body site together. The number of sequences in each sample, after removing ambiguous and chimera sequences, varied from 10953 to 162541 and data sets were rarified to the minimum library size (10953) (Figure 9A). Good's coverage was always above 98.00% at this sequencing depth. In general, the dominant bacterial phyla were *Firmicutes, Bacteriodetes,* and *Proteobacteria* (Figure 9B). Alpha-diversity was analyzed using Chao richness index analysis. Chao score showed significantly higher diversity in Rumen samples followed by fecal samples and the least diverse group were milk samples (p-value < 0.0001) (Figure 9C).

A PCoA was performed to compare beta-diversity between milk, rumen, and fecal samples. The fecal samples clustered together with minimal variation represented between samples. Rumen samples presented a cluster subdivided in two groups, but each group was closely related to the other. In contrast, the milk samples presented a higher diversity between samples. Significant differences made it possible to discriminate between samples taken from each body site (Figure 9D). By bulk processing all 16s rRNA targeted amplicon sequences from this study together, it was possible to conduct a comparative analysis of the OTUs presented in the rumen, milk and feces, and it was shown that a few OTUs were present in each sample type from the majority of cows. The OTUs that were present in most milk, rumen, and fecal samples were identified as *Christensenellaceae, Lachnospiraceae, Ruminococcaceae,* and *Ruminococcus* at the genus level (Figure 9E).

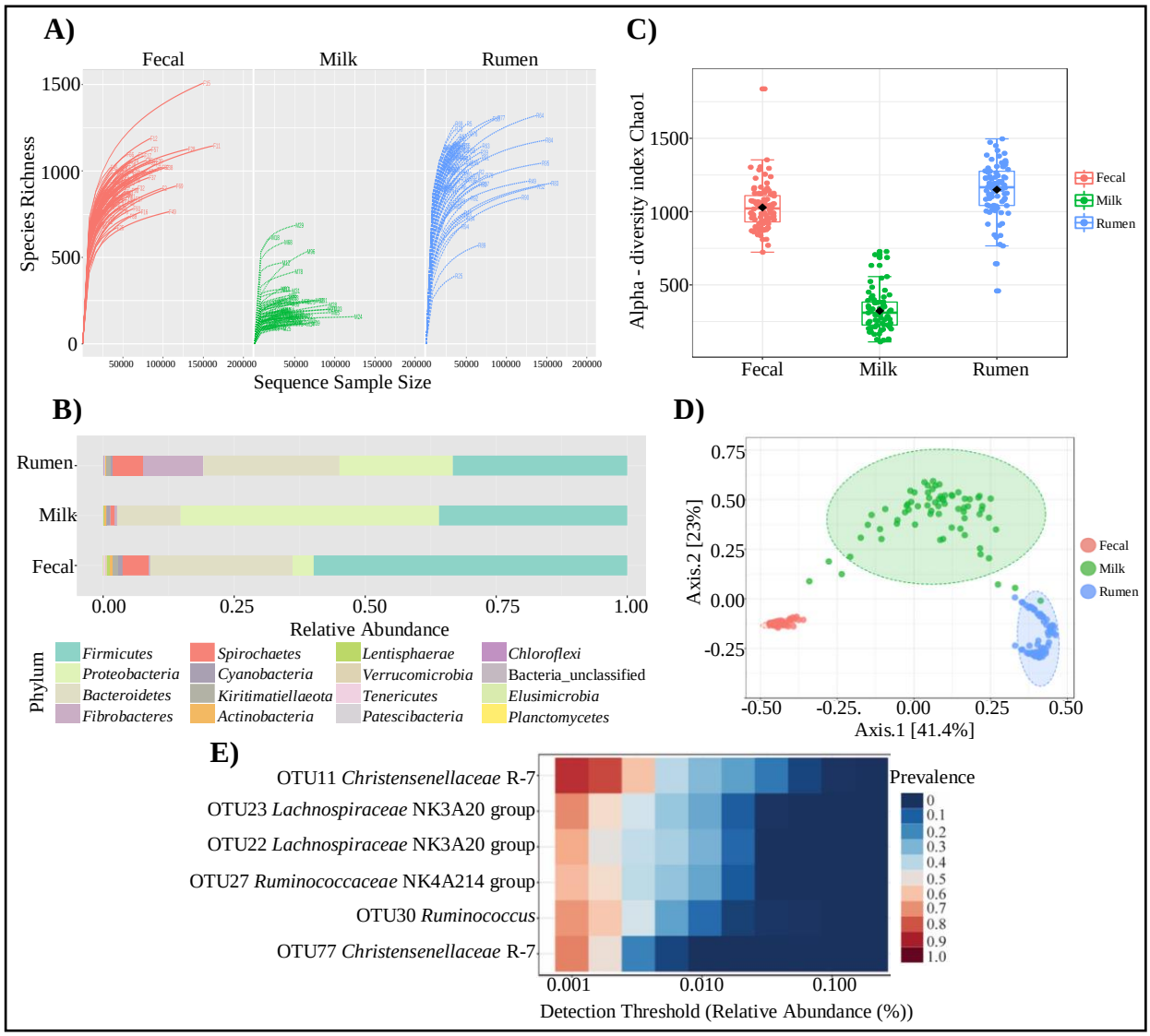


**Figure 9. Overview of the Bovine Microbiome.** A) Bovine microbiota rarefaction curves. Rarefaction curves demonstrate that appropriate Good's coverage of the population is being obtained for each sample. Milk (green) samples presented the lowest species richness, while rumen (blue) and fecal (red) samples had higher species richness. These curves were constructed using Microbiome Analyst (https://www.microbiomeanalyst.ca/) using un-rarified datasets (Dhariwal *et al.* 2017). B) Relative abundance of microbiota composition of rumen, milk and fecal samples at a phylum level indicated a high abundance of *Proteobacteria, Firmicutes, and Bacteroidetes*. C) Chao diversity Index showed that rumen samples presented the highest diversity followed by fecal samples, while the milk samples group was the least diverse. D) PCoA analysis of bovine microbiota. The PCoA analysis indicated that bacterial population clusters corresponded well with sample type. Fecal samples clustered together with minimum dispersion, rumen samples presented a closed cluster subdivided in two groups and milk samples presented the highest dispersion [PERMANOVA] F-value: 189.4: R-squared: 0.6; p-value < 0.001. E) A heat map displaying the detection threshold and prevalence across sample types for OTUs that occurred in >60% of total samples. This heatmap was constructed using MicrobiomeAnalyst (Dhariwal *et al.* 2017).

### 4.3 Rumen Microbiota

To determine if the rumen microbiota was affected by lactation status, a multi-variate analysis was performed. Rumen samples were divided into 2 groups based on whether the cows were dry (n=14) or lactating (n=73). Dry cows were also fed a different diet than lactating cows, so differences in the microbiota of the rumen could be expected. Non-metric multidimensional scaling (NMDS) followed by PERMANOVA analysis was used to determine that rumen microbial composition was significantly different between dry and lactating cows ($p<0.001$) (Figure 10A). *Proteobacteria* was shown to be more abundant in lactating cows than dry cows (Figure 10B).

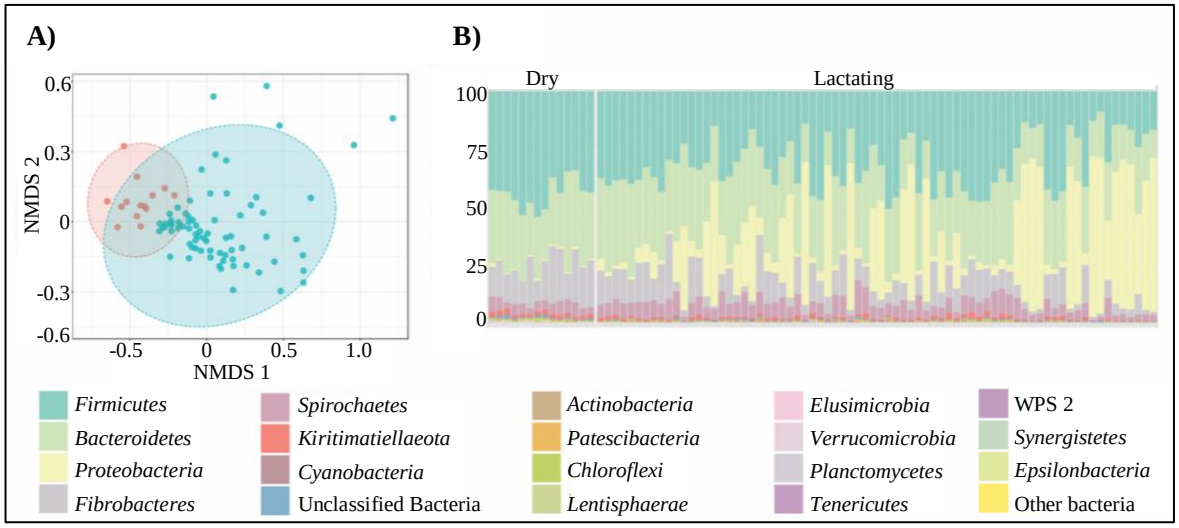


**Figure 10. Rumen Microbiota from Lactating and Dry Cows.** A) NMDS plot illustrates that lactating and dry cows have significantly different ruminal bacterial populations. The bacterial populations present in lactating animals clustered with a high dispersion, while the populations present in dry animals showed low dispersion ([PERMANOVA] F-value: 13.503; R-squared: 0.13708; p-value <0.001; NMDS Stress = 0.10992). B) Taxonomic composition of rumen samples from lactating cows and dry cows showed that *Proteobacteria* was present in a greater abundance in lactating cows than dry cows.

A Student's *t*-Test analysis (unpaired t-test) was conducted to determine if there were significant differences between the vitamin B12 concentration in lactating cows (n=73) and dry cows (n=14), and a significant difference was found (p= 0.0014). Since the dry cows had a

significantly different rumen microbiota and vitamin B12 concentrations than lactating cows, all samples (rumen, feces, blood, and milk) taken from dry cows were removed from the dataset for each of the following analyses.

### 4.3.1 Microbes Correlated to High Vitamin B12 Ruminal Concentration

To identify microbes correlated to high vitamin B12 concentration, rumen samples from lactating cows were divided according to their vitamin B12 concentration (Figure 8). Rumen samples with > 735 ng/g of vitamin B12 were classified as having a high-concentration (N=22), and samples with < 565 ng/g were classified as having low vitamin B12 (N=24) (Figure 8). Samples with an average vitamin B12 concentration between 566-734 ng/g (N=46) were excluded from this analysis to increase the probability of clearly identifying bacterial taxa correlated to high- and low- vitamin B12 concentrations. The samples associated with high and low groups (N=46) were rarified to 17274 sequences – the number of sequences in the sample with the lowest number of sequences in either of these groups, and Good's coverage ranged from 98.5-99.5. The dominant phyla in both groups were *Firmicutes, Proteobacteria, Bacteriodetes, Fibrobacteres,* and *Spirochaetes,* however, *Proteobacteria* was significantly more prominent in the high vitamin B12 concentration group when compared to the low concentration group (p-value < 0.001), and *Fibrobacteres* were observed to be more abundant in samples with low vitamin B12 concentration (p-value < 0.001).

LEfSe analysis of high and low vitamin B12 concentration samples identified forty-five genera that were significantly correlated to either high or low concentrations of vitamin B12 (p-value: <0.05, FDR < 0.05*)* (Appendix 2*).* Of those forty-five genera, only three; *Prevotella, Succinivibrionaceae,* and *Shuttleworthia* were associated with high concentrations of vitamin B12,

while *Fibrobacter*, and *Butyrivibrio* were the two genera highly correlated to low concentrations of vitamin B12 (Figure 11B).

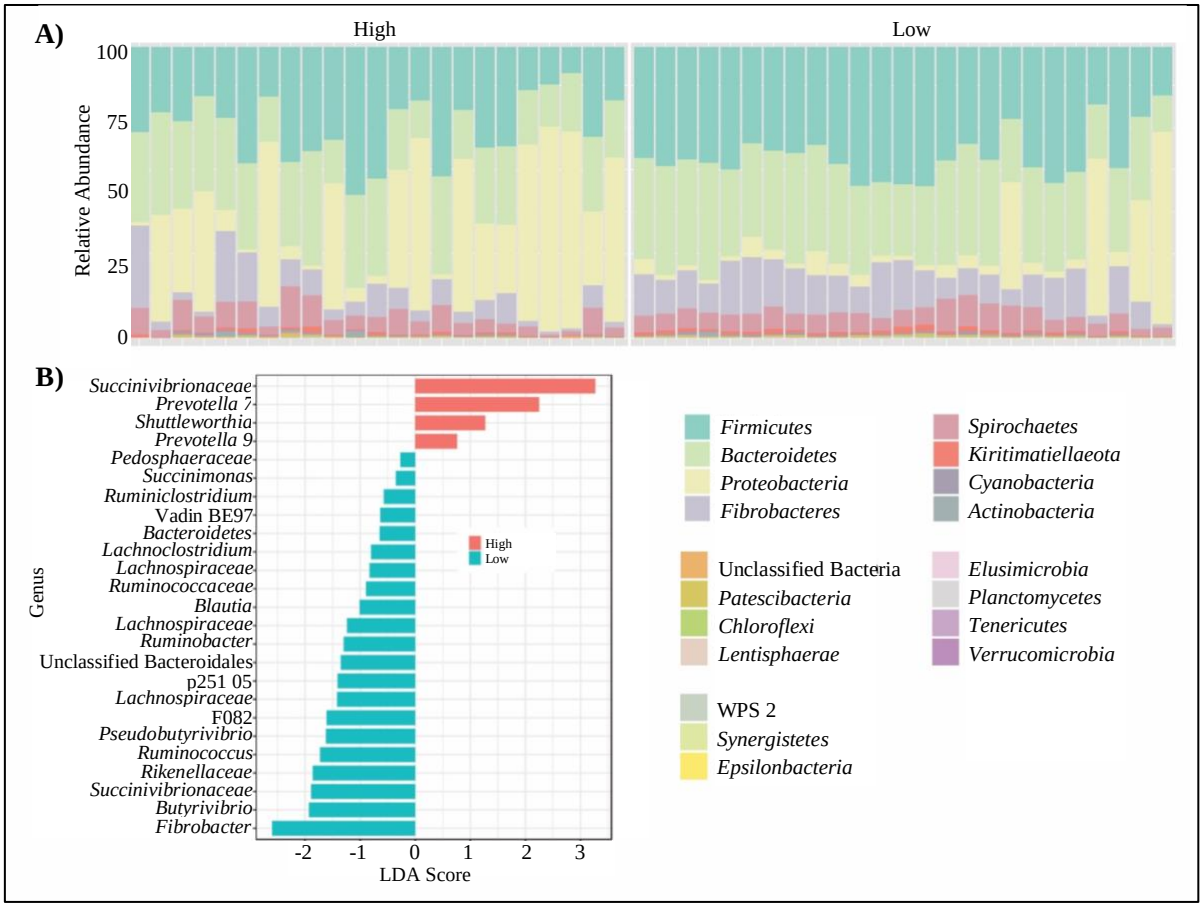


**Figure 11. Overview of Microbes Correlated to High Concentrations of Vitamin B12**. A) Taxonomic composition of rumen samples from high vitamin B12 concentration samples and low vitamin B12 concentration samples. *Proteobacteria* presented a higher abundance level in cows with a high ruminal concentration of vitamin B12. B) LEfSe analysis detected genera with significant differential abundance with regard to high and low vitamin B12 rumen samples.

### 4.4 Feces microbiota

To identify bacterial vitamin B12 consumption in the intestine, fecal samples from lactating cows were divided into 2 groups. These groups were designed following the logic that as vitamin B12 moves from the rumen into the small intestine, where it is absorbed into the blood circulation, it can also be consumed by bacteria or excreted in the feces. Therefore, the first group of fecal

samples included in this analysis came from animals that had high concentrations of vitamin B12 in the rumen followed by both high vitamin B12 in the serum and feces. In these animals very little vitamin B12 was lost in the intestine (N=4). This group was compared against samples where there was high concentration of vitamin B12, but this was followed by both low vitamin B12 concentrations in the feces and in the serum. In these animals, most vitamin was consumed in the intestine (N=6). The ten samples included in this analysis were rarified to 23073 sequences per sample, and Good's coverage was > 99.0. LEfSe analysis between constant vitamin B12 and loss of vitamin B12 groups did not show any significant bacterial taxa correlated with any group of samples (*p-value:* <0.05, *FDR < 0.05).*

### 4.5 Milk Microbiota

It is known that serum vitamin B12 is partially absorbed by the mammary gland and secreted in milk. To determine if the microbial population in the mammary gland was correlated with vitamin B12 consumption, we considered only samples from animals that had a high serum concentrations of vitamin B12, and divided these samples into two groups where vitamin B12 was lost in the udder (N=7), and a group where vitamin B12 was not lost in the udder (N=8). The fifteen samples included in this analysis were rarified to 18164 sequences per sample, and Good's coverage was > 99.8. However, LEfSe analysis between loss of vitamin B12 in the udder, and no lost of vitamin B12 concentration in the milk samples did not identify any significant bacterial taxa correlated with any group of samples (*p-value:* <0.05, *FDR < 0.05).*

## Chapter 5. Discussion

This study found that the concentration of vitamin B12 in the rumen is not strongly predictive of the concentration ultimately found in milk. A Pearson correlation analysis between concentrations of vitamin B12 found in the different biological compartments of the dairy cow (rumen, mammary gland, large intestine, and blood) showed a null correlation between them, with the exception of high vitamin B12 in the rumen being correlated with elevated levels of vitamin B12 in the feces. There are several factors that could result in these discrepancies through these different body sites. Microbial consumption of vitamin B12 in the digestive tract could be a factor in the lack of a correlation, although, in this study we did not identify a specific genus linked to over consumption (Girard *et al.* 2009). Another factor could be the absorption rate of vitamin B12 as well as its continued circulation through the animal. The absorption of the vitamin B12 produced in the rumen is not immediately reflected in the blood circulation due to the sometimes-slow absorption process present in the small intestine, and vitamin B12 net flux from the gastrointestinal tract to the portal vein blood can take more than 24 hours (Girard *et al.* 2001). In this study, samples were collected from the same animal at approximately the same time point. Our sampling strategy did not consider the time that the vitamin B12 from rumen took to reach other body sites or possible variations in the ruminal synthesis of vitamin B12. However, the range of daily variation of vitamin B12 synthesis in the rumen is unknown, and if it is somewhat consistent, our sampling strategy would have been adequate. The lack of a correlation between the concentration of vitamin B12 in milk and plasma has also been observed in another study and suggests that bovine genetics may play a strong role in absorption by gut epithelial cells, blood transport, and absorption efficiency of the mammary gland (Duplessis *et al.* 2018 & Rutten *et al.* 2013). This idea is supported by a

calculated heritability value of vitamin B12 concentrations in milk of 0.23, which suggests a role for bovine genetics (Rutten *et al*. 2013 & Duplessis *et al.* 2016).

In this study, we did not identify a specific bacterial genus linked to increased consumption of vitamin B12 in the intestinal tract. A possible explanation for this finding, or lack thereof, is that of the thousands of bacterial species that reside in the bovine intestine, approximately 80% require exogenous vitamin B12 (Degnan *et al*. 2014a). There is substantial evidence that although specific taxa can vary greatly between individuals, functionality is mostly conserved (Huttenhower *et al.* 2012). Since several different taxa are likely contributing to vitamin B12 depletion in the intestine, in different animals, it may make sense that we were unable to link increased consumption to specific taxa. While an *over consumer* was not identified by this study, it is likely that there is high-consumption by the intestinal population as a whole. Another possible explanation for not identifying specific taxa linked to consumption of vitamin B12, may have been our sampling strategy. It is known that the fecal microbiota is significantly different from the microbiota at other sites along the intestinal tract (He *et al.* 2018), and therefore crucial consumers of vitamin B12 may have been missed based on only collecting fecal samples, as opposed to sampling at several sites along the lower intestine.

In this investigation, we measured highly variable vitamin B12 concentrations in milk (1496 to 10612 pg/mL). The range observed in this study was significantly larger than the previous range reported in the literature (1575 to 4781 pg/mL) (Preynat *et al*. 2009; Akins *et al*. 2013; Rutten *et al*. 2013; Duplessis *et al.* 2016). This observation is somewhat surprising, since the animals in our study were from the same herd, all Holsteins, and were eating the same highly controlled diet, while the other studies included several different herds, diets and even different species of cows (Holsteins and Jerseys). An essential difference between milk samples from this

study and other studies was the days in milk (DIM) of the cows analyzed. In this study, the samples were collected in a wide range of 10-568 DIM (with an average of 180 DIM), in comparison with other studies where the range was only between 27-67 DIM (Akins *et al*. 2013; Duplessis *et al*. 2016). These differences suggested that high variations of vitamin B12 are occurring during the lactation periods of the cattle. The current dogma is that several external and internal factors, which are far from being fully understood, regulate and control the production and secretion of vitamin B12 in milk, and this current study supports this understanding.

Bovine microbiota observed in this study was similar to the taxonomic compositions reported by others (Jami *et al*. 2014; Deusch *et al.* 2017; Li *et al*. 2018; Mao *et al.* 2015). The different types of bovine microbiota investigated here (rumen, milk, and feces) also had distinct populations, and major differences in overall diversity. Milk samples showed the lowest diversity. It is known that a healthy bovine mammary gland can have between $10^1$ to $10^4$ CFU/mL with low microbial diversity (Quigley *et al.* 2013), while rumen and fecal samples are more densely populated at approximately $10^{10}$ CFU/mL and have complex bacterial communities with high species richness (Mackie *et al.* 2000).

Specific OTUs that correspond to the *Christensenellaceae*, *Lachnospiraceae*, *Ruminococcaceae,* and *Ruminococcus* genera were found in each sample type in almost every animal. Given the OTU resolution of this observation, the shared presence of these anaerobic bacteria provides additional evidence of the existence of an entero-mammary pathway. The theory of the presence of an entero-mammary pathway in cows is still controversial, however, our study supports the existence of the entero-mammary pathway (Addis *et al*. 2016; Young *et al*. 2015).

The average rumen taxonomic composition demonstrated that *Firmicutes, Bacteroidetes,* and *Proteobacteria* were the three dominant phyla, and these results agreed with the composition

presented in other studies (Plaizier *et al.* 2017; Jewell *et al.* 2015). However, we found that there were significant differences in population structure between lactating cows and dry cows. Lactating animals had a higher abundance of the *Proteobacteria* phylum than dry cows, an average of 23.3% and 0.4%, respectively. These taxonomic composition changes are likely related to the different diets used in lactating and dry cows (Appendix 3). Dry cows received a diet based on 64% Total Mixed Ration (TMR) and 36% hay, in contrast, lactating cows received only a TMR. TMR diets high in grains have been associated with an increased abundance of *Proteobacteria* when compared to forage-based diets (Petri *et al.* 2013). Rumen samples from lactating cows presented a significantly higher concentration of vitamin B12 than that of dry cows (p-value= 0.0014). This result agrees with previous work where high values of vitamin B12 were correlated to an increase of *Proteobacteria* phylum in the human gut (Xu *et al.* 2018). When analyzing the rumen samples from lactating cows at a higher taxonomic resolution, it was found that the high concentrations of vitamin B12 in the rumen were correlated with increased abundance of *Succinivibrionacea, Prevotella,* and *Shuttleworthia.* However, it is still unclear if these genera are producing vitamin B12 at higher levels or if they are merely benefiting from elevated levels of vitamin B12. Vitamin B12 studies related to each of these specific taxa are limited. Details about the relationship between *Succinivibrionaceae* and vitamin B12 production are not known. Increases in the abundance of *Succinivibrionaceae* in the bovine rumen is related to high-starch diets, rapidly fermentable carbohydrates, and corn silage-based diet (Stackebrandt *et al.* 2006; Deusch *et al.* 2017). The increased presence of *Succinivibrionaceae* in the high concentration of vitamin B12 group may be as a result of the high percentage of corn in the lactating cows' diet (Deusch *et al.* 2017) (Appendix 3). *Prevotella* is not known to have the ability to produce vitamin B12, and has very specific media requirements for vitamin B12 supplementation when it is grown

in the lab (Shin *et al.* 2004; Strobel, 1992), so it is likely that this genus is taking advantage of high vitamin B12 concentrations for increased growth. However, some *Prevotella* species found in the human gut have been reported to be capable of vitamin B12 *de novo* synthesis (Degnan *et al.* 2014a), again making the exact relationship between this genus and vitamin B12 synthesis ambiguous. Finally, *Shuttleworthia*, although a common inhabitant of the bovine rumen known to ferment starch and sugar, has not previously been linked to vitamin B12 and it is unclear what relationship exists between this organism and this vitamin (Xu *et al.* 2018; Neubauer *et al.* 2018). *Fibrobacter*, *Butyrivibrio*, *Ruminococcus*, and *Lachnospiraceae* were significatively correlated to low vitamin B12 in the rumen ($p$-value $< 0.05$). These genera include species with both: vitamin B12-dependent and biosynthetic genes (Degnan *et al.* 2014a). This vitamin B12 dependency could explain the low vitamin B12 for these groups of samples. However, specific genetic analysis at a species level is required for a thorough understanding of the relationships between these bacteria and vitamin B12 concentrations in the rumen environment.

The next step for this study will be to perform a metagenomic sequence analysis of the most representative samples associated with low and high concentrations of vitamin B12 in the rumen. The complete characterization of the microbiome of these samples will allow us to better identify genes and pathways involved in vitamin B12-related processes. A bioinformatic analysis of vitamin B12-related genes in the rumen microbiome will confirm and support our results, showing new information about bacterial producers and consumers of vitamin B12 in the dairy cattle's rumen. Next, bacteria taxa associated with the production or consumption of vitamin B12 can be isolated for both metabolic characterization and whole genome sequencing. Eventually, it is our hope that we can design a nutritional and probiotic strategy to increase high bacterial production of vitamin B12 in cattle – as well as increase the absorption capacity of the cattle.

Ultimately our current work has provided the first steps towards identifying targets for modifying the bovine microbiome to provide high and constant levels of natural vitamin B12 in milk.

# Chapter 6. Conclusion

Certain bacterial taxa were correlated with high concentrations of vitamin B12 in the bovine rumen, including *Succinivibrio, Prevotella,* and *Shuttleworthia* which were identified. Specific bacterial taxa correlated with higher than average consumption of vitamin B12 in the intestine and mammary gland could not be clearly identified. High concentrations of vitamin B12 in the rumen were not predictive of increased concentrations of vitamin B12 in milk.

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# Appendices

## Appendix 1. Mothur commands pipeline

1)make.contigs(file=stability.files, processors=7)
2)summary.seqs(fasta=stability.trim.contigs.fasta, processors=7)
3)screen.seqs(fasta=stability.trim.contigs.fasta, group=stability.contigs.groups, summary=stability.trim.contigs.summary, maxambig=0, maxlength=253, minlength=253, processors=7)
4)summary.seqs(fasta=stability.trim.contigs.good.fasta, processors=7)
5)unique.seqs(fasta=stability.trim.contigs.good.fasta)
6)count.seqs(name=stability.trim.contigs.good.names, group=stability.contigs.good.groups)
7)summary.seqs(fasta=stability.trim.contigs.good.unique.fasta, count=stability.trim.contigs.good.count_table, processors=7)
8)align.seqs(fasta=stability.trim.contigs.good.unique.fasta, reference=silva.v4.fasta, processors=7)
9)summary.seqs(fasta=stability.trim.contigs.good.unique.align, count=stability.trim.contigs.good.count_table, processors=7)
10)screen.seqs(fasta=stability.trim.contigs.good.unique.align, count=stability.trim.contigs.good.count_table, summary=stability.trim.contigs.good.unique.summary, start=1968, end=11550, maxhomop=8, processors=7)
11)summary.seqs(fasta=stability.trim.contigs.good.unique.good.align, count=stability.trim.contigs.good.good.count_table, processors=7)
12)filter.seqs(fasta=stability.trim.contigs.good.unique.good.align, vertical=T, trump=., processors=7)
13)unique.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.fasta, count=stability.trim.contigs.good.good.abund.count_table)
14)pre.cluster(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.fasta, count=stability.trim.contigs.good.unique.good.filter.abund.count_table, diffs=2, processors=7)
15)split.abund(fasta=stability.trim.contigs.good.unique.good.filter.fasta, count=stability.trim.contigs.good.good.count_table, cutoff=20, accnos=true)
16)summary.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.fasta, count=stability.trim.contigs.good.good.abund.count_table, processors=7)
17)chimera.uchime(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.fasta, count=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.count_table, dereplicate=t, processors=7)
18)remove.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.fasta, accnos=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.denovo.uchime.accnos)
19)summary.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.pick.fasta, count=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.denovo.uchime.pick.count_table, processors=7)
20)classify.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.pick.fasta, count=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.denovo.uchime.pick.count_table, reference=silva.v4.fasta, taxonomy=silva.nr_v132.tax, cutoff=80, processors=7)
21)remove.lineage(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.pick.fasta, count=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.denovo.uchime.pick.count_table, taxonomy=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.pick.nr_v132.wang.taxonomy, taxon=Mitochondria-Archaea-Eukaryota)
22)dist.seqs(fasta=stability.trim.contigs.good.unique.good.filter.abund.unique.precluster.pick.pick.fasta, cutoff=0.10)
23)cluster(column=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.pick.dist, count=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.denovo.uchime.pick.pick.count_table)
24)make.shared(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.pick.an.unique_list.list, count=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.denovo.uchime.pick.pick.count_table, label=0.03)
25)classify.otu(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.pick.an.unique_list.list, count=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.denovo.uchime.pick.pick.count_table, taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.nr_v132.wang.pick.taxonomy, label=0.03)
26)phylotype(taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.nr_v132.wang.pick.taxonomy)
27)make.shared(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.nr_v132.wang.pick.tx.list, count=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.denovo.uchime.pick.pick.count_table, label=1)
28)classify.otu(list=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.nr_v132.wang.pick.tx.list, count=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.denovo.uchime.pick.pick.count_table, taxonomy=stability.trim.contigs.good.unique.good.filter.unique.precluster.abund.pick.nr_v132.wang.pick.taxonomy, label=1)

## Appendix 2. Linear discriminant analysis Effect Size between rumen samples from lactating cows with high & low concentrations of vitamin B12.

| | Pvalues | FDR | High | Low | LDAscore |
|---|---|---|---|---|---|
| *Prevotella_7* | 1.4467e-06 | 0.00015914 | 61392.8262043163 | 2359.25056391843 | 4.47 |
| *Shuttleworthia* | 8.993e-05 | 0.004065 | 34211.7487539391 | 16687.3820374718 | 3.94 |
| *Butyrivibrio_2* | 0.00014202 | 0.004065 | 139891.526785602 | 259114.76315426 | -4.78 |
| *Pseudobutyrivibrio* | 0.00023993 | 0.004065 | 56119.822726645 | 114740.137181789 | -4.47 |
| *Clostridiales_unclassified* | 0.00028352 | 0.004065 | 16697.8443459592 | 35820.3286838834 | -3.98 |
| *probable_genus_10* | 0.00032312 | 0.004065 | 24638.7364879286 | 48681.1213920729 | -4.08 |
| *Succinivibrionaceae_UCG_001* | 0.00032353 | 0.004065 | 3303693.61338841 | 662661.694977673 | 6.12 |
| *Ruminococcus_1* | 0.00035212 | 0.004065 | 145415.625666972 | 205283.570409244 | -4.48 |
| *Ruminococcaceae_UCG_004* | 0.00037092 | 0.004065 | 5273.00347767134 | 9523.31630069512 | -3.33 |
| *Lachnospiraceae_XPB1014_group* | 0.00039855 | 0.004065 | 25925.6004318841 | 70777.5169175528 | -4.35 |
| *Succinivibrionaceae_UCG_002* | 0.0004065 | 0.004065 | 30382.5438475349 | 130995.948994154 | -4.7 |
| *Rikenellaceae_RC9_gut_group* | 0.00045232 | 0.0041463 | 145572.560294284 | 252353.49629425 | -4.73 |
| *Lachnospiraceae_ND3007_group* | 0.0005442 | 0.00436 | 2102.92400597607 | 5552.87022971045 | -3.24 |
| *uncultured* | 0.00055491 | 0.00436 | 33301.5279155315 | 100584.633798278 | -4.53 |
| *Lachnospiraceae_NK3A20_group* | 0.00076692 | 0.0056241 | 442618.422869769 | 656188.141601068 | -5.03 |
| *Christensenellaceae_R_7_group* | 0.00097169 | 0.0066804 | 398048.988713262 | 713500.667495282 | -5.2 |
| *Bacteroidales_unclassified* | 0.0015606 | 0.010098 | 6810.96282532548 | 17234.0376559407 | -3.72 |
| *Flexilinea* | 0.001832 | 0.011196 | 6089.06353969191 | 11450.9966395065 | -3.43 |
| *Ruminobacter* | 0.002039 | 0.011439 | 18392.738320925 | 44020.162960917 | -4.11 |
| *Fibrobacter* | 0.0020798 | 0.011439 | 867126.589747775 | 1419520.78442204 | -5.44 |
| *Ruminococcaceae_unclassified* | 0.0022731 | 0.011907 | 5429.93810498299 | 10674.1702343139 | -3.42 |
| *WCHB1_41_ge* | 0.0024017 | 0.012008 | 49559.9553050181 | 88471.896146941 | -4.29 |
| *Christensenellaceae_unclassified* | 0.0029169 | 0.013575 | 3923.36568279118 | 8286.14832205497 | -3.34 |
| *Lachnoclostridium_10* | 0.0029618 | 0.013575 | 7815.34444012002 | 13781.4758550845 | -3.47 |
| *Lachnospiraceae_unclassified* | 0.0044 | 0.018503 | 228653.75199307 | 292259.35644248 | -4.5 |
| *Gastranaerophilales_ge* | 0.0045519 | 0.018503 | 29315.3883818157 | 48824.9781337753 | -3.99 |
| *Ruminococcaceae_UCG_005* | 0.0046983 | 0.018503 | 27714.6551832369 | 41603.3697003176 | -3.84 |
| *Bacteroidales_UCG_001_ge* | 0.0047099 | 0.018503 | 29284.0014563533 | 51788.4270128435 | -4.05 |
| *Bacteroidia_unclassified* | 0.0053884 | 0.019958 | 12586.1571103941 | 21492.1972103301 | -3.65 |
| *Prevotellaceae_NK3B31_group* | 0.0057651 | 0.019958 | 22629.9732583395 | 37431.5241909497 | -3.87 |
| *Saccharofermentans* | 0.0057817 | 0.019958 | 113463.735546321 | 172110.205772683 | -4.47 |
| *Desulfovibrio* | 0.0058059 | 0.019958 | 3703.65720455487 | 6358.46798324357 | -3.12 |
| *Moryella* | 0.0061196 | 0.020399 | 16478.1358677229 | 23132.1640657368 | -3.52 |
| *Ruminococcaceae_ge* | 0.0072563 | 0.023476 | 13873.0210543496 | 19852.2303549234 | -3.48 |
| *Bacteroidales_BS11_gut_group_ge* | 0.0077522 | 0.024364 | 9259.14301138718 | 15968.0983289601 | -3.53 |
| *Syntrophococcus* | 0.0095892 | 0.0293 | 6434.31971977753 | 10213.8286608664 | -3.28 |
| *Ruminococcaceae_UCG_010* | 0.010057 | 0.0299 | 17859.1605880654 | 29116.6045205543 | -3.75 |
| *F082_ge* | 0.011805 | 0.034171 | 67481.8897440082 | 99520.093909681 | -4.2 |
| *Lachnospiraceae_UCG_008* | 0.01274 | 0.035273 | 9384.69071323649 | 13810.2472034249 | -3.35 |
| *Absconditabacteriales_(SR1)_ge* | 0.012826 | 0.035273 | 14720.4680418325 | 23391.106200801 | -3.64 |
| *Lachnospiraceae_UCG_002* | 0.01429 | 0.038339 | 3013.14484438362 | 5293.92809464623 | -3.06 |
| *Prevotellaceae_unclassified* | 0.015066 | 0.039458 | 60043.1884094362 | 81365.3731068453 | -4.03 |
| *Lachnospiraceae_FCS020_group* | 0.015956 | 0.040819 | 9447.46456416115 | 15594.070800534 | -3.49 |
| *Lachnospiraceae_AC2044_group* | 0.017525 | 0.043813 | 27338.0120776889 | 44796.9893661097 | -3.94 |
| *Blautia* | 0.020443 | 0.049972 | 3107.30562077061 | 5552.87022971045 | -3.09 |

**Appendix 3. Diet composition of dry cow, dry cow transition (close-up) and lactating cows (Group 1 and 2).**

| Item | Dry | Close-up | Group 1 | Group 2 |
|---|---|---|---|---|
| **Ingredient (% of DM)** | | | | |
| Hay for dry cows | 35.8 | - | - | - |
| Grass hay | 33.52 | 36.8 | 2.6 | 2.0 |
| Legume-grass silage | - | - | 23.0 | 39.2 |
| Corn silage | 11.47 | 34.0 | 34.1 | 26.1 |
| Cracked corn | - | - | 16.4 | 15.1 |
| Soybean meal | 13.42 | 16.3 | 8.4 | 9.2 |
| Beet pulp | 3.5 | 10.0 | 3.5 | - |
| Mineral and vitamin premix | 1.40 | 1.4 | 1.5 | 1.4 |
| Calcium carbonate | 0.51 | 1.2 | 1.0 | 1.0 |
| Distiller grain (corn) | - | - | 2.6 | 1.8 |
| Corn gluten meal | - | - | 2.6 | 1.8 |
| Canola meal | - | - | 1.7 | 1.2 |
| Micronized soybean | - | - | 1.7 | 1.2 |
| Urea | 0.39 | 0.3 | - | - |
| Megalac | - | - | 0.97 | - |