A metagenomic approach to understanding relationships between microbial communities in the bovine rumen and vitamin B12 abundance

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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 this thesis. I conducted DNA extraction from bovine rumen samples, performed data processing and analysis, and wrote this thesis under the supervision of Dr. Jennifer Ronholm. Bridget O'Brien participated in the design of several figures, and Elysse Magnusson provided information for designing a pipeline for metagenomic data processing.

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ABSTRACT

Vitamin B12 is an essential molecule required by many eukaryotes, but only produced by a few bacteria and archaea. Humans acquire the vitamin solely through their diet, as vitamin B12-producing bacteria and archaea are located downstream of the ileum, where nutrient absorption occurs. Milk is an excellent source of vitamin B12; however, there is large variability in vitamin B12 concentrations in milk, with a 250 mL glass containing anywhere from 16-57% of the daily recommended vitamin B12 intake. Because vitamin B12 is a vital cofactor required by several human enzymes, a deficiency in vitamin B12 can result in hematologic and neurological symptoms. The microbial composition of the bovine rumen is likely the greatest contributor to the variability of vitamin B12 concentrations in milk but this is largely influenced by host-management and diet. This study utilizes metagenomics to characterize the abundance and diversity of bacteria and archaea in the bovine rumen and analyzes their influence on the abundance of vitamin B12 in the rumen, and by proxy in milk. Many bacterial and archaeal genomes encode many pathways for both the synthesis and consumption of vitamin B12; however, some prokaryotes seem to benefit entirely from the vitamin B12 produced by other microbes. Nearly 61% of the genomes we identified contained vitamin-B12 dependent genes, while most of them lacked the presence of a complete vitamin B12 biosynthetic pathway. Many *Prevotella* genomes appeared to be major consumers of vitamin B12 and seemed to have the ability to utilize exogenous corrinoids for their own benefit. Contrarily, species of *Methanobrevibacter* and *Methanosphaera* were found to be solely involved in the production of vitamin B12.

RÉSUMÉ

Le vitamine B12 est une molécule essentielle requise par nombreux eucaryotes, mais produite uniquement par quelques bactéries et archées. Les humains acquièrent la vitamine exclusivement par leur régime alimentaire, car les bactéries et les archées productrices de vitamine B12 sont situées en dessous de l'iléon, où s'arrive l'absorption des nutriments. Le lait est une excellente source de vitamine B12, cependant il existe une grande variabilité des concentrations, allant de 16 à 57% de l'apport quotidien recommandé en vitamine B12. Parce que la vitamine B12 est un cofacteur vital requis par plusieurs enzymes, une carence en vitamine B12 peut entrainer des symptômes hématologiques et neurologiques. La composition microbienne du rumen bovine est probablement le plus grand contributeur a la variabilité des concentrations de vitamine B12, mais elle est largement influencée par la gestion de l'hôte et le régime alimentaire. Cette étude utilise la métagénomique pour caractériser l'abondance et la diversité des bactéries et des archées dans le rumen bovin et analyse leur influence sur les concentrations de vitamine B12 dans le rumen, et par procuration dans le lait. Il est évident que de nombreux génomes bactériens et archéens codent à la fois pour des gènes de synthèse et de consommation de vitamine B12, mais certains semblent bénéficier entièrement d'autres microbes producteurs de vitamine B12. Pres de 61% des génomes étudies contenaient des gènes dépendants de la vitamine B12, alors que la plupart d'entre eux n'avaient pas la capacité de produire de la vitamine B12. De nombreux génomes de Prevotella semblaient être de grands consommateurs de vitamine B12 et semblaient avoir l'aptitude d'utiliser des corrinoïdes exogènes pour leur propre bénéfice. Au contraire, les espèces de *Methanobrevibacter* et *Methanosphaera* semble être uniquement impliquées dans la production de vitamine B12.

CHAPTER 1. INTRODUCTION

1.1 General Introduction

Vitamin B12, also known as cobalamin, is a complex molecule that is synthesized only by certain bacteria and archaea but is essential for cell metabolism within many eukaryotes. Humans lack the ability to synthesize vitamin B12; however, it is critical for DNA production, and the prevention of megaloblastic anemia and neuropathy (Duplessis, Pellerin, Robichaud, Fadul-Pacheco, & Girard, 2019). Animal products, especially dairy products, are a good source of dietary vitamin B12 for humans since vitamin B12 is not destroyed by the pasteurization process and survives for extended periods at refrigeration temperature (Duplessis, Pellerin, Cue, & Girard, 2016). Additionally, vitamin B12 from dairy products is more readily absorbed than synthetic vitamin B12 supplements (Matte, Guay, & Girard, 2012). However, there is a large variability in the vitamin B12 concentrations of Canadian milk products. For example, a 250 mL glass of milk can range from providing 16-57% of the daily recommend vitamin B12 intake (Duplessis et al., 2016).

The ruminal archaea and bacteria that synthesize vitamin B12, do so via one of two pathways: the *de novo* pathway or the salvage pathway. Both pathways require the utilization of cobalt, an essential precursor; however, the pathways vary drastically in terms of metabolic cost (Matte, Britten, & Girard, 2014). Once the vitamin is produced in the rumen, it is then absorbed by the cow via her small intestine, although a significant portion is excreted in the feces, urine, and milk (Girard, Santschi, Stabler, & Allen, 2009).

The microbial composition of the bovine rumen is likely the greatest contributor to the variability of vitamin B12 concentrations in the rumen; however, the bacterial population in this environment is considerably influenced by many intrinsic and extrinsic factors (Duplessis et al., 2016). The rumen consists of three dominant bacterial phyla: *Firmicutes*, *Bacteriodetes*, and *Proteobacteria* (Doxey, Kurtz, Lynch, Sauder, & Neufeld, 2015; Jami, Israel, Kotser, & Mizrahi, 2013; Salfer, Staley, Johnson, Sadowsky, & Stern, 2018). However, at higher taxonomic resolution, the composition can be altered by host variability, host-management, diet, and geography. In addition to the synthesis of vitamin B12, some microbes actively consume vitamin B12. Certain bacterial populations that lack the capacity of vitamin B12 biosynthesis are correlated with high levels of vitamin B12 in the rumen, which indicates that microbial consumption may also play an important role in concentration variability (Lopez-Franco, 2019).

Furthermore, few studies have analyzed the impact of archaeal communities on the concentrations of vitamin B12 in the rumen and by proxy in milk. Despite their limited abundance in the rumen, they are primal and resilient organisms that are predominantly methanogenic. They are essential to rumen function. The archaeal rumen population remains largely uncharacterized, however recent studies are finding that uncultured groups consist of low abundances of non-methanogenic archaea, and that current methods possibly contribute to the limited diversity of archaea (Tajima, Nagamine, Matsui, Nakamura, & Aminov, 2001). This project will look to characterize the abundance and diversity of archaea in the rumen and establish how the archaeal and bacterial community composition is correlated to vitamin B12 concentrations.

1.2 Rationale

Many factors have been shown to influence the microbial population in the rumen; however, the influence that certain archaeal and bacterial phylotypes have on vitamin B12 concentrations remains under-explored. In this investigation we will attempt to further define the relationships between microbial communities and vitamin B12 concentrations. The goal is to understand exactly which archaea and bacteria influence the levels of vitamin B12 in milk, so that in the future we can naturally manipulate the microbial composition in the rumen to produce and maintain high concentrations of vitamin B12 in milk. This would help create a cost-effective approach to provide a reliable source of vitamin B12 for human nutrition.

1.3 Hypothesis

The abundance of certain phylotypes of archaea and bacteria, as well as abundance of prokaryotic genes involved in the production and consumption of vitamin B12, in the bovine rumen, are correlated to the abundance of vitamin B12.

1.4 Objectives

1. Preform 16S rRNA targeted amplicon sequencing of the rumen archaea on 96 rumen samples and correlate the archaeal community composition with vitamin B12 concentrations.
2. Conduct metagenomic sequencing of twelve rumen samples and determine which microbial populations and which metabolic pathways contribute to vitamin B12 production and consumption in the rumen

CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

2.1 Vitamin B12

Vitamin B12, known as one of the most structurally complex molecules in nature (Warren, Raux, Schubert, & Escalante-Semerena, 2002), became significant in the 1920s when it was discovered that pernicious anemia could be cured by including crude liver extract in a patient's diet. However, the crucial compound was only identified in 1948, where scientists isolated and crystallized the liver factor, which became known as vitamin B12 (Scott & Molloy, 2012).

2.1.1 Corrinoid Chemistry

Vitamin B12 is a water-soluble molecule that is also known as cobalamin (Cbl). It contains three main components: a corrin ring with a centered cobalt ion, an upper (Co-b) axial ligand, and a lower (Co-a) ligand containing a nucleotide loop (Woodson, Reynolds, & Escalante-Semerena, 2005). The lower ligand consists of 5,6-dimethylbenzimidazole (DMB), and the upper ligand is comprised of a 5'-deoxyadenosyl group; therefore, vitamin B12 is sometimes also referred to as adenosylcobalamin or coenzyme B12 (Woodson et al., 2005). Cobamides are complete corrinoids, which essentially are molecules with a cobalt-containing corrin ring, with an upper ligand that comprises of an adenosine, methyl, hydroxyl or a cyano-group, which yields the four vitamers/forms of vitamin B12 (Woodson et al., 2005).

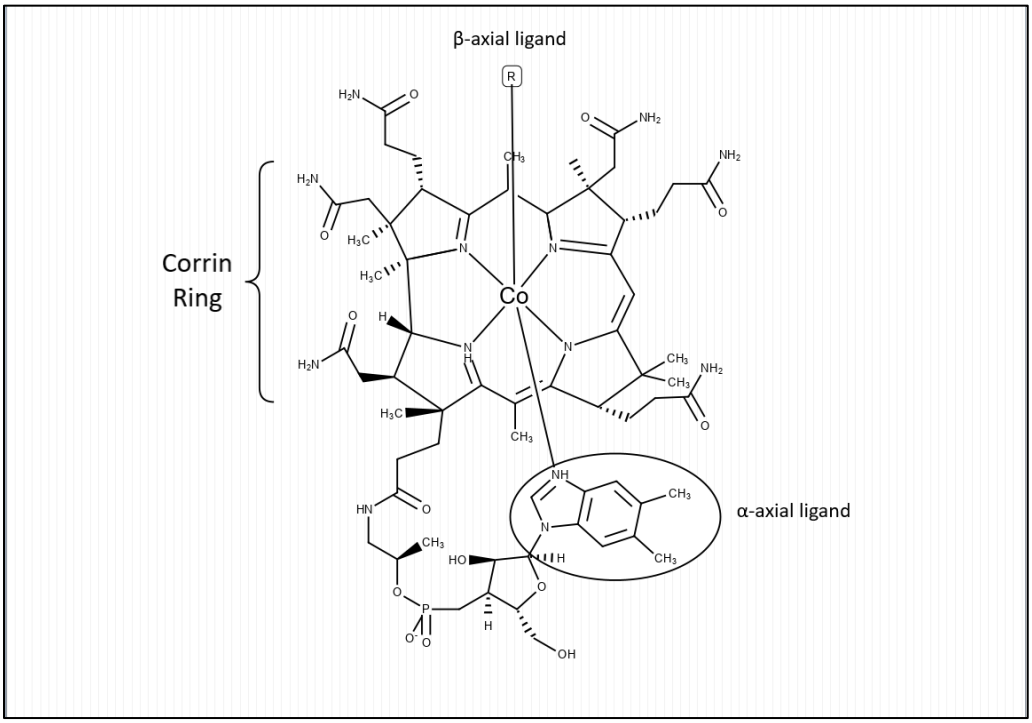


Figure 1. Vitamin B12. Corrin ring with a centered cobalt ion, and a dimethylbenzimidazole (DMB) lower, α-axial ligand.

2.1.2 Eukaryotic Vitamin B12 Absorption

Despite being an essential nutrient in humans, vitamin B12 has been shown to be extremely scarce in the environment and shown to be synthesized by only a few bacteria and archaea species (Scott & Molloy, 2012). Nevertheless, most bacteria, archaea, and eukaryotes exhibit requirements for vitamin B12 and other corrinoids. Humans only acquire vitamin B12 through diet, specifically via the consumption of animal products. The bacteria and archaea that synthesize vitamin B12 are located downstream from the ileum in the colon, but vitamin B12 is absorbed in the ileum meaning humans are unable to make use of the vitamin B12 synthesized in their own colons (Albert, Mathan, & Baker, 1980). Less than 2% of corrinoids produced by microbes are vitamin B12 (Degnan, Taga, & Goodman, 2014), making vitamin B12 scarce in the

environment and therefore much sought after. Additionally, high numbers of gut microbes in humans have been correlated with lower levels of vitamin B12 (Degnan, Taga, et al., 2014), suggesting direct competition between the host and the microbiome for scarce vitamin B12 resources, as both are dependent on exogenous corrinoids. Although it has been discovered that 80-86% of identified human gut bacteria metabolize corrinoids, fewer than 40% have the genetic ability to synthesize these molecules *de novo*, again highlighting the competition for this important nutrient (Degnan, Taga, et al., 2014; Shelton et al., 2019).

Ruminants and mammals that practice coprophagy are exceptions to the lack of self-producing bioavailable vitamin B12. Animals that practice coprophagy produce vitamin B12 in their colons and then consume their excreted fecal material so that the nutrients produced in the colon can be absorbed in the ileum. Ruminants, contain a wide range of microorganisms in their rumen, which is located upstream of the small intestine (Fig. 2), including vitamin B12 producing bacteria and archaea. Vitamin B12 and other nutrients are absorbed across the ruminal wall into the bloodstream and transported to the liver and other tissues for storage; however, most nutrient absorption occurs in the small intestine, specifically the ileum (Girard et al., 2009). Cows primarily excrete excess vitamin B12 in their feces; however, a small portion is also present in urine, and milk (Smith & Marston, 1970). The mechanisms of intestinal absorption in dairy cows is not completely understood but is thought to be similar to that found in humans (Girard et al., 2009).

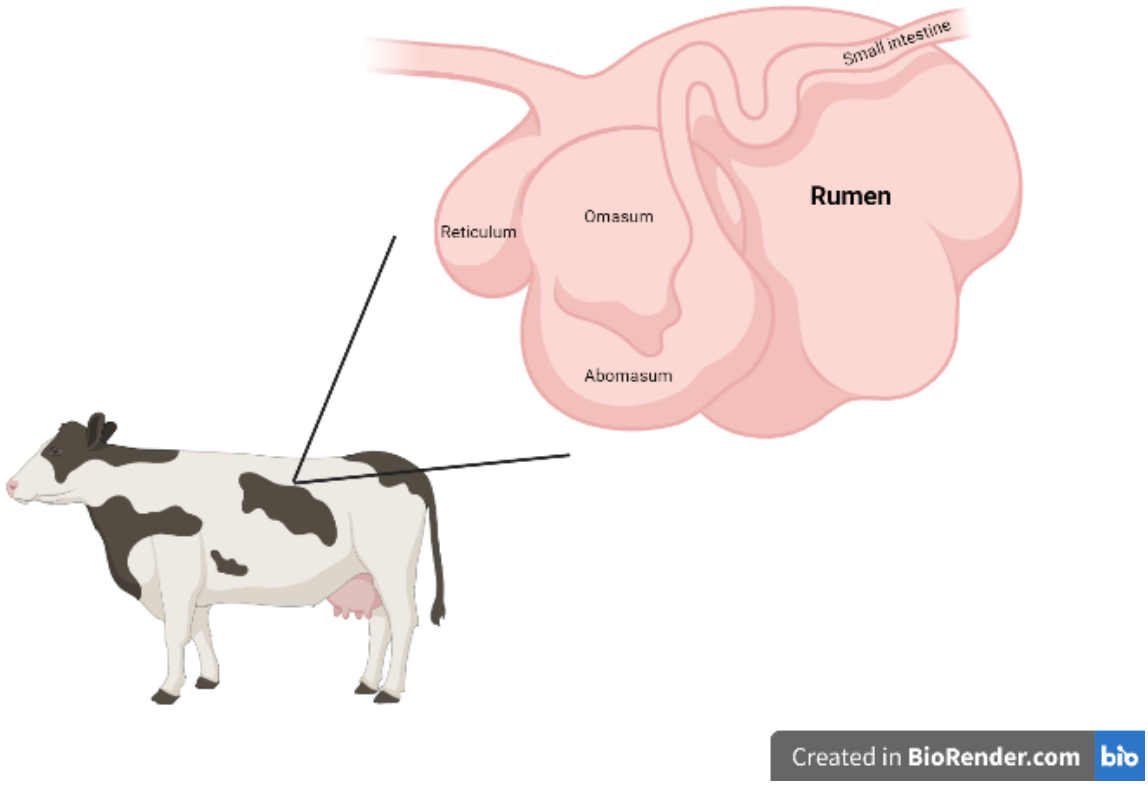


Figure 2. Ruminant digestion. The ingested food leads down the esophagus into the rumen, followed by the omasum, the abomasum, and finally to the small intestine, where most nutrients are absorbed. The reticulum functions to regurgitate the food for further chewing.

In humans, after ingestion, vitamin B12 is released from food by the enzyme pepsin (Gille & Schmid, 2015). Once in the upper gastrointestinal tract, vitamin B12 binds to the saliva-secreted glycoprotein haptocorrin, which protects the vitamin from hydrolysis. Vitamin B12 is translocated to the duodenum where pancreatic proteases breakdown haptocorrin releasing vitamin B12 into the gastric environment (Gille & Schmid, 2015; Nielsen, Rasmussen, Andersen, Nexo, & Moestrup, 2012). The presence of the intrinsic factor (IF), a glycoprotein secreted by the parietal cells in the stomach, is essential to absorption of vitamin B12 (McDowell, 1989). Expressed on the enterocytes of the ileum, IF binds vitamin B12 to form the IF-B12 complex which is recognized by the cubillin receptor, located on the apical surface of

cells of the terminal ileum, which absorbs the IF-B12 complex by receptor-mediated endocytosis (Figure 3) (Gille & Schmid, 2015; Nielsen et al., 2012).

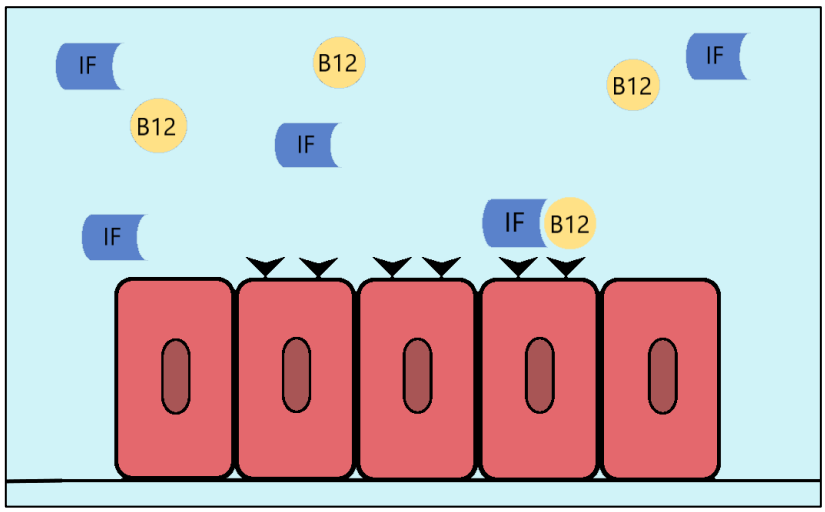


Figure 3. Receptor-mediated endocytosis of vitamin B12. IF and vitamin B12 form a complex which is recognized by the cubillin receptor on the apical surface of cells in the ileum.

When internalized, the complex is degraded via lysosome proteases, which liberates the vitamin B12 from IF for lysosomal export through the LMBD1 receptor. Vitamin B12 is passed between proteins and trafficked to the cell where it undergoes endocytosis after being recognized by the transcobalamin receptor (Green, 2010). From here, vitamin B12 is escorted to the mitochondrion then modified by the vitamin B protein into active cofactors deoxyadenosylcobalamin and methylcobalamin. Studies have shown that the vitamin B12 in bovine milk primarily binds transcobalamin whereas vitamin B12 in human milk binds haptocorrin (Watanabe & Bito, 2018). The free vitamin B12 that binds haptocorrin, is transported to the liver, where vitamin B12 is stored with a half life of 1-4 years (Gille & Schmid, 2015). Significant amounts of vitamin B12 are collected in the liver compared to other water-soluble vitamins, and this provides a reserve when cobalt levels are low.

2.1.3 Physiological Functions of Vitamin B12

The vitamin B12 cofactor is required by three enzymes in the human body: Methylmalonyl-CoA mutase, which consists of a small subunit *mcm*, and a large subunit *mutB*, and methionine synthase (*metH*) (Nielsen et al., 2012). Methylmalonyl-CoA mutase uses deoxyadenosylcobalamin for the interconversion of R-methylmalonyl-CoA and succinyl-CoA, which function in the digestion of organic compounds (fatty acids, sugars, cholesterol, etc.) in the mitochondria. Methionine synthase is involved in nucleotide synthesis, by utilizing methylcobalamin to catalyze the final step in the biosynthesis of methionine, which converts homocysteine to methionine (Degnan, Taga, et al., 2014; Scott & Molloy, 2012). Furthermore, the genetic composition of some eukaryotes encodes a corrinoid-dependent ribonucleotide reductase, essential for DNA synthesis (Degnan, Taga, et al., 2014).

Vegans and vegetarians are frequent victims of vitamin B12 deficiency because most plant-derived foods do not produce nor require vitamin B12. Unfortunately, even with the concomitant aerial and soil bacteria, plants rarely encompass significant amounts of vitamin B12 (Watanabe & Bito, 2018). This reiterates that the predominant dietary sources of vitamin B12 are limited to animal products.

Vitamin B12 deficiency, defined by serum vitamin B12 levels <150pmol/l and clinical anomalies, can be caused by pernicious anemia and food-vitamin B12 malabsorption (Dali-Youcef & Andrès, 2009). The latter is much more common and is characterized by proteins unable to extract vitamin B12 from food or binding proteins, and from gene mutated receptors on the ileum, responsible for vitamin B12 absorption (Dali-Youcef & Andrès, 2009). Pernicious anemia is much less common and is predominantly limited to elderly patients (Dali-Youcef & Andrès, 2009), and is defined by a reduction in red blood cells caused by the inability to properly

absorb vitamin B12. It is caused by a lack of intrinsic factor (IF) and destruction of gastric parietal cells (Stabler, 2013). Vitamin B12 deficiency can cause neurological disorders due to its involvement in the central nervous system, specifically the development of initial myelination (Stabler, 2013), responsible for the quick transmission of electrical impulses.

Vitamin B12 deficiency clinical indicators are delayed by 5-10 years due to the efficient enterohepatic circulation and reuptake in kidneys, thus creating long-term storage of vitamin B12 (Dali-Youcef & Andrès, 2009; Nielsen et al., 2012). Furthermore, the intestine absorbs 1-5% of free vitamin B12 by passive diffusion (Ellenbogen & Cooper, 1991), but only at very high concentrations. Vitamin B12 deficiency can be treated specifically with high-dose oral supplements, as only 1% of the oral dose is absorbed (Johnson, 2008).

2.2 Bacterial and Archaeal Synthesis of Vitamin B12

A cobalt-rich diet helps prevent vitamin B12-deficiency in ruminants, as cobalt is essential for the biosynthesis of vitamin B12 (Watanabe & Bito, 2018). The ruminal microflora utilizes 11% of the average daily intake of cobalt for corrinoid synthesis, of which 3-4% is converted into vitamin B12 (Girard et al., 2009). Ruminants, such as cattle and sheep, exhibit a vitamin B12 content of 38% of corrinoids in the rumen, which is notably higher than levels found in human feces, suggesting that coprophagic and ruminant animals may select for vitamin B12-producing gut microbes (Degnan, Taga, et al., 2014). Coprophagic species fulfill their requirements by ingesting their vitamin B12-containing feces, which transports vitamin B12 to the upper part of the digestive tract where the essential molecule can be utilized (Degnan, Taga, et al., 2014). Barnes and Fiala (1958) determined that rats fed a vitamin B12 deficient diet and

prevented from eating their feces resulted in growth depression, correlated to a lack of vitamin B12, illustrating its essential function in mammalian development.

Bacteria from the phyla *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Firmicutes* are predicted to be most involved in *de novo* vitamin B12 production (Magnúsdóttir, Ravcheev, de Crécy-Lagard, & Thiele, 2015; Shelton et al., 2019). It is also apparent that several bacterial genomes have also been shown to encode only parts of the biosynthetic pathway, which could be an indication of their involvement in the acquisition of exogenous corrinoids via the salvage pathway (Shelton et al., 2019). Few studies have explored the biosynthesis of vitamin B12 in archaea, however Zhang, Rodionov, Gelfand, and Gladyshev (2009) discovered that 75% of archaeal species that utilize cobalt also possess the vitamin B12 biosynthetic pathway. Furthermore, Makarova et al. (1999) have shown that archaea and bacteria share genome organization and expression, including vitamin B12 biosynthesis genes (Table 1). There are two pathways for the biosynthesis of vitamin B12: the *de novo* pathway and the salvage pathway (Fang, Kang, & Zhang, 2017).

Table 1. Orthologous genes present in archaeal and bacterial species essential for vitamin B12 biosynthesis, adapted by Makarova et al., 1999.

Pathway	**Orthologs of bacterial genes found in archaeal genome subjects**	**Genes missing in archaeal genome subjects**
Vitamin B12 Biosynthesis	***cysG2, cbiH, cbiF, cbiE, cbiC, cbiA, cbiP, cbiB, cobS***	***cobA, cobD***

2.2.1 *De Novo* Pathway

The *de novo* pathway is metabolically very costly, as synthesis of the complex vitamin B12 molecule requires the expression of a minimum of 30 genes (Warren et al., 2002). Two iterations of this pathway: aerobic and anaerobic exist, and these variations are based primarily in the timing of the cobalt insertion (Escalante-Semerena, 2007) and in the requirement of oxygen for the corrin ring contraction (Martens, Barg, Warren, & Jahn, 2002). The mechanisms of the pathway have been predominantly studied in the bacterial species *Salmonella enterica* ser. Typhimurium, *Propionibacterium freudenreichii* subsp. shermannii, and *Paracoccus denitrificans* (Fang et al., 2017).

The *de novo* pathway begins with the synthesis of aminolevulinic acid (ALA), a precursor to tetrapyrrole compounds, including vitamin B12, by the C4 or C5 pathway (Fang et al., 2017) (Figure 4). Within the C4 pathway, glycine and succinyl-CoA produce an ALA synthase enzyme that catalyzes the formation of ALA. In the C5 pathway, ALA is established from glutamate via three enzymatic reactions. Two ALA molecules condense and are polymerized by HemBCD enzymes to form uroporphyrinogen III, the first macrocyclic intermediate (Martens et al., 2002). From here, *cobA* (*P. denitrificans*) or *cysG* (*S. enterica* ser. Typhirumium *and Escherichia coli*) catalyze methylation of uroporphyrinogen III, which results in precorrin-2, the precursor of vitamin B12, and the diverging point for the anaerobic and aerobic pathways.

The anaerobic pathway begins with the chelation of precorrin-2 with cobalt, catalyzed by *cbiK*, whereas the cobalt insertion in the aerobic pathway occurs at a much later step and is ATP-dependent (Martens et al., 2002). This pathway consists of 10 *cbi* methyltransferases that

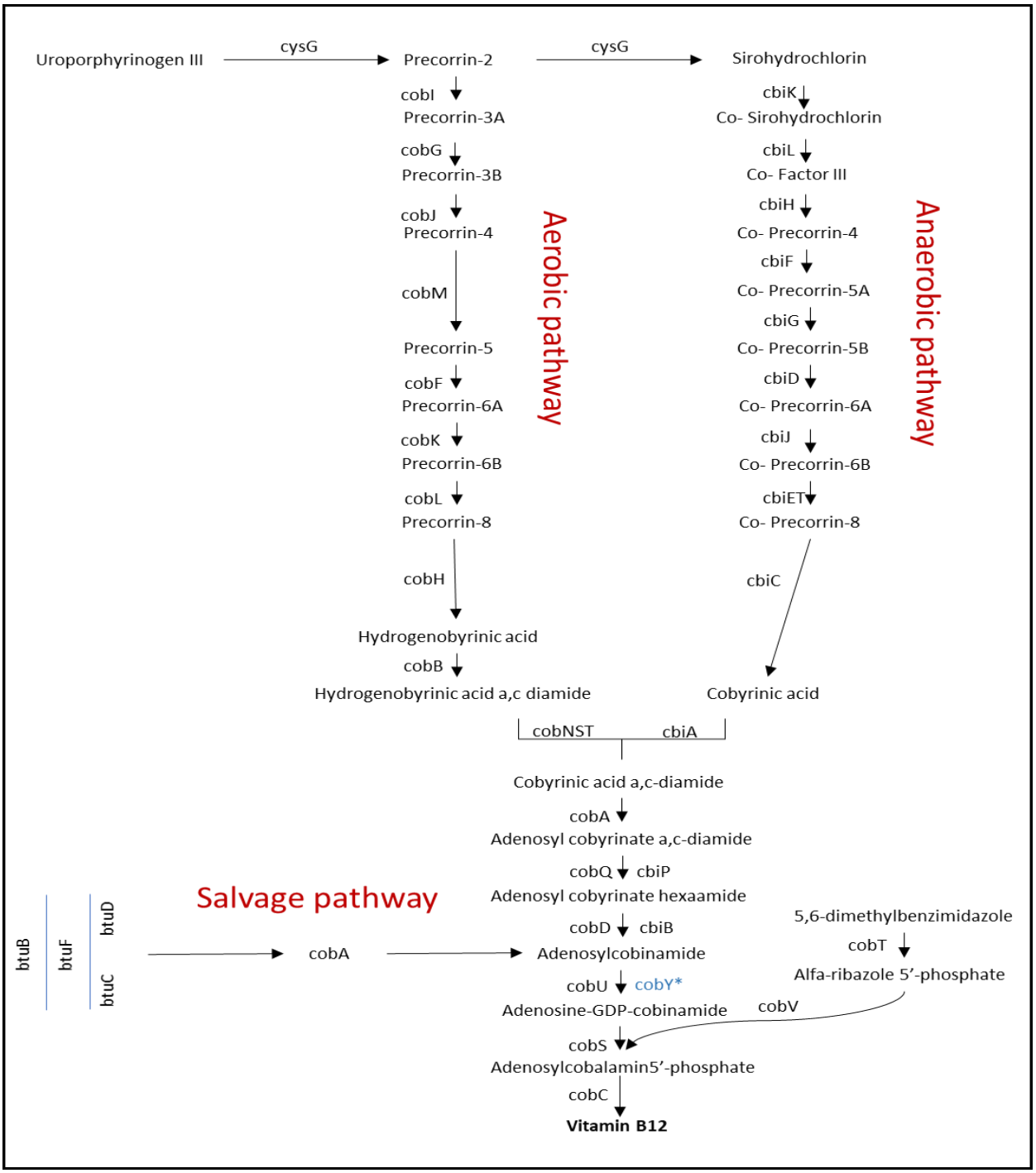


Figure 4. Bacterial Biosynthesis of vitamin B12, adapted from Fang et al., 2017 and Doxey et al. 2015. The anaerobic pathway is represented by the *cbi* genes, while the aerobic pathway is depicted by the *cob* genes. Genes *cobA, cobS, cobC* and *cobU* are found in both aerobic and anaerobic organisms, whereas genes *cobQ_cbiP* and *cobD_cbiB* are specific to their desginated pathway. The cobY gene (indicated by *) is the archaeal substitute for the bacterial *cobU* gene. The salvage pathway includes the ABC transporter that is represented by genes *btuB, btuF, btuC* and *btuD.* Gene *btuB* is located in the outer membrane, *btuF* in the periplasm, and the remaining *btuC* and *btuD* genes are found in the inner membrane.

ultimately form cobyrinic acid, which is where it converges with the aerobic pathway. The molecule is phosphorylated by *cobU*, a bifunctional enzyme that has AdoCbi kinase and AdoCbi-P guanylyltransferase activity, which converts AdoCbi to AdoCbi-GDP (Warren et al., 2002). The last reaction is the addition of the nucleotide loop to the lower ligand, manufacturing coenzyme B12 (Fang et al., 2017).

Archaea have been found to synthesize abundant amounts of cobamides and are thought to share similar mechanisms as bacteria in the biosynthetic pathways of vitamin B12 (Thomas & Escalante-Semerena, 2000). However, archaea possess certain distinctive non-orthologous gene replacements (Warren et al., 2002). More specifically, archaea do not contain a *cobU* orthologue, but instead a unique *cobY* gene that encodes for a protein with GTP:AdoCbi-P guanylyltransferase activity, but lacks NTP:AdoCbi kinase activity. Moreover, open reading frames Vng1576G and Vng1578H which encode for the *cbiP* and *cbiB* genes of *S. enterica* have been identified in *Halobacterium* sp. strain NRC-1 (Woodson, Peck, Krebs, & Escalante-Semerena, 2003). These archaeal orthologs function as AdoCby and AdoCbi-P synthases, which catalyze the final steps in corrin ring biosynthesis, essential for the *de novo* biosynthesis pathway. The gene *cbiP* is located downstream of ORF Vng1574G and ORF Vng1573G (Figure 5), which encode the bacterial *cobA* and *cbiA* in *S. enterica*, and function to modify the corrinoid prior to the cbiP-catalyzed step (Woodson, Peck, et al., 2003).

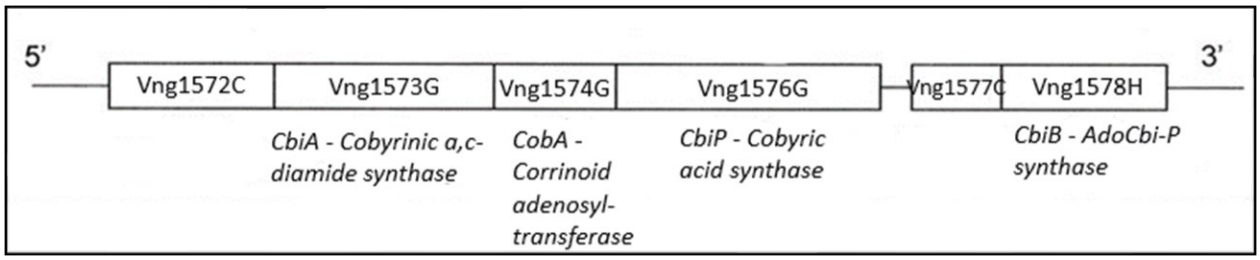


Figure 5. Open-reading frames in archaea essential for cobamide biosynthesis, adapted from Woodson et al., 2003. The bacterial orthologos *cobA, cbiA, cbiP and cbiB* are present in archaeal ORFs, and essential to the biosynthesis of vitamin B12 in archaea.

2.2.2 Salvage pathway

Prokaryotes also have the ability to salvage the precursor cobinamide (Cbi) from the environment and convert it to AdoCba (Escalante-Semerena, 2007). Cobinamide is an incomplete corrinoid lacking the nucleotide loop as the 5'-deoxyadenosine (Ado) ligand. This process is much more energy efficient, as it does not require prokaryotes to maintain the genetic information to make the corrin ring (Escalante-Semerena, 2007). The kinase activity found in the bacterial enzyme encoded by *cobU* is required for cobinamide salvaging in bacteria (Woodson, Peck, et al., 2003); however, studies have also shown that archaea are capable of salvaging exogenous cobinamide by using an alternative pathway, distinct from the one used by bacteria (Woodson et al., 2005).

Few studies have detailed evidence for the synthesis of vitamin B12 by an archaeal species. The first discovery of an archaeal corrinoid transport system was found after genetic analysis of *Halobacterium sp*. strain NRC-1 (Woodson et al., 2005). Strain NRC-1 contains open reading frames (ORFs) Vng1370G, Vng1371Gm, and Vng1369G encoding components of an ortholog of the bacterial vitamin B12 ABC transporter (ATPase-binding cassette transporter), essential for the salvage pathway. Furthermore, it was discovered that ORF Vng1578H, which

encodes the AdoCbi-P synthase enzyme *cbiB*, is needed for retrieving cobyric acid, an intermediate precursor in the synthesis of vitamin B12, from the environment (Woodson, Zayas, & Escalante-Semerena, 2003). The enzymatic activity of this ORF demonstrates the variation in the archaeal salvaging pathway, as bacteria do not require this enzyme because the kinase activity of *cobU* directly converts AdoCbi to AdoCbi-P, the product of the *cbiB* enzyme found in bacteria (Woodson, Zayas, et al., 2003).

The regulation of vitamin B12 biosynthesis and transportation is controlled by the vitamin B12 riboswitch, located in the 5′ untranslated regions (UTR) of the corresponding genes (Nahvi, Barrick, & Breaker, 2004). When levels of vitamin B12 are elevated in bacteria the *btuB* mRNAs contain a coenzyme B12-dependent riboswitch that represses translation of encoded vitamin B12 transport proteins (Nahvi et al., 2004). Specifically, when concentrations of vitamin B12 are too high, Rho-independent termination causes RNA transcription to halt. The mRNA contains a sequence that base pairs with itself to form a stem-loop that inhibits the polymerase reading from the transcription site. Translation is also inhibited by sequestration of the ribosome binding site (RBS). Once levels of vitamin B12 drop, formation of an anti-terminator hairpin occurs, allowing transcription of downstream genes, and the RBS is no longer sequestered, facilitating translation (Fang et al., 2017).

2.2.3 Biosynthetic Pathway Design

The vitamin B12 biosynthetic pathway has been studied numerous times in different organisms due to its complexity, meaning that there are varying possibilities concerning the enzymes required in these pathways. The present study formed unique *de novo* and salvage

pathways adapted from Fang et al. (2017) and Doxey et al. (2015) that encompasses the genes common to both pieces of literature (Figure 4.). Fang et al. (2017) designed an overview of the anaerobic and aerobic pathways, originating from *P. denitrificans* and *S. enterica* ser. Typhimurim, whereby Doxey et al. (2015) utilized the pathways described by Moore et al. (2013) and Raux, Schubert, Roper, Wilson, and Warren (1999) which are based on the pathways from commonly studied bacterial species *Bacillus megaterium*, *Propioni-bacterium shermanii*, and *S. enterica* ser. Typhimurim. Despite Doxey et al. (2015) interpreting the influence of *Thaumaerchaeota* in cobalamin production in marine environments, their selected marker genes are adapted from bacterial-associated pathways due to the limited research on these pathways in archaea. However, studies have shown that archaeal genomes encode a similar vitamin B12 biosynthetic pathway to bacteria (Makarova et al., 1999). The genes applied in this present study were selected based on their recurrence in literature, and their distribution throughout the pathways. The anaerobic pathway consists of the genes *cysG, cbiK, cbiL, cbiH, cbiF, cbiG, cbiD, cbiJ, cbiET, cbiC, cbiA, cobA, cbiP, cbiB, cobU, cobT, cobS, cobV,* and *cobC*, whereas the aerobic pathway is made up of the genes *cysG, cobI, cobG, cobJ, cobM, cobF, cobK, cobL, cobH, cobB, cobNST, cobA, cobQ, cobD, cobU, cobT, cobS, cobV,* and *cobC.* The anaerobic and aerobic *de novo* pathways overlap with the salvage pathway, which is composed of the genes *btuB, btuF, btuC, btuD, cobA, cobU, cobT, cobS, cobV* and *cobC.*

2.3 Synthetic Production of Vitamin B12

In 1973 Woodward and Eschenmoser achieved the full chemical synthesis of vitamin B12. However, the production is quite complex as it requires about 70 steps, thus making it very expensive (Eschenmoser & Wintner, 1977; Martens et al., 2002). The large-scale industrial

production involved involves biosynthetic fermentation, by utilizing B12-producing bacterial species (Fang et al., 2017). Unfortunately, no genera of archaea are considered as genetically optimal microorganisms. Bacteria selected for biosynthetic production of vitamin B12 naturally generate high yields of the vitamin, in addition to containing rapid growth rates and resistance to toxic intermediates in the media (Martens et al., 2002). The two primary strains used in the industry are *Propionibacterium freudenrechii*, and *Pseudomonas denitrificans* (Ma, Wang, Zhang, & Yi, 2008).

2.4 Bovine Rumen Microbiome

2.4.1 Rumen Microbial Composition

The bovine rumen houses bacteria, archaea, protozoa, fungi, and viruses. The microbial components of the rumen reveal a symbiotic relationship with the bovine host. The indigestible plant material is broken down and transformed into energy and nutrients by the microbial flora for the host. Specifically, mammals cannot digest cellulose, a main component of a cow's diet, but cellulolytic bacteria and other microbes secrete essential enzymes responsible for the breakdown of consumed products (Janssen & Kirs, 2008). Furthermore, the components of the ingested commodities are utilized by the microbes for metabolism and cellular growth.

Archaea

Archaea were established as one of the three domains of life, alongside Bacteria and Eurkarya, in the late 1970s when a new classification system based on molecular comparisons

was proposed (Woese, Kandler, & Wheelis, 1990). The new method of differentiation determined that the metabolic pathways and genetic composition of archaea resemble that of the eukaryotes rather than bacteria (Woese et al., 1990). They are single-celled organisms with an undeveloped nucleus and have a cell wall made up of pseudopeptidoglycan. Archaea divide asexually (budding, fragmentation or binary fission), and contain a plasmid-shaped single-circular chromosome. Archaea are often characterized as primitive organisms and classified as extremophiles; however, they have been discovered in nearly all niches occupied by bacteria. Species of archaea can survive and remain metabolically active in environments of high acidity, high temperature or high salt concentrations (Albers & Meyer, 2011). Some of the most common groups within the archaeal domain are Methanogens, Halophiles, and Thermoacidophiles.

Archaea are limited to 0.3-3.3% of the microbial community present in bovine rumen and consist primarily of methanogens (Janssen & Kirs, 2008). Despite their minimal capacity in the microbial biomass, methanogens are essential in rumen function (Janssen & Kirs, 2008). Methanogens are obligate anaerobes that utilize hydrogen, to reduce carbon dioxide, acetate and other methyl compounds, to methane. The hydrogen is primarily produced by fermentation, and its removal helps complete the oxidation of fermented substrates (Sharp, Ziemer, Stern, & Stahl, 1998). The fermentation of feed forms volatile fatty acids (VFAs), a significant energy source in ruminants (Henderson et al., 2015).

Most studies have characterized methanogens, specifically the family *Methanobacteriaceae*, as the rumen's sole archaeal inhabitants and have discovered that there is limited diversity in the dominant archaeal groups worldwide (Henderson et al., 2015; Skillman, Evans, Strömpl, & Joblin, 2006). The most common species of methanogens isolated from the rumen are strains of genera *Methanobrevibacter, Methanomicrobium, Methanobacterium,* and

Methanosarcina (Whitford, Teather, & Forster, 2001). Two clades, one defined by the species *Methanobrevibacter gottschalkii*, *Methanobrevibacter thaueri* and *Methanobrevibacter millerae*, and the other represented by *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae* account for 60-75% of all ruminal archaea (Henderson et al., 2015; Janssen & Kirs, 2008; Whitford et al., 2001), which suggests that a minimal range of substrates are used by the rumen archaeal population. Of the archaea discovered, nearly 80% consist of hydrogenotrophic methanogens which use hydrogen as a growth substrate. The remaining archaea either utilize a combination of hydrogen and methyl groups, and even more rare are the methanogens that form methane from acetate (Henderson et al., 2015).

The dominant genus-level groups *Methanobrevibacter* and *Methanomicrobium* make up roughly 75-77% of archaea detected in the rumen (Chaucheyras-Durand & Ossa, 2014; Janssen & Kirs, 2008). The remaining rumen archaea are classified as a large uncultured group of archaea that display considerable sequence variation, commonly referred to as the rumen cluster C (RCC). Other studies have found the RCC to comprise of 17-50% of the rumen archaea and contain sequence differentiation greater than 3% between members of distinct subgroups (Janssen & Kirs, 2008; Wright, Auckland, & Lynn, 2007).

Like previous studies, Tajima et al. (2001) also found methanogens to be the dominant archaeal group in the rumen. Their experiment consisted of two different archaea-specific primer sets; the first library yielded methanogen species *Methanobacterium formicicum*, *Methanobacterium ruminantium*, *Methanosarcina barkeri*, and *Methanobacterium mobile*. The second archaeal library; however, discovered a novel group of sequences that are distantly related to the thermoacidophilic archaea *Thermoplasma acidophilum* and *Picrophilus oshimae*. Unfortunately, no closely related isolates have been cultivated, however 94% similarity exists in

a conserved region to that of *Thermoplasma* sequences. *Thermoplasma* species belong to the family *Thermoplasmata* and thrive in acidic and high-temperature environments. Current archaeal primer designs appear to be concealing the real phylogenetic diversity present in the rumen, but it may also be related to difficulties in isolation, culturing or maintenance (Tajima et al., 2001).

All available genomes within the phylum *Thaumarchaeota* appear to contain vitamin B12 synthesis genes (Lu, Heal, Ingalls, Doxey, & Neufeld, 2020). *Thaumarcheota* encode the *cbiA/cob* and *cbiC/cobH* genes, which are reliable indicators of the entire biosynthetic pathway, and have shown predominant use of the aerobic process (Doxey et al., 2015). Considered the most representative archaea on the planet, *Thaumarcheota* have been identified in low abundance in the bovine rumen (Shin et al., 2004; Wang et al., 2016).

Moreover, sequences of Thermophilic-*Crenarchaeota* and non-thermophilic-*Crenarchaeota* were identified in rumen fluid and epithelium (Shin et al., 2004). The ruminal fluid fraction consisted primarily of members belonging to the *Methanomicrobiaceae* family; however, one sequence was related to non-typical rumen archaea *Methanobrevibacter smithii* and another sequence (2.2% of ruminal fluid fraction) related to the *Sulfolobus acidophilum* family. Similar to the ruminal fluid fraction, the majority of sequences within the rumen epithelium belonged to the *Methanomicrobiaceae* family, however 5.1% of clones related to the *Thermoproteus* genera (Shin et al., 2004). Wang et al. (2016) also discovered that 15% of goat rumen consists of the phyla *Thaumarchaeota*, and 0.68% of *Crenarchaeota*, while the remaining archaeal community is made up of species from the phylum *Euryarchaeota*.

Bacteria

Bacteria are the major microbes in the rumen as they comprise of 10^{10}-10^{12} bacteria per ml, and account for approximately 60-90% of total rumen rRNA (Lin, Raskin, & Stahl, 1997). The bovine rumen microbiota exhibits a core bacterial community shared by all cows; however, these bacterial groups are not uniformly abundant in all species (Henderson et al., 2015; Lin et al., 1997; Zhu et al., 2018). The rumen generally consists of three dominant bacterial phyla: *Bacteroidetes, Firmicutes,* and *Proteobacteria* (Doxey et al., 2015; Jami et al., 2013; Salfer et al., 2018). *Firmicutes* is the major phylum comprising of 58%-70% of ruminal sequences and can be further divided into members of the genera *Butyrivibrio*, *Ruminococcus*, *Lachnobacterium*, *Oribacterium*, *Selenomonas*, *Streptococcus*, and *Succiniclasticum* (Creevey, Kelly, Henderson, & Leahy, 2014; Kim, Morrison, & Yu, 2011).

The genus *Prevotella* is the most abundant genus in the rumen in the *Bacteroidetes* phyla, consisting of 40-72% of total reads (Jami et al., 2013; Salfer et al., 2018). Creevey et al. (2014) found that the core *Bacteroidetes* OTUs shared >96% gene sequence similarity with the *Prevotellaceae* family 16s rRNA genes. The most commonly isolated *Prevotella* spp. are *P. ruminicola, P. brevis,* and *P. bryantii,*(Stevenson & Weimer, 2007). Importantly, the phylum *Bacteroidetes* has been affiliated with being the largest vitamin B12 dependent taxon (Shelton et al., 2019; Wexler et al., 2018).

Proteobacteria is the lesser of the three phyla, as it comprises of roughly 5.5 – 11% of the bacterial biomass in the rumen (Creevey et al., 2014; Jami & Mizrahi, 2012). This phylum is usually comprised of *E. coli, Ruminobacter*, *Campylobacter*, and *Desulfovibrio*, (Zhong, Xue, & Liu, 2018). However, like the *Bacteroidetes* and *Firmicutes* phyla, the abundance of *Proteobacteria* is prone to great variation in response to dietary change (Jami & Mizrahi, 2012).

Finally, the phyla *Tenericutes* and *Spirochaetes* are also common in the bovine rumen, but are noticeably less abundant, making up roughly 1.5-2% of the microbial community (Zhong et al., 2018).

Protozoa

Protozoa are single-celled organisms that display much greater variability between animal species, compared to bacteria and archaea. They can contribute up to 50% of the rumen biomass (Newbold, de la Fuente, Belanche, Ramos-Morales, & McEwan, 2015); however, nearly all ruminal protozoan sequences can be assigned to 12 genera: *Isotricha*, *Dasytricha*, *Entodinium*, *Ostracodinium*, *Epidinium*, *Diplodinium*, *Metadinium*, *Osphyoscolex*, *Polyplastron*, *Eudiplodinium*, *Diploplastron*, and *Metadinium*. Notably, Henderson et al. (2015) determined that over half of their studied ruminal samples (54.7%) were attributed to the genera *Entodinium* and *Epidinium*. The primary role of protozoa remains controversial; however, it is known that they contribute to digestion and methanogenesis, and that most rumen protozoa are vitamin-dependent, while some have acquired vitamin B12 specificity for substrate metabolism (Arnstein & White, 1961).

Fungi

More than 18 species of rumen fungi have been identified and encompass up to 10% of total microbial biomass within the rumen (Chaucheyras-Durand & Ossa, 2014). The rumen anaerobic fungi can be classified into six genera: *Neocallimastix*, *Piryomyces*, *Anaeromyces*, *Caecomyces*, *Orpinomyces*, and *Cyllamyces*. The most represented genus is *Piromyces*, which

consists of 36% of the fungi community, while the genera *Orpinomyces* and *Callamyces* make up about 0.7-1.1% (Chaucheyras-Durand & Ossa, 2014). Fungal species have been shown to primarily encode the vitamin B12-independent methionine synthase (Wheatley, Ng, & Kapoor, 2016); however, some suggestions that zoosporic marine fungi have vitamin B12 requirements have been made (Goldstein, 1973). Limited information has been found on the association of fungus and vitamin B12, although, fungi seem to have little to no dependence for vitamin B12.

2.4.2 Microbe-Rumen Associations

Under anoxic conditions, methanogenesis occurs as a biproduct of a bacteria, fungi, and protozoa and methanogen metabolism (Thauer, Kaster, Seedorf, Buckel, & Hedderich, 2008). Methanogenic substrates are produced by anaerobic bacteria, protozoa, and fungi via the hydrolysis of polymers to glycerol and fatty acids, which are then fermented to acetic acid, carbon dioxide, and hydrogen (Thauer et al., 2008).

Within the rumen, methanogens can be free-living or associated with protozoa as a protozoa associated methanogen (PAM), or with fungi as a fungi associated methanogen (FAM). This endosymbiotic relationship improves methanogen exposure to H_2. It has been estimated that 9-25% of ruminal methanogens are associated with protozoa, which are found in concentrations of 10^6 ml^{-1} within the rumen (Janssen & Kirs, 2008). Specifically, Janssen and Kirs (2008) found that the genera *Methanobrevibacter,* and *Methanomicrobium* roughly account for 30% of PAM interactions. However, Tymensen, Beauchemin, and McAllister (2012) discovered that *Methanobrevibacter* represented up to 79% of archaea in the PAM community, while *Methanomicrobium* species are predominantly free-living. Furthermore, switching the

composition of the diet affects the overall community structure, altering methanogen and protozoan associations.

Methanogens have been discovered within the cytoplasm and attached to the external surface of ciliates where interspecies hydrogen transfer is performed (Finlay et al., 1994). Protozoa and bacteria produce hydrogen through fermentation-based systems that breakdown organic matter. The built-up of hydrogen is inhibitory to protozoa metabolism and can be removed by methanogens as a substrate for methanogenesis. Therefore, selective attachment enables increased cellular growth for both affiliates (Sharp et al., 1998). *Entodinium* spp. and *Dasytricha ruminantium*, which account for more than 90% of rumen ciliates, average 96-520 endosymbiotic methanogens per microorganism (Finlay et al., 1994). Methanogens can be found in association with ruminal ciliates of genera *Diplodinium*, *Epidinium*, *Dipoplastron*, *Enoplopastron*, *Entodinium*, *Eremoplastron*, *Ostrcodinium*, *Eudiplodinium*, and *Polyplastron* (Vogels, Hoppe, & Stumm, 1980). However, some studies argue that associations between archaea and protozoa are not convincingly proven. Henderson et al. (2015) detected no strong correlations between archaea and protozoa, indicating that these vital interactions are perhaps non-specific (Henderson et al., 2015).

The most abundant bacteria at the genus level are *Prevotella*, *Butyrivibrio*, and *Ruminococcus*. The genus *Ruminococcus* and species within phylum *Firmicutes* are known to be active H_2 producers, while other species of bacteria are H_2 users. Kittelmann et al. (2014) discovered a high number of *Ruminococcus* species associated with high-methane emissions, which indicates the presence of syntrophic communities. Interspecies hydrogen transfer occurs by diffusion and when hydrogen levels are elevated, hydrogen-utilizing methanogens inhibit the metabolism of hydrogen-forming bacteria, and vice versa (Stams & Plugge, 2009). The rate of

diffusion is greatly enhanced by the aggregation of anaerobic bacteria and methanogenic archaea. Finally, limited research has been conducted on the physical associations between methanogens and fungi (Lan & Yang, 2019).

2.4.3 Factors that Alter Rumen Composition

The overall microbial composition of the rumen has been shown to be influenced by variations in diet, environment, host management practice, and host genome, age, and health status (Janssen & Kirs, 2008). More specifically, bacteria have shown to be the leading microbes behind observed differences in rumen microbial community structure. This can be partially explained by the large diversity in metabolism specificity amongst bacterial species. Like archaea, dominant groups of rumen bacteria are common around the world, despite the high numbers of rumen bacteria, and the impact of internal and external factors.

Ruminants display lower abundances of archaea in reflection to a diet characterized by high energy and protein, while cows with 100%-hay diets demonstrated the highest abundance of archaea, specifically methanogens, in the GI tract (Lin et al., 1997). The effects of transitioning dairy cattle to a high concentrate diet and reducing ruminal pH were examined and resulted in a change of methanogen composition and increased protozoal concentration in the rumen fluid due to a high concentrate feed (Hook, Steele, Northwood, Wright, & McBride, 2011). A reduction in ruminal pH selected *Methanosphaera stadimanae*, which can be characterized by a wider pH range optimum compared to other methanogens, or perhaps acid-tolerant methanogens acquire less competition in low pH environments (Hook et al., 2011).

The impacts of diet on the microbial community in bovine rumen were assessed, as well as variabilities between primiparous and multiparous cows (Kumar, Indugu, Vecchiarelli, & Pitta, 2015). The findings were comparable with other reports that found minimal to no changes in the archaeal composition, despite altered external and internal factors. The minimal diversity in archaea and their resilience can possibly be explained by their early establishment in the rumen at an early age or due to their low density in the rumen (Su, Bian, Zhu, Smidt, & Zhu, 2014). Similarly, unlike bacteria, ruminal archaea do not display an age-related pattern, indicating that they are less sensitive to age and diet (Wang et al., 2016). Yet, in addition to *Methanobrevibacter* maintaining a high and constant abundance, methylotrophic *Methanosphaera* exhibited an increase in quantity during the transition period (Zhu et al., 2018). However, this response may be explained by a change in postpartum diet, containing a higher source of pectin, which facilitates the growth of methylotrophic methanogens (Zhu et al., 2018). Moreover, 11 phylotypes were identified in Ontario feedlot cattle fed a corn-based diet, that were not present in the feedlot cattle from Prince Edward Island fed potato by-products (Wright et al., 2007).

2.5 Prokaryotic Vitamin B12 Consumption

Along with vitamin B12 synthesis, prokaryotes are also responsible for the consumption of vitamin B12 in the rumen. Studies have shown a direct competition for vitamin B12 between the host and its microbial flora, thus acting as a regulator of the gut microbiome (Degnan, Barry, Mok, Taga, & Goodman, 2014). Vitamin B12 represents 38% of corrinoids synthesized in the rumen, and only half of synthesized corrinoids reach the duodenum. Nearly 80% of bacteria in the gastrointestinal tract have vitamin B12-dependent enzymes, however only 25% of these

bacteria contain the capacity for *de novo* vitamin B12 biosynthesis (Girard et al., 2009). These vitamin B12-depdendent genes include *metH*, *mutB* and *mcm*, which can be used as a indicators for the metabolism of vitamin B12 among bacteria and archaea (Shelton et al., 2019).

Many bacterial genomes encode multiple vitamin B12 transporters, providing them with a competitive advantage for vitamin B12 (Degnan, Barry, et al., 2014). The *btuBFCD* transporter is a predominant route for vitamin B12 within Gram-negative species. The enzyme *btuB* serves as the outer membrane receptor, along with the inner membrane ABC transporter, and the periplasmic binding protein *btuF* (Chimento, Mohanty, Kadner, & Wiener, 2003). Gram-positive species exhibit a similar transporter; however, lack the outer membrane receptor *btuB*. Species from the *Bacteroidetes* phylum can encode up to four copies of the corrinoid transporter *btuB* (Degnan, Barry, et al., 2014). Different homologs of *btuB* exist, but all present different corrinoid affinities. Most importantly, the *btuB3* homolog seems to provide *Bacteroides thetaiotaomicron*, whose genome encodes 3 functional *btuB* proteins, with the greatest competitive advantage (Degnan, Barry, et al., 2014). Additionally, *Bacteroides* species encode a supplementary surface lipoprotein *btuG*, which has femtomolar affinity binding for vitamin B12. The strong *btuG* affinity for vitamin B12 has the capability of removing the vitamin from the mammalian intrinsic factor (Wexler et al., 2018).

Bacterial overgrowth, which is the abnormal increase of bacteria in the small intestine, has been correlated with significantly lower levels of vitamin B12 absorption. Small intestinal bacterial growth (SIBO) is a condition characterized by bacterial populations exceeding 10^5-10^6 organisms/mL in the small intestine, while usual bacterial abundance in the small intestine is below 10^3 organisms/mL. The competitive uptake of vitamin B12 by the enteric flora has led to nutrient deficiencies, including vitamin B12 malabsorption (Dukowicz, Lacy, & Levine, 2007;

Zaidel & Lin, 2003). The use of antibiotics that specifically target *Bacteroidetes* have shown to be extremely effective in reversing vitamin B12 deficiency (Schjonsby, Halvorsen, Hofstad, & Hovdenak, 1977). Gut microbes have also been found to convert dietary vitamin B12 into analogs that cannot be used by humans (Allen & Stabler, 2008).

The rate of vitamin B12 consumption varies substantially between Gram-positive and Gram-negative strains of bacteria. Gram-positive bacteria such as *Bacillus subtilis* has shown much greater uptake values, nearing 24,000ng/hour, compared to Gram-negative bacteria with uptake values of roughly 255ng/hour (Giannella, Broitman, & Zamcheck, 1971). Furthermore, *B. subtilis* demonstrated these measurable quantities of vitamin B12 with 10,000 times fewer organisms than these Gram-negative species (Giannella et al., 1971).

Correlations between the rumen taxonomic composition and high levels of vitamin B12 have been identified. An increased abundance of *Succinivibrionacea*, *Prevotella*, and *Shuttleworthia* were found in the rumen, with measurably elevated concentrations of vitamin B12 (Lopez-Franco, 2019). Studies have not identified *Prevotella* as a species capable of *de novo* synthesis of vitamin B12. It is possible *Prevotella* is present at higher levels of vitamin B12 to benefit metabolically from the increased vitamin B12 concentrations (Lopez-Franco, 2019). The relationships between bacteria and vitamin B12 concentrations in the rumen remain ambiguous; however, findings imply that vitamin B12 concentrations may drive the community composition, and bacterial consumption of the vitamin may play a larger role than bacterial production.

Current literature is not available on the role of archaea in vitamin B12 consumption. However, vitamin B12 ABC transporters are ubiquitous among archaea, as nearly 3% of archaeal genomes encode these particular transporters, which are significant for the uptake of corrinoids (Wilkens, 2015). Furthermore, these archaeal transporters have demonstrated high substrate

affinity, allowing them to scavenge substrates at low concentrations (Albers, Koning, Konings, & Driessen, 2004). Therefore, this work was undertaken to further elucidate the relationships between bacteria and vitamin B12 in the bovine rumen – as well as to attempt to understand how archaea are involved in vitamin B12 cycling in the rumen.

CHAPTER 3. METHODS

3.1 Sample Collection

Seventy-five rumen samples were collected at the Sherbrooke Research and Development Centre by staff at the centre with the help of a summer undergraduate student. Each sample was a composite sample composed of sub-samples collected from different sections of the rumen of lactating dairy cattle: cranial dorsal, cranial ventral, central, caudal dorsal, and caudal ventral, which were freeze dried after mixing and stored at -20°C until processing. Vitamin B12 quantification was conducted by members of Agriculture and Agri-Food Canada (AAFC) Research Centre Team (Sherbrooke, Quebec, Canada).

3.2 16S rRNA Targeted Amplicon Sequencing

3.2.1 DNA Extraction

Extraction of bacterial DNA from the rumen samples was done using the QIAamp PowerFecal DNA Kit, following the manufacturer's instructions for a 200 mg sample. The quality of the extracted DNA was measured using a Nanodrop™ 2000/2000c spectrophotometer, and purified DNA was stored at −20°C until 16S rRNA PCR amplification.

3.2.2 Library Prep

The purified DNA from each sample was sequenced by a 16S rRNA targeted-amplicon sequencing approach that was optimized to characterize the archaeal communities present in the rumen. Archaea-specific and universal primers from literature were selected and compiled together to compare the coverage, amplicon length, and sequenced hypervariable region (Table

3). The V3 region of the 16S rRNA gene was chosen for sequencing since it appeared to offer good coverage of archaea and because the amplicon was <250 bp (which is optimal for reducing bias in the paired end 16S rRNA targeted amplicon sequencing approach). The archaeal 16S rDNA from sample was amplified by PCR using barcoded primers 349F and 519R. The primers consisted of 8 different forward and 12 different reverse primers, that generated 96 unique sequence combinations with an amplicon length of 170bp (Table 4). Custom primers 349F and 519R were selected because they provide the best overall archaeal coverage, with high specificity for the archaeal domain (Table 4) (Klindworth et al., 2013). The samples were sent to the Metagenom Bio Life Sciences lab in Waterloo, Ontario, for library preparation and sequencing.

Table 2. Archaea Specific PCR Primers

Name	Secondary Assigned Name	Sequence 5'→3'	Individual Coverage	Amplicon length	Hypervariable region	Reference
519F	S-D-Arch-0519-a-S-15	CAGCMGCCGCGGTAA	79.3%			Klindworth et al. (2013) Cannot be used to target archaea specifically (Pausan)
1041R		GGCCATGCACCWCCTCTC				
349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	76.8%	170bp	3	Klindworth et al. (2013)
519R	S-D-Arch-0519-a-A-16	TTACCGCGGCKGCTG				
SSU1ArF		TCCGGTTGATCCYGCBRG		Longer amplicon	1 & 2	Bahram et al.
SSU520R		GCTACGRRYGYTTTAARC				
340F		CCCTAYGGGYGCASCAG		Shorter amplicon	4 & 5	Bahram et al.
806rB		GGACTACNVGGGTWTCTAAT				
SSU666ArR		HGCYTTCGCCACHGGTRG			1 & 2	Bahram et al.
SSU1000ArR		GGCCATGCAMYWCCTCTC				
344F	S-D-Arch-0344-a-S-20	ACGGGGYGCAGCAGGCGCGA		150-300bp	3	Pausan et al. (2018)
1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC				
519F	S-D-Arch-0519-a-S-15	CAGCMGCCGCGGTAA		150-300bp	3	Pausan et al. (2018)
806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT				
787F	S-D-Arch00787-a-S-20	ATTAG ATACC CSBGT AGTCC	60-70% Methanogens only	273bp		Yu et al., (2005)
1059R		GCCAT GCACC WCCTC T				
D30		ATTCCGGTTGATCCTGC				Tajima et al.
D33		TCGCGCCTGCGCCCCGT				
0025e		CTGGTTGATCCTGCCAG				Tajima et al.
1492		GGTTACCTTGTTACGACTT				
340f		CCCTACGGGGYGCASCAG	64.5%		Specific approach	Koskinen et al.
519ar		TTACCGCGGCKGCTG				
344F		ACG GGG YGC AGC AGG CGC GA	Methanogens only	191bp	3	Yu et al., 2008
519R		GWA TTA CCG CGG CKG CTG				
515F		GTGCCAGCMGCCGCGGTAA	Soil archaea – primarily crenarchaeota	~250bp		Bates et al. (2011)
806T		GGACTACVSGGGTATCTAAT				

Table 3. Archaea Specific 16S rRNA Targeted Amplicon Sequencing Primer Sequences

Primer Name	Sequences (5'-3')	Size (bp)
F349-SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGYGCASCAGKCGMGAAW	66
F349-SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGYGCASCAGKCGMGAAW	66
R519-SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCTTACCGCGGCKGCTG	59
R519-SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCTTACCGCGGCKGCTG	59
V4-Read-1	TATGGTAATTGTGYGCASCAGKCGMGAAW	29
V4-Read-2	AGTCAGTCAGCCTTACCGCGGCKGCTG	27
V4-Index	CAGCMGCCGCGGTAAGGCTGACTGACT	27

3.2.3 Amplicon Sequencing

The 16S rRNA amplicons were sequenced using a 500-cycle V2 reagent kit (Illumina), at the Metagenom Bio Life Sciences lab in Waterloo, Ontario.

3.3 Amplicon Sequencing Data Analysis

3.3.1 16S rRNA Targeted Amplicons

The 16S rRNA sequencing reads were processed by the Metagenom Bio Life Science (Waterloo, Canada). The sequences were demultiplexed and processed using DADA2 v1.8 (Callahan et al., 2016), managed through the microbiome bioinformatics platform QIIME2 v.2019.7 (Bolyen et al., 2019), to merge paired ends, trim primers and remove chimeras.

Sequences were referenced to the SILVA v.134 database to generate a table with operational taxonomic units (OTUs) for downstream analysis. All raw sequence read data were up-loaded to the NCBI SRA database and can be found under the accession number SUB9247324.

3.3.2 Metadata Correlation Analysis

Marker Data Profiling (MDP) was used in the web platform MicrobiomeAnalyst for data analysis (Chong, Liu, Zhou, & Xia, 2020). Two output files generated from data processing were inputted into the system: the feature table with taxonomy file and the phylogenetic tree file. Further processing was done to remove low quality OTUs. OTUs with counts below 20 and present in less than 10% of samples were removed from downstream analysis, and data rarefication was performed to generate an even sequencing depth.

Metadata files were designed for vitamin B12 concentration, parity, days in milk (DIM), and production. Ruminal vitamin B12 concentrations were divided into two groups: high (>735ng/g) and low (<535ng/g). The metadata for Days in Milk was split into 3 groups labelled low (<125), mid (125-220), and high (>220). Production levels were categorized as high (>18kg), mid (14-18kg) low (<14kg), and Parity ranged between 1-6. Linear discriminant analysis effect size (LEfSe) was used to determine biologically significant features in specific groups of samples. This was done by using a combination of a Kruskal-Wallis rank sum test, which identifies features with a differential abundance, followed by a Linear Discriminant Analysis (LDA) which assesses the effect size of the differential abundance (Dhariwal et al., 2017). The default settings of LefSe were used: adjusted p-value cut-off: 0.05, Log LDA score of 1.0 and a False Discovery Rate (FDR) of 0.05.

3.4 Metagenomic Samples

Twelve DNA extracted rumen samples were selected for metagenomic sequencing based on the sample from which they were extracted also having particularly high (>735ng/g) or particularly low (<535ng/g) ruminal vitamin B12 concentration. Samples grouped as "high concentration" are 2, 33, 34, 58, 62, 83, while samples grouped as "low concentration" are 13, 36, 37, 80, 85, 95. In addition to vitamin B12 concentrations, the samples were chosen based on having no prior *Staphylococcus* infections and having a small range of DIM (Table 5).

Table 4. Metagenomic sample parameters.

Sample ID	Staph	DIM	Production (kg)	Parity	Ruminal Vitamin B12 Concentration (ng/g)	Classification
2	0	13	23.80	6	1003.2563	High
13	0	277	9.20	2	467.69441	Low
33	0	284	20.63	2	810.28239	High
34	0	140	20.98	5	809.22262	High
36	0	175	21.98	3	421.63164	Low
37	0	169	7.53	3	519.60596	Low
58	0	179	19.47	4	735.89497	High
62	0	133	15.58	6	890.99589	High
80	0	188	17.30	4	382.25359	Low
83	0	202	15.79	5	776.77237	High
85	0	137	16.57	4	470.94529	Low
95	0	13	14.57	5	534.9753	Low

3.4.1 Library Preparation & Sequencing

The DNA concentration of the selected samples were calculated using the QuantiT^{TM} dsDNA High-Sensitivity Assay Kit (ThermoFisher). Following quantification, samples were diluted with water to adjust the concentration to >150 ng/μL to meet the Genome Quebec

requirements, and subsequently aliquoted into a 96-well plate. The samples were shipped to Genome Quebec (Montreal, Canada) for shotgun sequencing via NovaSeq 6000 with 70M reads PE 150bp. The sequence read data were up-loaded to the NCBI SRA database and can be found under the accession number PRJNA691977.

3.5 Metagenomic Data Analysis

3.5.1 Reference Based Analysis

The sequencing reads received from Genome Quebec were inputted into MG-RAST, a metagenomics analysis server, which generates a taxa distribution and a distribution of functional categories using the SEED pipeline (Keegan, Glass, & Meyer, 2016). After uploading the data, sequences were normalized to produce unique IDs, and artificial duplicate reads and low-quality sequences were removed (Meyer et al., 2008). Sequences were clustered at 97% to compute the abundance profile per lowest common ancestor (LCA). Amino acid sequences were filtered and clustered at 90% identity. The output was compared against the M5NR protein database to identify protein features.

Processed sequencing reads were also inputted into eggNOG-mapper, a functional annotator that uses precomputed orthologous groups from the eggNOG database to generate gene catalogs (Huerta-Cepas et al., 2017; Huerta-Cepas et al., 2019).

3.5.2 Genome Binning

A unique pipeline was designed that encompassed quality control, assembly, binning, taxonomic profiling, and functional annotation. Initially, the raw reads were preprocessed in the quality filtering step using FASTQC and BBDUK. FASTQC generates statistics of the raw data to help determine the quality filtering steps to apply to the sequence data (Pérez-Cobas, Gomez-Valero, & Buchrieser, 2020). Contaminants that cause errors in quality control can be removed accordingly by sequence trimming and filtering using BBDUK, which refines reads using Kmers (Bushnell, 2021). Specifically, five different commands were used to optimize the quality of the raw reads: adapter-trimming, removal of phiX and human contamination, quality trimming, and removal of short reads.

Following trimming, the clean reads were then assembled using the simple approach assembler, MEGAHIT, to reconstruct the original metagenomic data (D. Li, Liu, Luo, Sadakane, & Lam, 2015; Pérez-Cobas et al., 2020). BAM files were then generated to map the reads to the assembled contigs using the short-read aligner BBMAP. The minimum ID required for the read to map was set at 95%. These bam files were then sorted using samtools to be compatible for use during binning (H. Li et al., 2009).

Similar reads were then classified into taxonomic bins using Metabat2, to be used for taxonomic classification and downstream functional characterization (Kang et al., 2019). Following binning, the reads were run through CheckM, a collection of tools that measures the quality of genomes (Parks, Imelfort, Skennerton, Hugenholtz, & Tyson, 2015), with quality parameters set at ≥50% completeness and ≤10% contamination. Any bins that did not meet the above-mentioned quality standards were discarded, which ultimately generated a pool of metagenome-assembled genomes (MAGs) to be used for further processing (Table 6).

The krona tool in RefineM was used to produce a visual taxonomic distribution of the finalized MAGs (Ondov, Bergman, & Phillippy, 2011; Parks et al., 2017). The MAGs were then run through the Quant_bin module in metaWRAP, which uses Salmon, a tool for transcript quantitation, to calculate the abundances of each bin within a sample (Patro, Duggal, Love, Irizarry, & Kingsford, 2017). Following quantitation, MAGs associated with *Prevotella* were processed in PROKKA, a prokaryotic genome annotator (Seemann, 2014), to identify the encoded genes and their functions.

3.5.3 NCBI Database

A total of 33 genomes were downloaded from the NCBI Sequence Set Browser and were run through the Prokka functional annotator to be used as comparisons to the MAGs generated from the twelve managed samples. Thirteen genomes were classified as uncultured *Methanobrevibacter*, and isolated from the bovine rumen. The remaining twenty genomes consisted of four different *Prevotella* spp.: *Prevotella ruminicola, Prevotella copri, Prevotella oris,* and *Prevotella bryantii.* The selected strains of *P. copri* and *P.oris* were isolated from human sources, as no available bovine rumen strains existed.

CHAPTER 4. RESULTS

4.1 16S rRNA Targeted Amplicon Sequencing

From the 75 samples sequenced to characterize correlations between the overall archaea populations and the abundance of vitamin B12, the genera *Methanobrevibacter, Methanosphaera,* and *Candidatus Methanomethylophilus* were the only notable archaea identified (Figure 6A). Uncultured archaea are also present in several of the samples but at a lower abundance than the above-mentioned genera.

4.2 Archaeal Correlations with Selected Features

The LefSe analysis demonstrated that the abundance of the genera *Methanobrevibacter, Methanosphaera*, and *Candidatus Methanomethylophilus* do not show any significant correlations with vitamin B12 concentrations in the rumen (Figure 6B). The abundance chart shows a general pattern of *Methanobrevibacter* as the predominant archaeal genus in the rumen, followed by *Methanosphaera*, and finally *Candidatus Methanomethylophilus*. Uncultured archaea are sporadically distributed among both groups of samples.

4.2.2 Milk, Rumen, and Fecal Composition

The identified archaeal community was also analyzed based on its relationship on the fat, urea, lactose and protein composition in milk, rumen and fecal samples. Lactose, urea, protein, and fat levels were divided into “high” and “low”, which are represented by the upper and lower 33% of samples. The findings indicate that there are no significant archaeal features correlated with any of the important quality measures of milk (Figure 7).

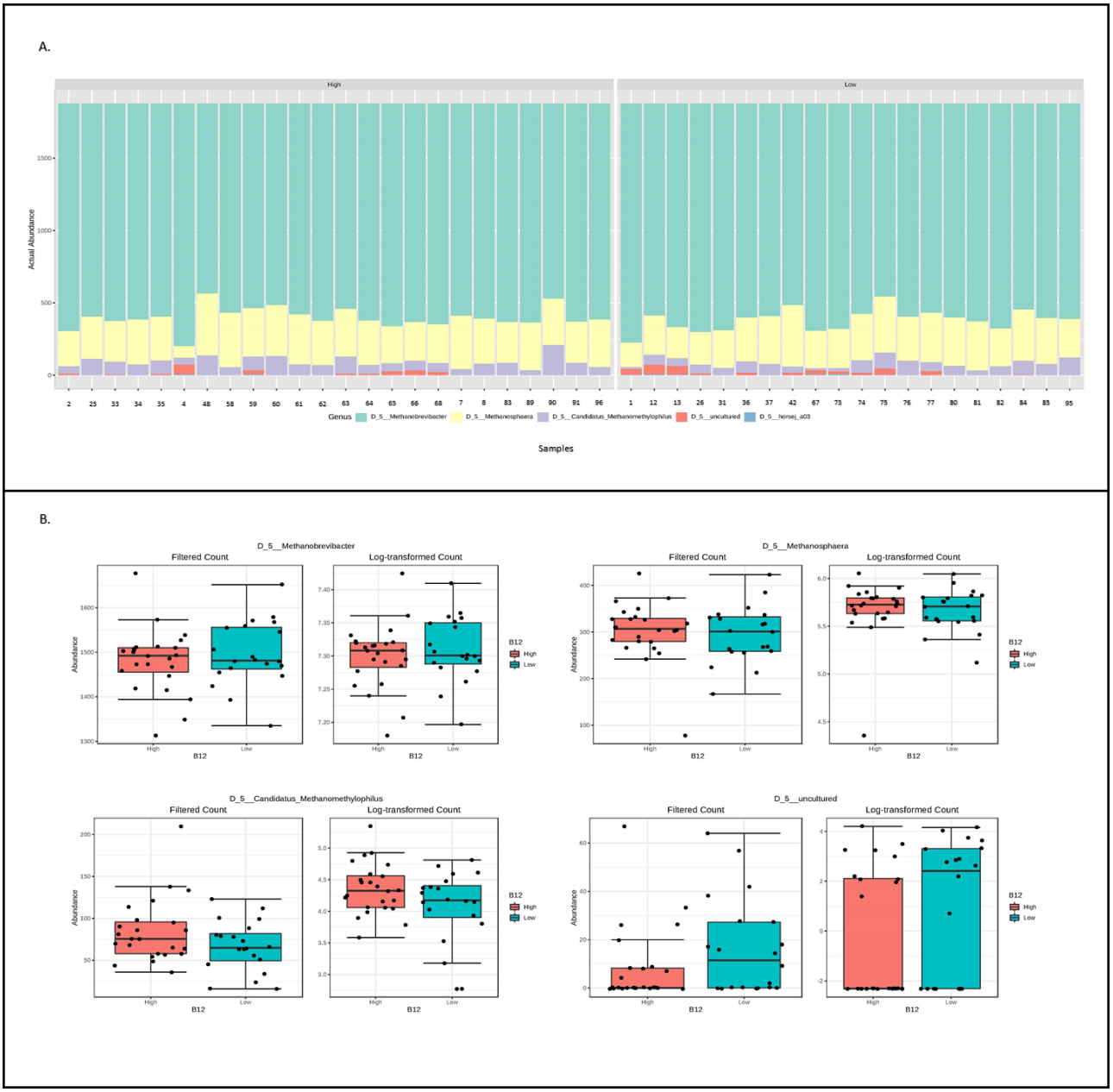


Figure 6. Archaea and Vitamin B12. A. Archaeal taxonomic composition of rumen samplesfrom high and low vitamin B12 concentration samples. Genera *Methanobrevibacter, Methanosphaera, and Candidatus Methanomethylophilus* are present in all samples, showing no obvious differences between the variant samples. B. LefSe plots of identified archaeal genera and ruminal vitamin B12 concentrations.

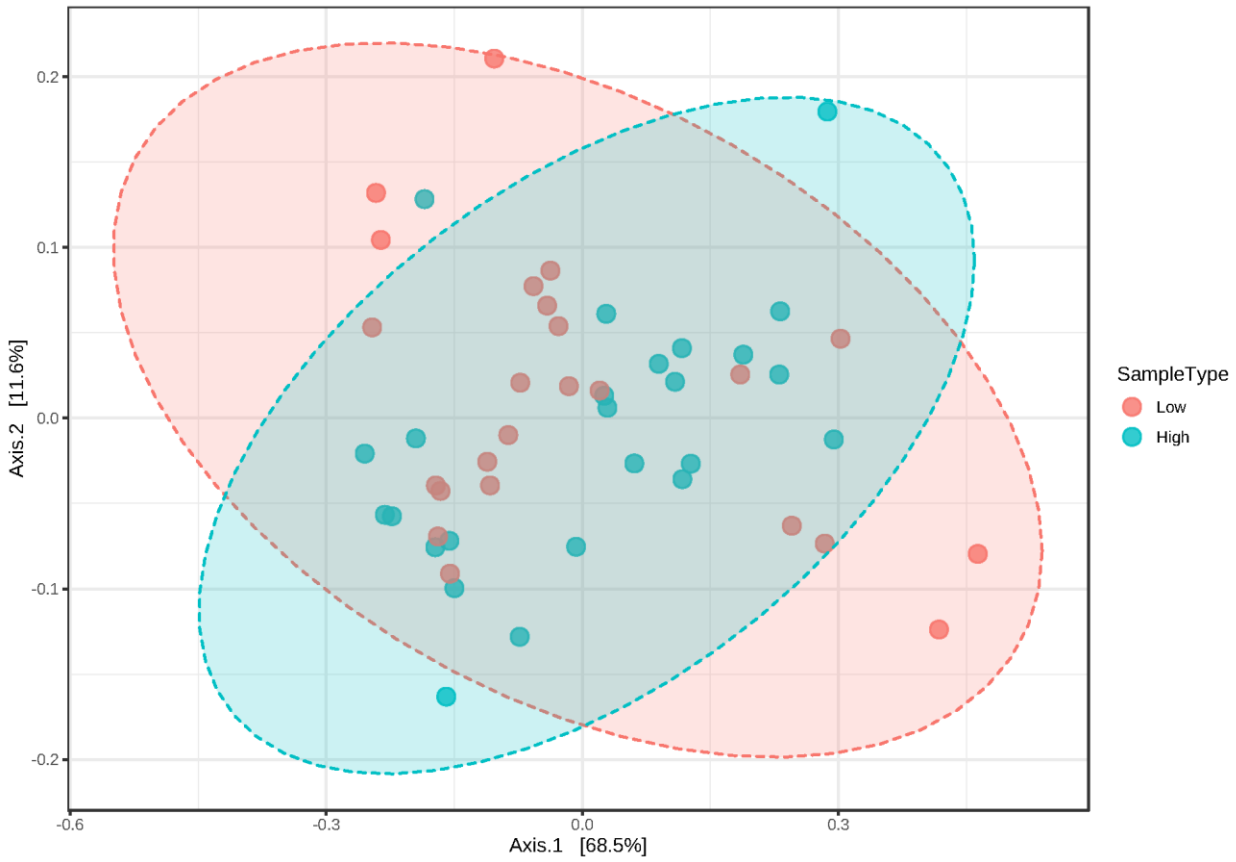


Figure 7. Archaeal Beta-Diversity in the Rumen. The composition of the archaeal community in the bovine rumen was not directly correlated to the abundance of vitamin B12 found in the rumen. PERMANOVA F-value: 1.8324; R-squared: 0.041804; p-value < 0.121.

4.3 Rumen Metagenome

4.3.1 Taxonomic Composition

The results from MG-RAST showed very similar taxonomic distributions and gene representations between each of the samples (Figure 8). Bacteria represented a significant portion of the community, ranging from 91.64-96.71% in the samples, and archaea comprised about 1.36-3.28% of the overall community.

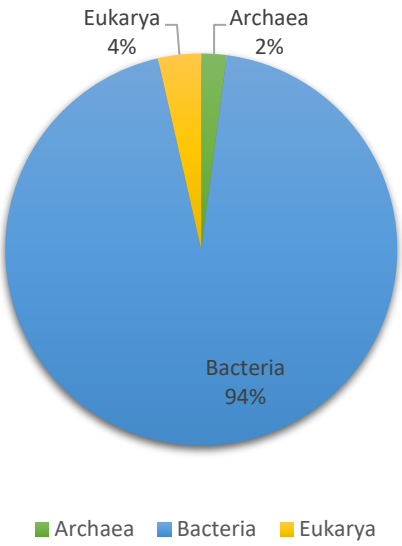


Figure 8. The Average Taxonomic Distribution. The taxonomic distribution of all twelve metagenomic samples were averaged at the domain level. The samples are represented by 94% Bacteria, 2% Archaea, and 4% Eukarya.

The number of sequence reads were normalized across samples and then the most abundant genera were identified. The results show that *Prevotella* is the most abundant genus, followed by *Bacteroides* and *Clostridium* (Figure 9). The absolute abundance of *Prevotella* seems to fluctuate notably between samples; however, there is no significant correlation to vitamin B12 abundance. *Prevotella* is the most abundant genus, followed by *Bacteroides*, *Clostridium*, *Eubacterium*, and *Ruminococcus*. Similar relative abundances were also established at the species level among all samples, and *Prevotella ruminicola* was the most abundant species (Figure 10).

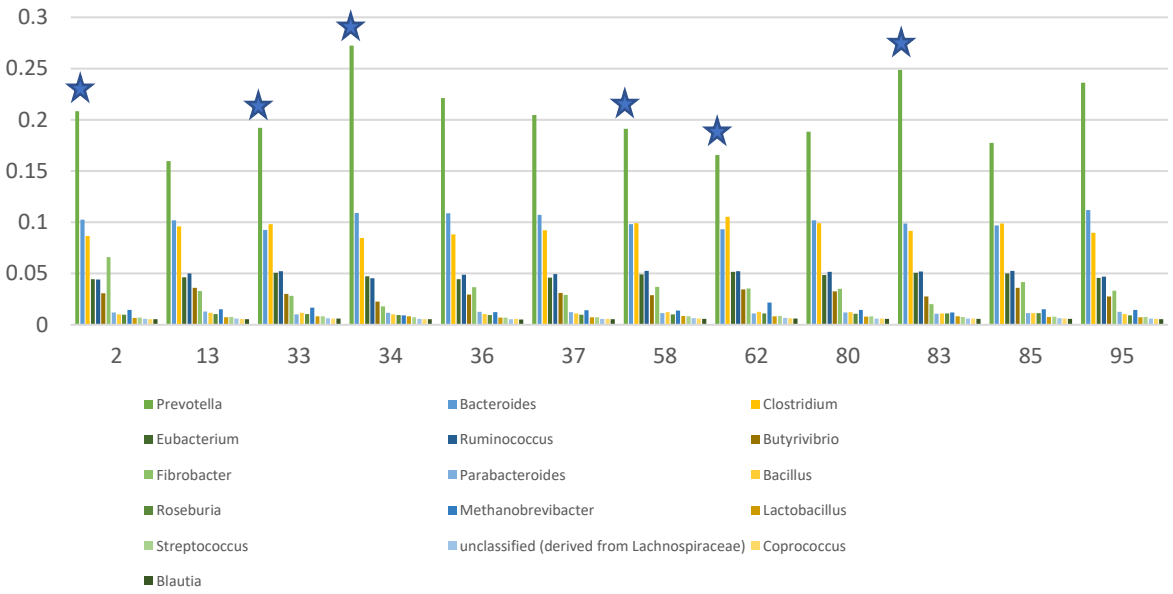


Figure 9. Genus Abundance. Samples with a high vitamin B12 concentration are indicated with a blue star. *Prevotella* is the most abundant genus, followed by *Bacteroidetes* and *Clostridium.*

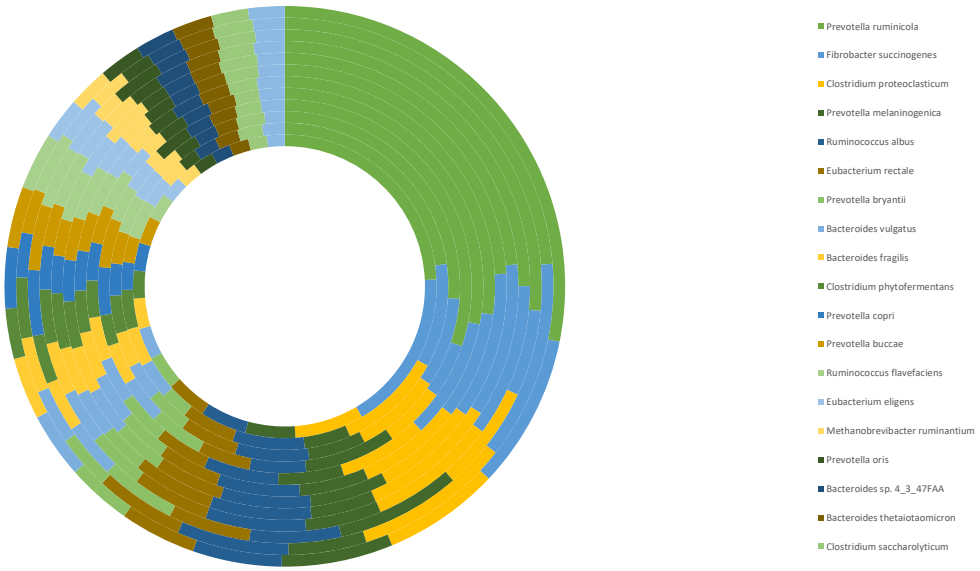


Figure 10. Species Abundance. The microbial community at the species level is represented by *P. ruminicola, F. succinogens, C. proteoclasticum P. melaninogenica, R. albus, Eubacterium rectale, Prevotella bryantii*, *Bacteroides vulgatus, Bacteroides fra*gilis*, Clostridium phytofermentains, Prevotella copri, Preovtella buccae, Ruminococcus flavefaciens, Eubacterium eligens, Methanobrevibacter ruminantium, Prevotella oris, Bacteroides* sp. 4_3_47FAA, *Bacteroides thetaiotaomicron, Clostridium saccharolyticum,* and *Bacteroides* sp. 1_1_6

The archaeal abundances were analyzed separately to create a better representation of the archaeal community present in the rumen samples (Figure 11). The genera discovered in the rumen samples include *Methanobrevibacter, Methanosphaera, Methanothermobacter, Methanosarcina, Methanococcus, Methanocaldococcus, Methanocorpusculum, Pyrococcus, Thermococcus,* and *Archaeoglobus.* The genus *Methanobrevibacter* makes up most of the archaeal population, with the remaining nine genera consisting of a negligeable amount.

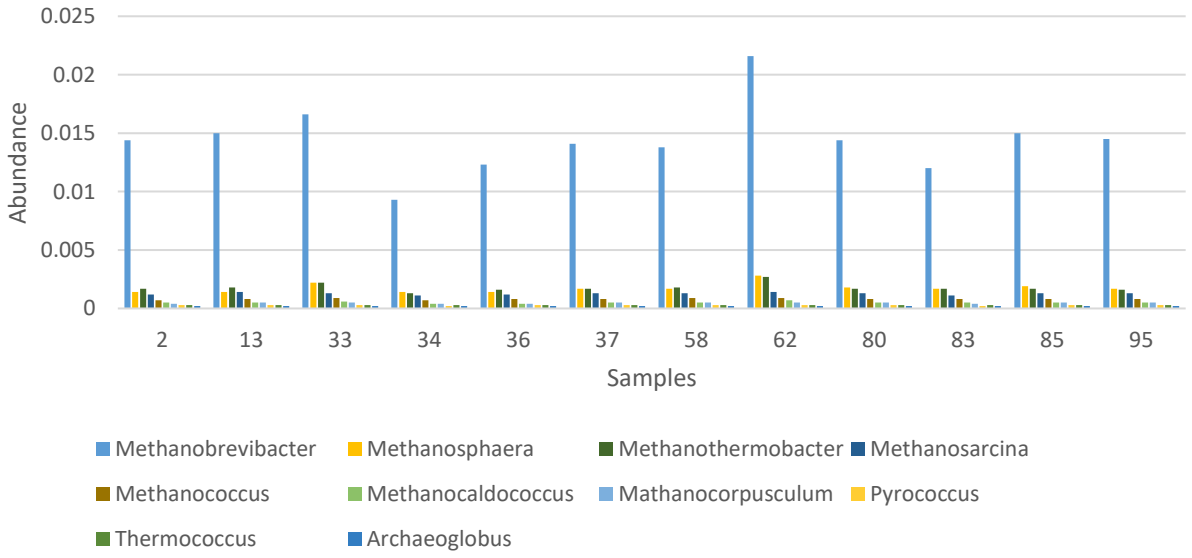


Figure 11. Archaeal Genus Abundance. The ten most abundant archaeal genera were selected and used to create a visual composition of the archaeal community.

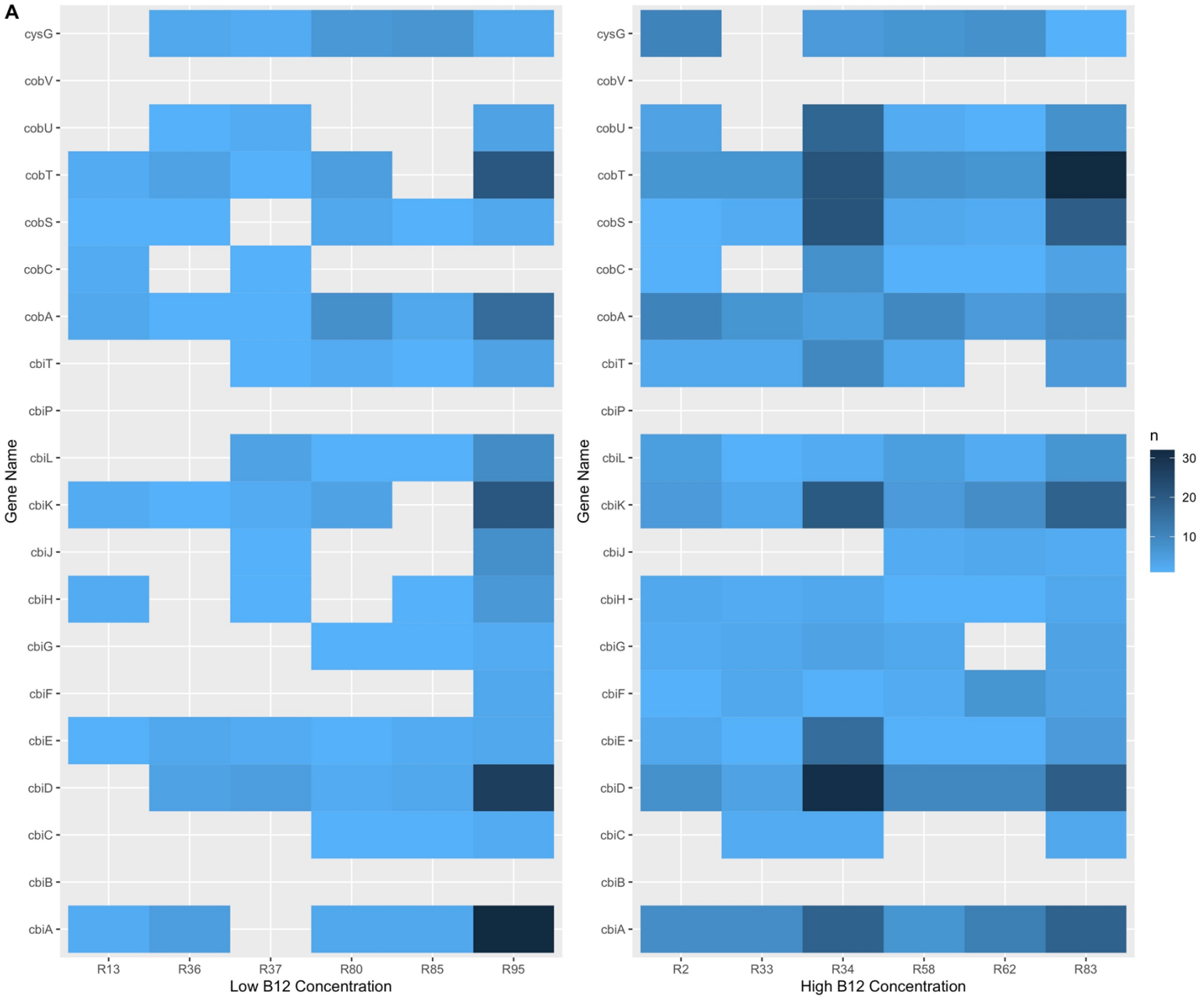
4.3.2 Functional Annotation of Vitamin B12 Production Related Genes in the Bulk Metagenome

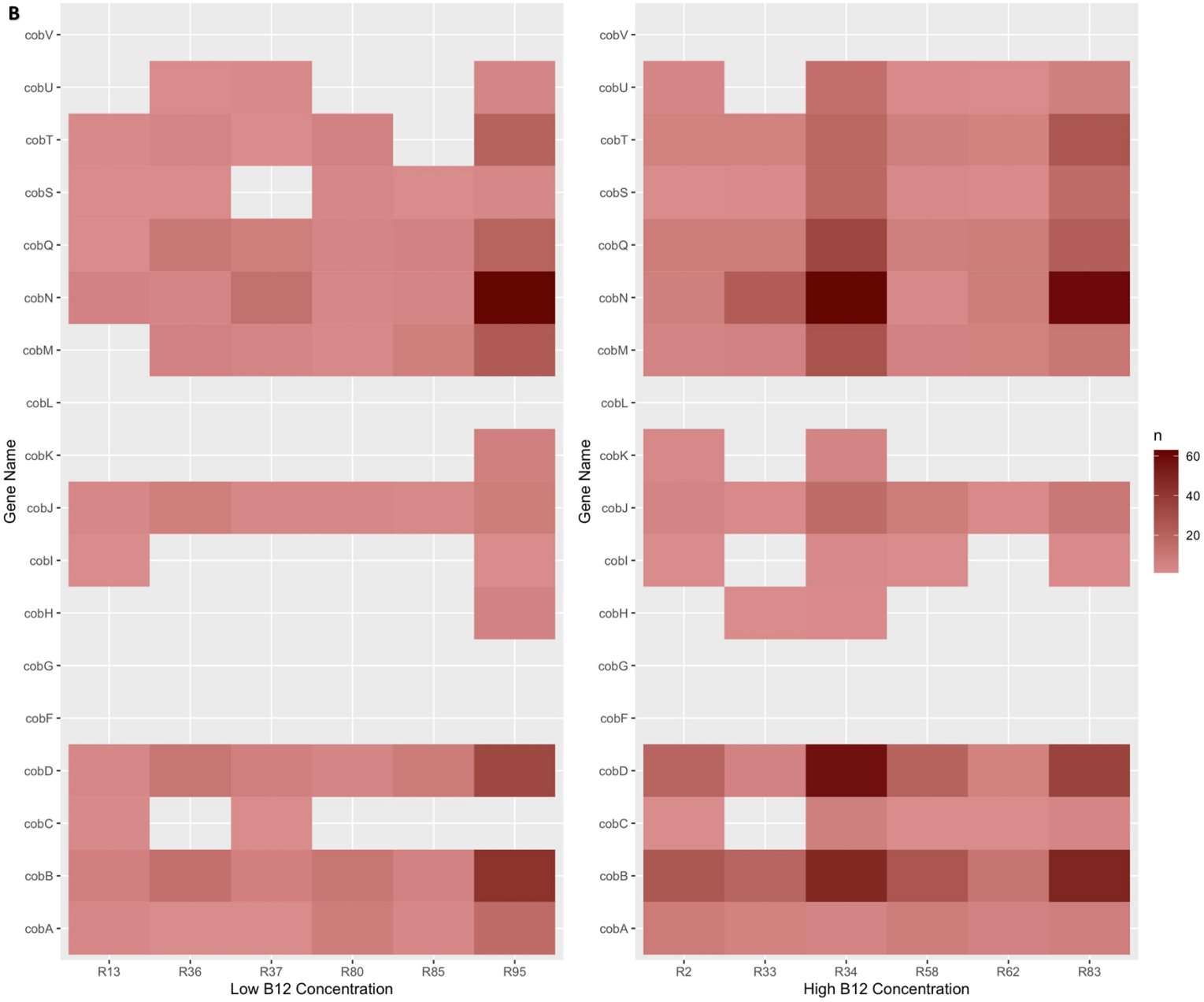
The eggNOG-produced genes were filtered into three categories: the anaerobic pathway, the aerobic pathway, and the salvage pathway (Figure 12). Several genes were not identified in any of the samples, indicating that they are likely not recognized by the eggNOG database. These genes include: *cbiB, cobR, cobV, cobL, cobG, cobF* and *btuD.* It is also important to note that genes *cobV, cobU, cobT, cobS* and *cobC* are involved in the three pathways and therefore overlap considerably.

Overall, samples with a high concentration of vitamin B12 seem to show a greater total prevalence of genes in all three pathways as well as vitamin B12-dependent genes. The anaerobic heatmap shows the largest discrepancy between samples with high and low vitamin B12 concentrations. Sample 95 demonstrates an extremely high abundance of the listed genes, however, is classified as having a low vitamin B12 concentration, rendering it an outlier from the remaining samples. The genes associated with the aerobic section of the *de novo* pathway include *cobA, cobI, cobG, cobJ, cobM, cobF, cobK, cobL, cobH, cobB, cobN, cobS, cobT,* and appear to be less abundant than those affiliated with the anaerobic section of the pathway, which is represented by genes *cysG, cbiL, cbiJ, cbiH, cbiF, cbiG, cbiT, cbiE, cbiD, cbiC, cbiB,* and *cbiA*. The salvage pathway depicts a similar trend to the aerobic and anaerobic pathways, with samples classified under a low concentration being more unfamiliar with the *cobC* gene, and samples 34 and 83 encoding the highest number of related genes.

Finally, methionine synthase (*metH)*, the large subunit of methylmalonyl-CoA mutase (*mutB)*, and the small subunit of methylmalonyl-CoA mutase (*mcm*), are vitamin B12-dependent genes that were selected as markers for microbes with vitamin B12-dependent metabolisms (Figure 12D). Samples with a higher vitamin B12 concentration appear to encode a greater

number of all three of the stated genes. Overall, samples with a greater abundance of vitamin B12-producing genes also encode a greater abundance of vitamin B12-dependent genes ($r(10)=0.96$, $p<0.05$).

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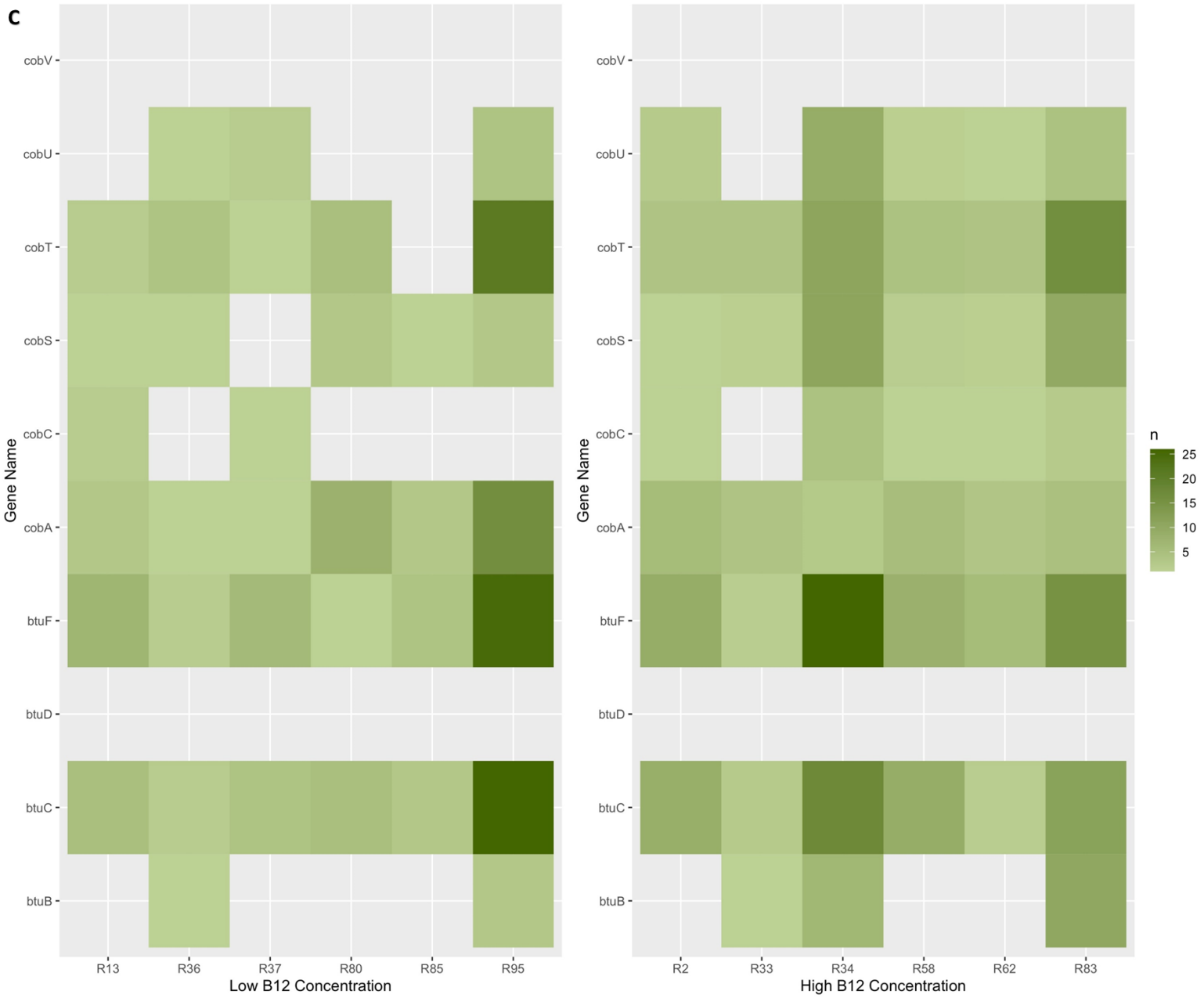
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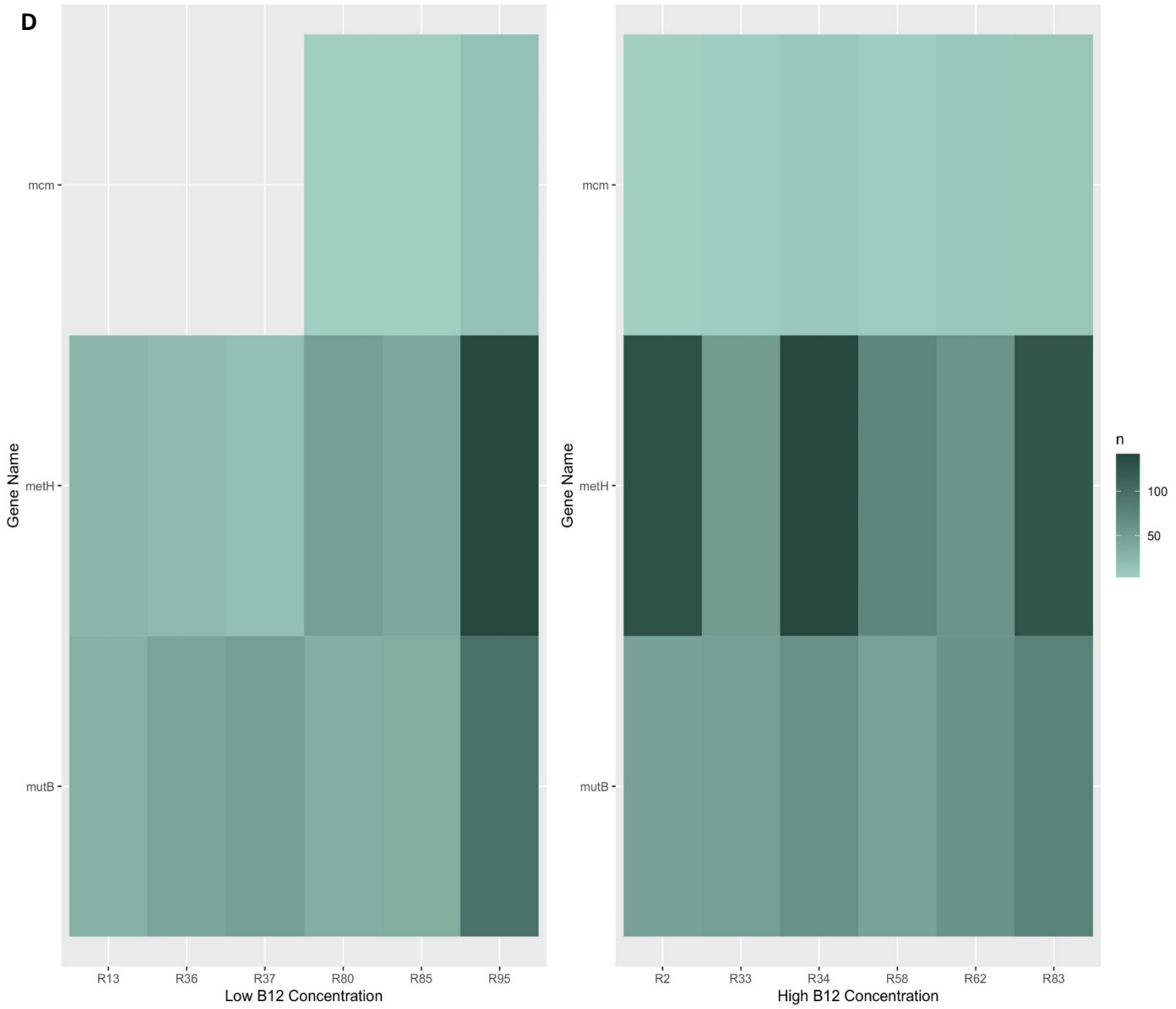


Figure 12. Abundance of Vitamin B12-Associated Genes. Genes affiliated with the vitamin B12 biosynthetic pathway were discovered in both sampled with low B12 concentration and high B12 concentration **A.** Genes involved in the anaerobic synthesis pathway include *cysG, cobV, cobU, cobT, cobS, cobR, cobQ, cobD, cobD, cobC, cobA, cbiL, cbiJ, cbiH, cbiF, cbiG, cbiT, cbiE, cbiD, cbiC, cbiB, cbiA.* **B.** Genes involved in the aerobic synthesis pathway include *cobA, cobI, cobG, cobJ, cobM, cobF, cobK, cobL, cobH, cobB, cobN, cobS, cobT, cobR, cobA, cobQ, cobC, cobD, cobU, cobT, cobV, cobS,* and *cobC*. **C.** Genes involved in the salvage pathway include *btuB, btuF, btuC, btuD, cobA, cobU, cobT, cobV, cobS,* and *cobC.* **D.** Vitamin B12-dependent genes include *metH, mutB,* and *mcm*

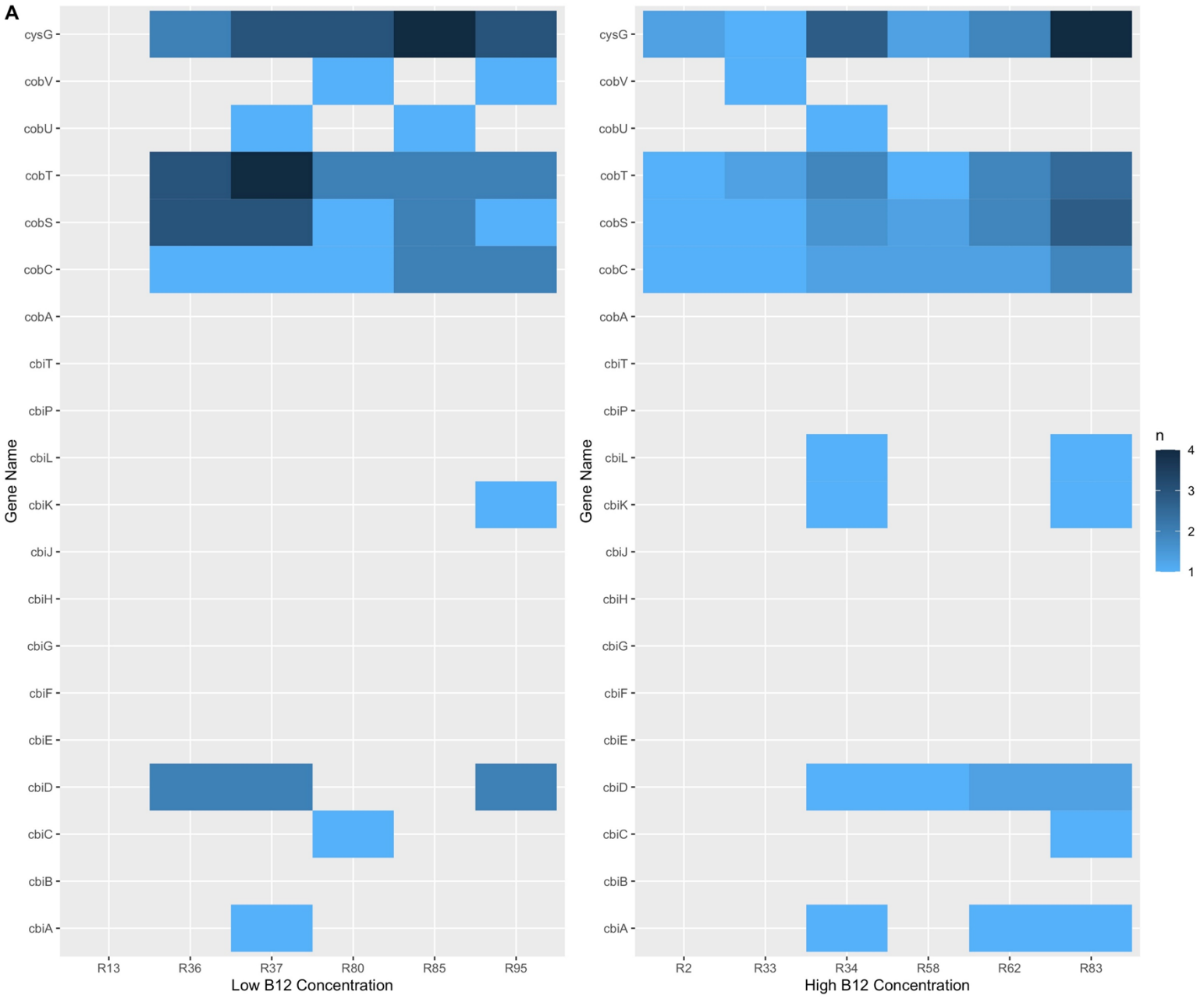
4.3.3 Functional Annotation of Vitamin B12 Related Genes in *Prevotella spp.* MAGs

MAGs generated from Metabat2 were predominantly identified as *Prevotella spp.*. *Prevotella* MAGs as well as any parent-related taxonomic group, were used for further examination to determine any associations between vitamin B12 concentrations and *Prevotella* spp.. This was a particularly important area of investigation since the presence of two different OTUs each identified as *Prevotella* at the genus level were found to be correlated with both high and low levels of vitamin B12 in the rumen (Lopez et al., 2020). The krona taxonomic distribution charts were used to identify the *Prevotella* spp. present in the samples. Only species that consisted of ≥10% of the entire bin set were used for analysis. *Prevotella* species discovered in the MAGs include *Prevotella byrantii, Prevotella albensis, Prevotella ruminicola,* and many uncultured species such as *Prevotella*_sp002373375, *Prevotella*_sp900318625, and *Prevotella_s*p002353555.

P. byrantii was detected in all twelve samples and represent a large portion (≥77%) of the identified MAGs. Uncultured *Prevotella*_sp900318625 is the next most notable species, as it is found in seven of the twelve metagenomic samples, and was distributed between samples with high and low vitamin B12 concentrations. The remaining relevant species are dispersed among the twelve samples, revealing no obvious patterns.

The taxonomic composition of all the *Prevotella*-associated bins, and their related functional annotation were collected and compiled into two heatmaps (Figure 13). One visualizes the genes associated with the anaerobic *de novo* pathway, the other displays the genes involved in the salvage pathway. It is apparent that members of the *Prevotella* genus lack several of the genes required for synthesizing vitamin B12 via the *de novo* pathway (Figure 13A), however, the

genes affiliated with the salvage pathway are notably more abundant in the *Prevotella* MAGs (Figure 13B). Samples 34, 37, 58, 83, and 95 seem to encode a larger number of *btuB* genes, which are an essential component of the ABC corrinoid transport system, that functions in the active translocation of vitamin B12 across the outer membrane into the periplasm (Lundrigan, Köster, & Kadner, 1991). The remaining genes *cobB, cobC, cobD, cobO, cobP, cobQ, cobS, cobT, cobU*, and *cobV*, are the residual components of the salvage pathway (Figure 13C), whereby corrinoids that are transported from the above-mentioned ABC transporter are modified into a vitamin B12 cofactor. The genes involved in the salvage pathway are relatively comparable between the two categories, except for sample 13, which only encodes 20% of the associated genes.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cobA
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cbiG
cbiF
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cbiD
cbiC
cbiB
cbiA
Gene Name
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R36
R37
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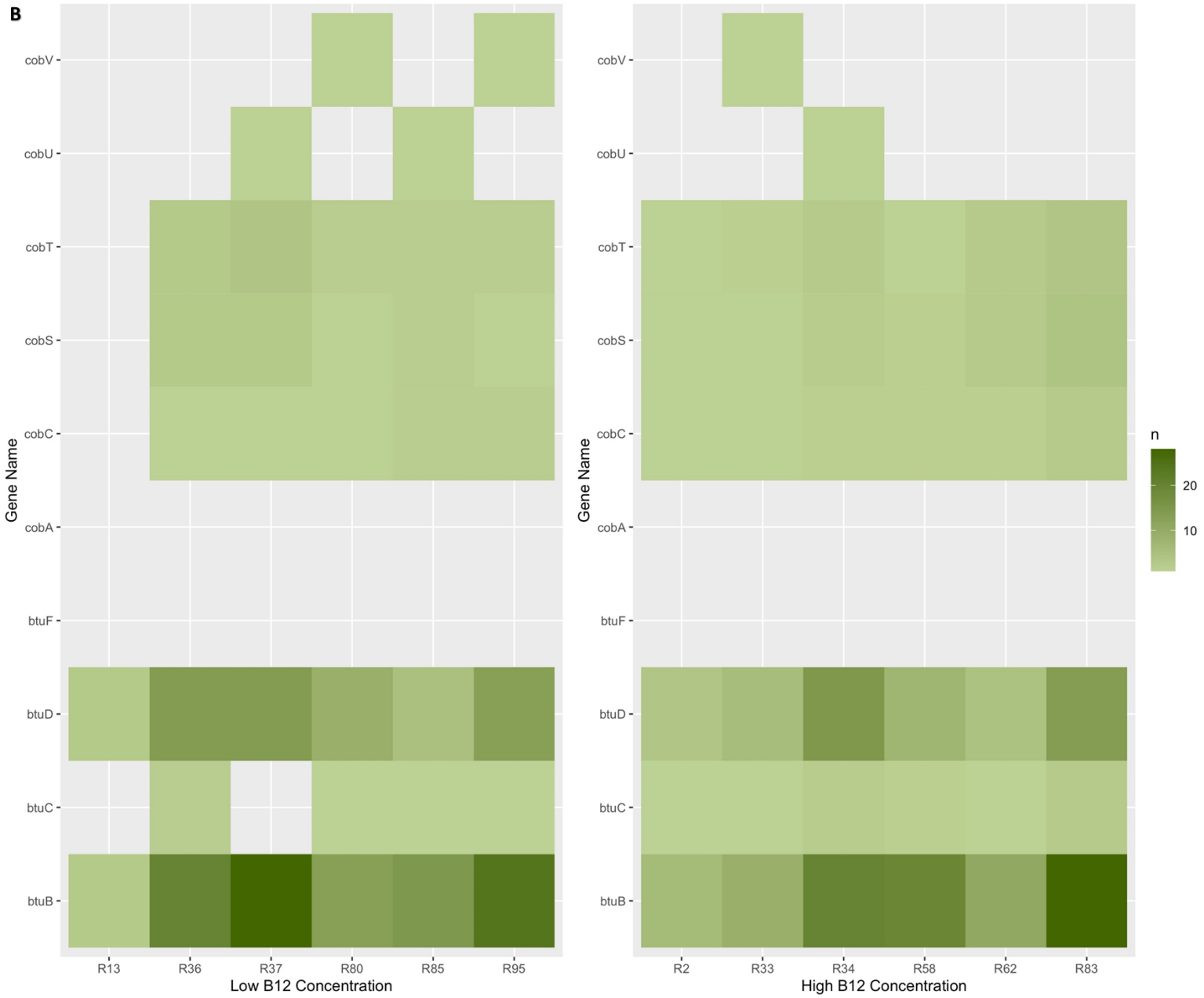
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Gene Name
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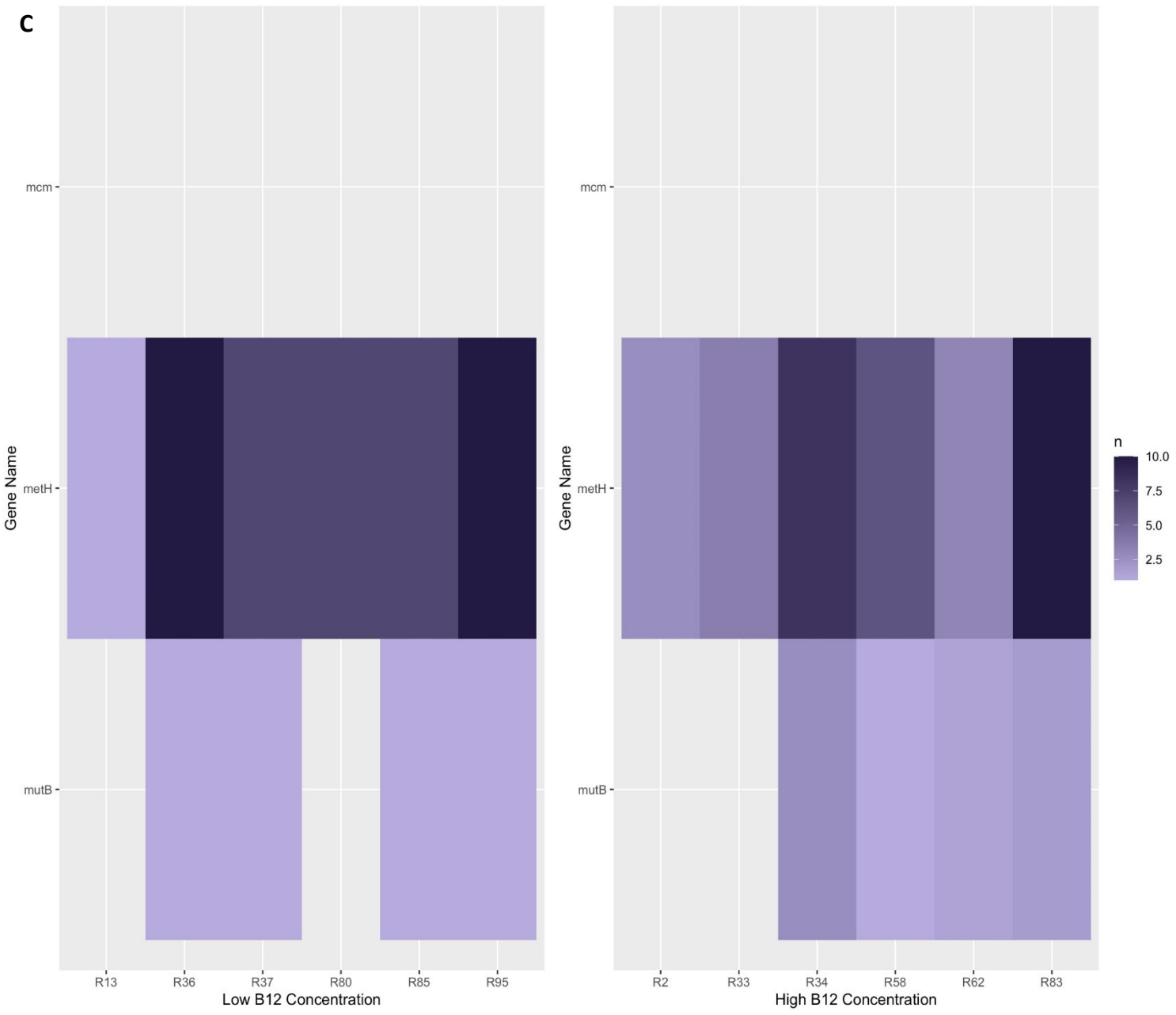
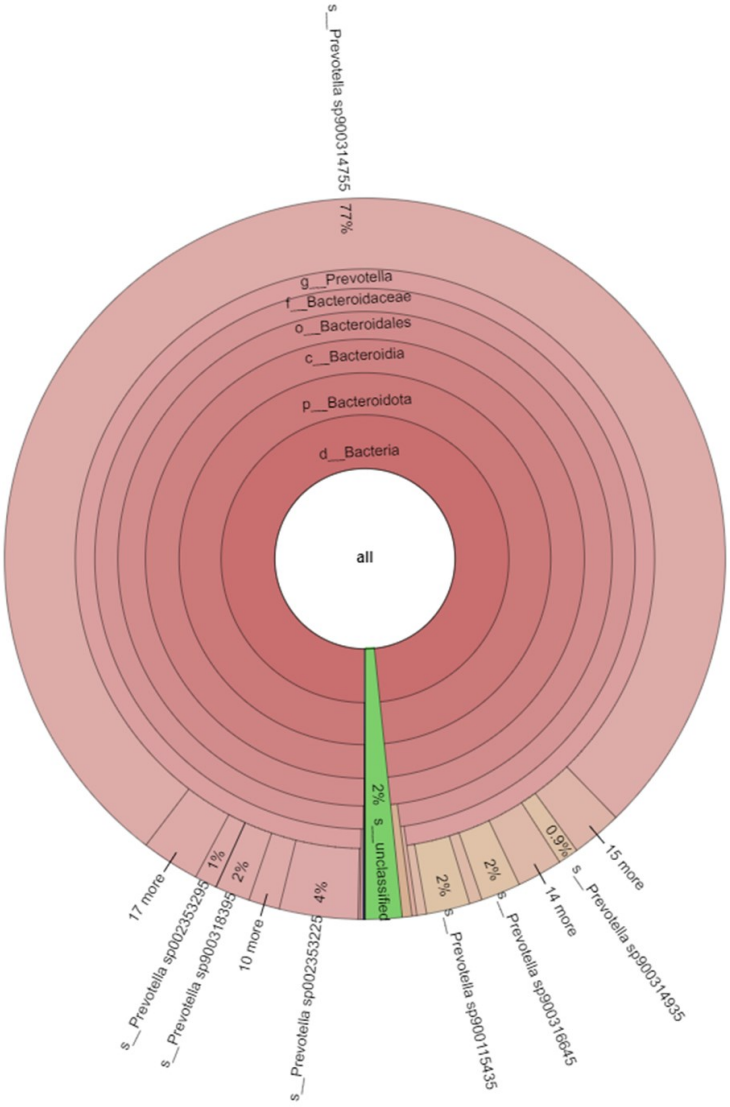


Figure 13. The *De Novo* Anaerobic and Salvage Pathways in *Prevotella*-Labelled MAGs. A. The genes identified from the twelve metagenomic samples, associated with the *de novo* anaerobic pathway. **B.** The genes identified from the twelve metagenomic samples, associated with the salvage pathway. **C.** Vitamin B12-dependent genes include *metH, mutB,* and *mcm*

Prevotella MAGs with a completeness ≥90% were further investigated to better establish relationship between this genus and vitamin B12. Selected MAGs were predominantly identified

as *Prevotella byrantii* (make up ≥80% of the entire bin), and were affiliated with the genes *btuB, btuC, btuD, cobC, cobS,* and *cobT*. These genes together represent 60% of the components required in the salvage pathway. Sixty-seven percent of the identified MAGs contained all six of the stated genes, while the residual 33% contained five. Furthermore, genes *metH* and *mutB* were discovered in 100% of the *Prevotella* MAGs, with eight of them containing two copies of *metH*. Additionally, MAG 3 of sample 36 is largely represented by a *Prevotella sp900314755* genome that encodes nine *btuB*, and 7 *btuD* genes, signifying the presence of multiple vitamin B12 transporters (Figure 14).



Gene	Product
btuB_1	Vitamin B12 transporter BtuB
btuB_2	Vitamin B12 transporter BtuB
btuB_3	Vitamin B12 transporter BtuB
btuB_4	Vitamin B12 transporter BtuB
btuB_5	Vitamin B12 transporter BtuB
btuB_6	Vitamin B12 transporter BtuB
btuB_7	Vitamin B12 transporter BtuB
btuB_8	Vitamin B12 transporter BtuB
btuB_9	Vitamin B12 transporter BtuB
btuD_1	Vitamin B12 import ATP-binding protein BtuD
btuD_2	Vitamin B12 import ATP-binding protein BtuD
btuD_3	Vitamin B12 import ATP-binding protein BtuD
btuD_4	Vitamin B12 import ATP-binding protein BtuD
btuD_5	Vitamin B12 import ATP-binding protein BtuD
btuD_6	Vitamin B12 import ATP-binding protein BtuD
btuD_7	Vitamin B12 import ATP-binding protein BtuD
cbiD	Cobalt-precorrin-5B C(1)-methyltransferase
cbiET	Cobalamin biosynthesis bifunctional protein CbiET
cobB_1	NAD-dependent protein deacylase
cobB_2	Hydrogenobyrinate a,c-diamide synthase
cobB_3	Hydrogenobyrinate a,c-diamide synthase
cobD	Cobalamin biosynthesis protein CobD
cobO	Cobalamin adenosyltransferase
cobP	Bifunctional adenosylcobalamin biosynthesis protein CobP
cobQ	Cobyric acid synthase
cobS	Adenosylcobinamide-GDP ribazoletransferase
cobT	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase
metH_1	Methionine synthase
metH_2	Methionine synthase

Figure 14. *Prevotella* Vitamin B12-Associated Genes. A. A breakdown of the taxonomic distribution of MAG 3 from sample 36 shows that the genus *Prevotella* represents 97% of the entire MAG, with uncultured species *Prevotella sp900314755* representing 77%. **B.** The gene btuB was recovered nine times in this genome, along with several other genes common to the salvage pathway.

4.3.4 Functional Annotation of Vitamin B12 Related Genes in *Prevotella spp.* genomes

Doxey et al. (2015) determined that 11 genes can be used as markers to establish the likelihood of the presence of a complete and utilized biosynthetic pathway: *cysG, cbiL, cbiH, cbiF, cbiT, cbiE, cbiC, cbiA, cbiB, cbiP, cobS* (Figure 15). To confirm the results of our MAG

analysis we collected representative whole genome sequences of *Prevotella spp.* from the NCBI database. These genomes lack the representative genes associated with the anaerobic pathway but contain a greater number of genes affiliated with the salvage pathway. There is a possibility that certain *Prevotella* spp. are capable of synthesizing vitamin B12 from exogenous corrinoid precursors. The genera *P. ruminicola*, *P. oris* and *P. bryantii* contained the greatest number of vitamin-B12 associated genes, and *P. copri* revealed the lowest (Figure 15).

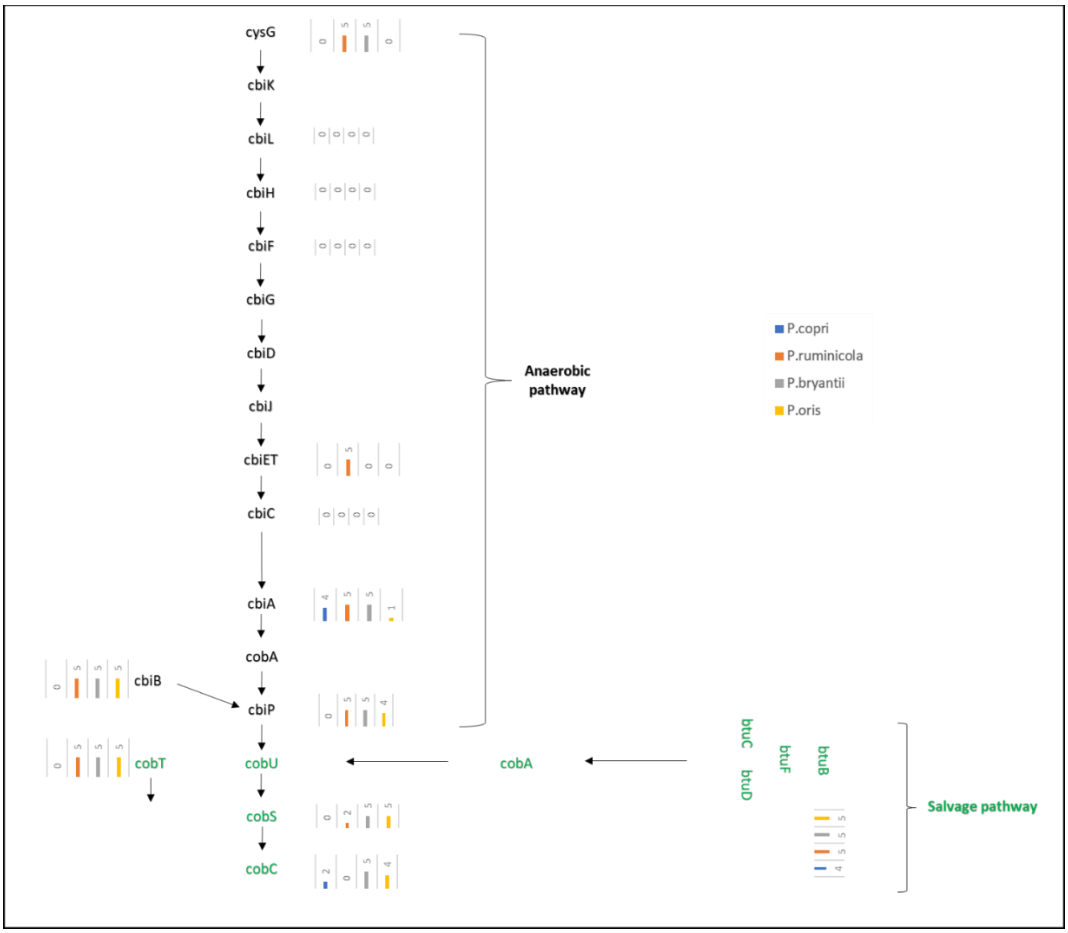


Figure 15. Anaerobic Vitamin B12 Synthesis Pathway and *Prevotella* Genomes. Genes affiliated with the anaerobic and salvage pathways were observed in twenty *Prevotella* spp. genomes extracted form the NCBI database. Five genomes of *Prevotella copri, Prevotella ruminicola, Prevotella bryantii, and Prevotella oris* were summarized and compared in the graphs situated among the marker genes adapted by Doxey et al. (2015). Charts for genes *cobT, cobC* and *btuB* were also included for reference.

4.3.5 Functional Annotation of Vitamin B12 Production Related Genes in *Methanobrevibacter* spp. MAGs

The genus *Methanobrevibacter* very often contains genes involved in the vitamin B12 *de novo* and salvage pathways but lacks vitamin B12-dependent genes. More specifically, MAGs identified as *Methanobrevibacter sp900314635* contain up to 80% of the genes required for the first section of the anaerobic vitamin B12 synthesis pathway. MAGs identified as *Methanosphaera* were uncommon; however, they displayed similar trends to those represented by *Methanobrevibacter sp900314635* in that they contained large parts of the vitamin B12 synthesis pathway, but lacked the genes indicating vitamin B12 consumption. The MAGs characterized by *Methanobrevibacter sp900314635* were found in the samples 33, 34, 36, 58, 62, and 83. To be used as a comparison, the functional annotation of 13 uncultured *Methanobrevibacter* genomes from NCBI were pooled together with 3 genomes generated from the metagenomic samples (Figure 16). The results confirm that isolated ruminal strains of *Methanobrevibacter* do not encode vitamin B12-dependent genes, but instead encode roughly 72% of the marker genes on average, with some containing up to 91% of the indicated genes. In addition to the stated marker genes, many of the genomes also comprised of supplementary genes *cbiG, cbiD, cbiJ, cobD*, common to the *de novo* pathway.

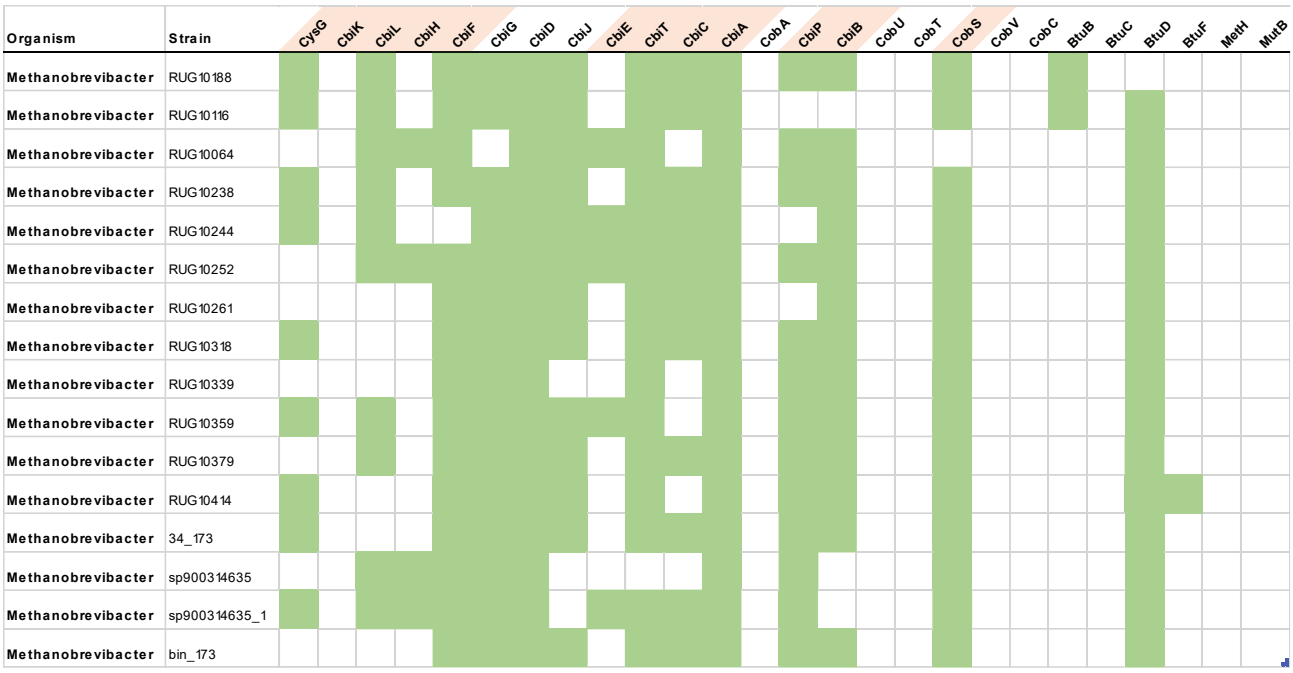


Figure 16. *Methanobrevibacter* Vitamin B12 Production and Consumption. Genes associated with the vitamin B12 biosynthetic pathway are identified in 16 different strains of uncultured *Methanobrevibacter* from the bovine rumen, with the indicator genes highlighted in pink. The first 13 strains are extracted from the NCBI database, with the last three representing three different MAGs from the metagenomic samples used in this project. Genes *btuB, btuC, btuD,* and *btuF* pertain to the salvage pathway, while genes *metH* and *mutB* are vitamin B12-dependent genes.

4.3.6 Functional Annotation of Vitamin B12 Production Related Genes in all MAGs

Very few MAGs contained more than 60% of the genes required for the first ten enzymatic reactions in the vitamin B12 biosynthetic pathway. More, specifically, only MAGs represented by the families *Lachnospiraceae*, *Acutalibacteraceae,* and *Anaerovoracaceae*, which all belong to the class *Clostridiales*, and the genera *Methanobrevibacter*, *Methanosphaera*, *Methanomethylophilus*, *Ruminococcus*, and *Succiniclasticum,* demonstrated a high probability of vitamin B12 production via the *de novo* pathway. The families *Acutalibacteraceae* and *Anaerovoracaceae* were primarily discovered in samples 62 and 83 and encoded between 70-90% of the ten genes found at the beginning of the anaerobic pathway. Samples 34 and 83 contained an uncultured species of *Succiniclasticum*, that encoded 90% of the first ten genes,

while *Ruminococcus* MAGs contained 60-70% and were distributed between samples 83 and 95. Lastly, the group *Lachnospiraceae*, which was present in all samples, had up to 100% of the first half of the anaerobic pathway, and between 55-85% of the entire pathway. Particularly, samples 34, 58 and 95 encompassed a *Lachnospiraceae*-labelled MAGs with the most complete pathways, however samples 34 and 83 had the greatest number of *Lachnospiraceae* MAGs.

4.3.7 Functional Annotation of Vitamin B12 Consuming Related Genes in all MAGs

It is apparent that many bacterial and archaeal genomes encode both vitamin B12 synthesizing and consuming genes; however, some benefit entirely from other vitamin B12 producing microbes. The *metH*, *mutb*, and *mcm* genes were identified in 61% of all MAGs, despite many of them lacking the presence of vitamin-B12 producing genes. Furthermore, twenty-two MAGs were contained to a minimum of six *btuB* genes. Fourteen of these were *Prevotella*, and the remaining eight were affiliated with the order *Bacteroidales*, and the family *Bacteroidaceae*. The genus labelled *Ruminococcus_E* was often limited to *btuD* and *metH* genes and indicated no obvious ability to produce vitamin B12. These MAGs were found in samples 2, 36, 58 and 95. Overall, MAGs attributed to the phyla *Firmicutes* and *Bacteroidetes* seemed to encode the greatest number of vitamin B12-depedendent genes.

CHAPTER 5. DISCUSSION

Analyzing the bovine rumen microbial community and its functions is a crucial step into understanding the relationship between microbes and the complex vitamin B12 biosynthetic pathway. Franco-Lopez et al. (2020) determined that certain bacterial populations in the rumen are present at higher levels of vitamin B12, implying that the rumen microbiota may have an influence on vitamin B12 concentrations in milk. This study utilized 16S rRNA targeted amplicon sequencing and shotgun metagenomic sequencing as well as publicly available whole genomes to characterize the bacterial and archaeal species in the rumen and attempt to elucidate their roles in the synthesis or consumption of vitamin B12.

Archaea

The selected archaeal primers for 16S rRNA amplicon sequencing generated information about the overall archaeal community present in seventy-five rumen samples. The only archaeal genera identified using this method were *Methanobrevibacter*, *Methanosphaera,* and *Candidatus Methanomethylophilus*. *Methanobrevibacter* was the most abundant, followed by *Methanosphaera*, providing evidence that methanogens make up a large portion for the rumen archaeal population. Fouts et al. (2012) found *Methanobrevibacter* and *Methanosphaera* to be the dominant genera in the rumen; however, also discovered sequences pertaining to *Thermogymnomonas* in 33% of their samples. There was concern that the limited diversity of archaea observed in the 16S rRNA targeted amplicon sequencing data was due to poor primer selection; however, when this data was compared to the metagenomic data similar patterns were observed. Despite MG-RAST finding a greater diversity of archaea, which included the addition of *Methanothermobacter, Methanosarcina,* and *Methanococcus*; the genus *Methanobrevibacter*

still made up 66% of the total archaeal community, with the additional genera all falling below 10%. Similarly, other literature has noted that *Methanobrevibacter* make up roughly 61%-64% of the total rumen archaea (Janssen & Kirs, 2008; Matthews et al., 2019). *Methanobrevibacter* is clearly the most abundant archaeal genus in the rumen. These findings suggest that the selected primers were adequate in identifying the archaeal taxonomic composition between the diverse samples.

Overall, the characterized archaea showed similar abundance trends between all samples, indicating that they have no obvious influence on ruminal vitamin B12 concentrations. Additionally, these genera did not share any relationships with the fat, lactose, protein or urea composition in the rumen, fecal and milk samples, concluding that archaeal species likely do not have any significant influence on vitamin B12 concentrations in the rumen.

However, the analysis of the combined MAGs and additional whole genomes from NCBI, indicate that strains of *Methanobrevibacter* have the potential to produce vitamin B12 via the anaerobic *de novo* pathway. *Methanobrevibacter* showed the greatest range and abundance of genes used in the synthesis of vitamin B12. In addition, *Methanobrevibacter* sequences do not encode the vitamin B12-dependent enzymes *metH, mutB,* or *mcm*, suggesting that they do not have a role in the consumption of vitamin B12, but instead are exclusively involved in the production of vitamin B12. Similarly, *Methanosphaera*-labelled MAGs also contain nearly complete anaerobic pathways but show no evidence of vitamin B12 consumption. Nevertheless, despite *Methanobrevibacter* being the most abundant archaea, making up 1.32% of the entire rumen microbiota in this study, there remains no obvious connections to indicate that it contributes significantly to total vitamin B12 concentrations.

Bacteria

The results presented indicate that bacteria make up roughly 94% of the total composition of the bovine rumen microbiome, which is comparable to other similar studies (Edwards, McEwan, Travis, & John Wallace, 2004; Jami & Mizrahi, 2012). The findings show that *Prevotella, Bacteroides, Clostridium, Eubacterium,* and *Ruminococcus* were the dominant bacterial genera in the rumen, and this is in agreement with existing literature (Chuang et al., 2020; Henderson et al., 2015; Zhong et al., 2018).

Establishing the presence of particular genes among the samples created the opportunity to outline the three distinct pathways for the biosynthesis of vitamin B12. The absence of a complete *de novo* and salvage pathway can be explained by the use of incomplete genomes, and functional annotators that contain limited databases. Using a pipeline that consisted of an assembly and binning module enabled the reconstruction of genomes which allowed for a more detailed analysis, specifically at the genus and species levels. However, due to the chosen parameters for bin completeness and contamination, there was an abundance of genomic information that was excluded from downstream analysis. Nevertheless, as previously mentioned, genomes with the stated 11 representative vitamin B12 biosynthesis genes can be hypothesized to encode the entire pathway (Doxey et al., 2015).

Quantifying the genes of entire read sets facilitated the understanding that samples with a high vitamin B12 concentration generally encode a greater abundance of both synthesizing and vitamin B12-dependent genes. In samples with a larger number of vitamin B12-producing genes, there were also a larger number of vitamin B12-dependent genes. The MAGs affiliated with the *metH* and *mutB* genes were largely represented by the phyla *Firmicutes* and *Bacteroidetes*, and many of them showed an absence of any B12-producing genes. Magnúsdóttir et al. (2015)

discovered that only 50% of *Firmicutes* and *Bacteroidetes* genomes were predicted to be vitamin B12 producers, signifying that are large proportion of genomes are indeed incapable of production. This study found that less than 10% of the refined MAGs contained ≥6 of the genes required for the first ten enzymatic reactions. These MAGs were almost entirely characterized by the phylum *Firmicutes*, with over half of the MAGs being attributed to the family *Lachnospiraceae*. The remaining MAGs were represented by the genera *Methanobrevibacter, Methanosphaera, Methanomethylophilus, Ruminococcus,* and *Succiniclasticum,* demonstrating the diverse contributions involved in vitamin B12 levels. However, despite the evident involvement of these taxa in vitamin B12 synthesis, the associated MAGs also showed an abundance of *metH* genes. Except for *Methanobrevibacter* and *Methanosphaera*, these groups seem to produce vitamin B12 for their own benefit, as their genomes encode at least one vitamin B12-depedent gene.

Prevotella

Overall, the functional annotations from the *Prevotella*-associated MAGs proved that *Prevotella* spp. are likely incapable of self-producing vitamin B12 via the *de novo* pathway. A complete depiction of the genes required for the entire anaerobic *de novo* pathway demonstrate that roughly 20 genes are necessary for the synthesis of vitamin B12. The results show that *Prevotella* spp. only encode between 30-50% of this total pathway. Even using the indicator genes determined by (Doxey et al., 2015), the selected *Prevotella* spp. lacked 80% of the marker genes found in the first ten steps of the anaerobic pathway, confirming that *Prevotella* benefits metabolically from environments with a high concentration of vitamin B12 (Franco-Lopez et al., 2020).

Furthermore, the analysis of 25 *Prevotella* genomes from the NCBI database helped establish the improbability of *Prevotella*'s involvement in the production of vitamin B12. The *Prevotella* species researched include: *Prevotella copri, Prevotella ruminicola, Prevotella bryantii, Prevotella oris*. These species were selected based on their occurrence in the bovine rumen, however due to some infrequent *Prevotella* genomes, some of the strains used were isolated from human sources. Literature has already discussed the possibility of *P. copri* as a vitamin B12 producer (Yoshii, Hosomi, Sawane, & Kunisawa, 2019), however, out of the species analyzed, it encoded the fewest genes that pertain to either the *de novo* or the salvage pathways. Instead, the results show that strains of *P. ruminicola* generally contain a greater number of genes affiliated with the *de novo* pathway, but are still missing genes *cbiL, cbiH, cbiF,* and *cbiC,* indicating the unlikelihood that any of the aforementioned *Prevotella* spp. contribute to the synthesis of vitamin B12 via the *de novo* pathway.

It is apparent that certain *Prevotella* spp. can perhaps exploit the more energy efficient salvage pathway. Nearly complete salvage pathways were compiled from the generated MAGs, indicating that the species of *Prevotella* can likely produce vitamin B12 by utilizing exogenous corrinoids. Furthermore, these genomes encoded multiple copies of the *btuB* gene, which implies that *Prevotella* may contain multiple vitamin B12 transporters, thus creating a higher affinity for corrinoids (Degnan, Barry, et al., 2014; Putnam & Goodman, 2020). Similarly, Wexler et al. (2018) determined that certain species of *Bacteroidetes* are capable of outcompeting other microbes for vitamin B12 by benefitting from a surface lipoprotein with high affinity for the vitamin. However, some *Prevotella*-labelled MAGs indicated no obvious connection with vitamin B12 production, meaning that some *Prevotella* spp. seemingly benefit entirely from other microbes in terms of vitamin B12 availability. More specifically, it is possible that particular strains of

Prevotella and other highly vitamin B12-dependent bacteria have forged nutritional symbiotic relationships with *Methanobrevibacter* and *Methanosphaera* spp (Figure 17).

The next steps would be to characterize and isolate strains involved in the production and consumption of vitamin B12 and perform whole genome sequencing to better understand the influence bacteria and archaea have on vitamin B12 concentrations in the rumen. This may eventually facilitate the production of natural methods to increase the abundance of high vitamin B12 producing organisms in the bovine rumen, to ultimately generate higher vitamin B12 concentrations in milk.

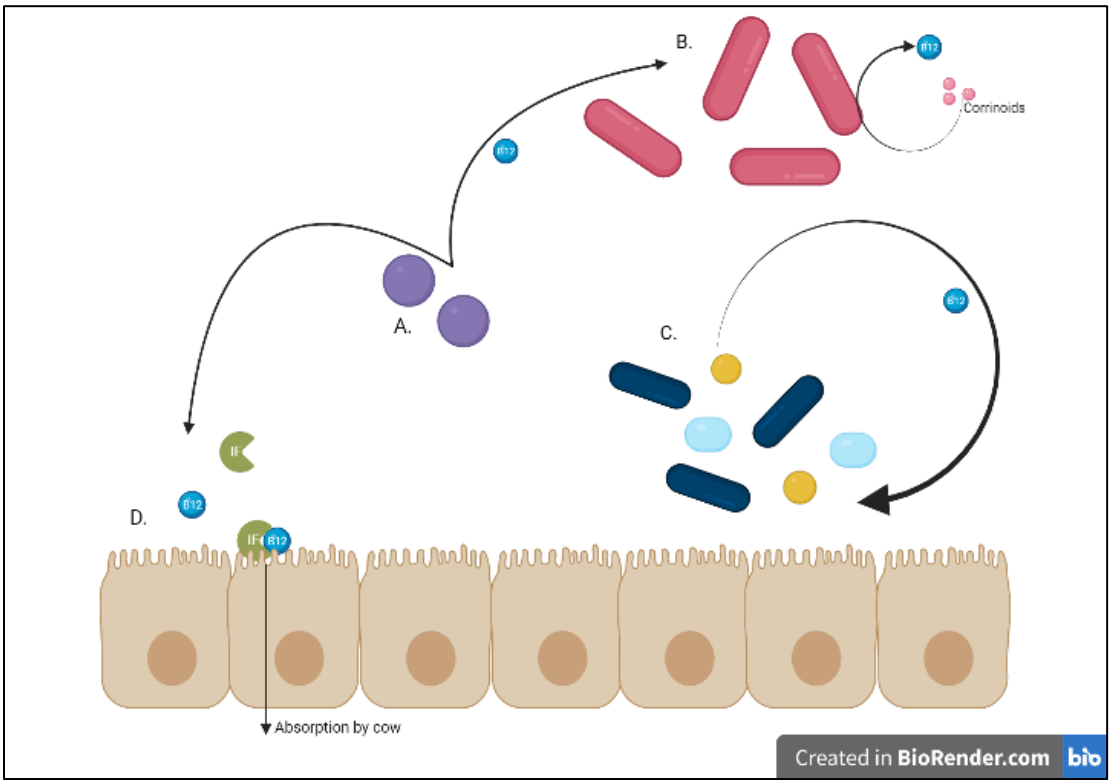


Figure 17. Microbial Competition for Vitamin B12 in the Rumen. A. Methanogens synthesize vitamin B12 to be secreted into the surrounding environment. **B.** Species of *Prevotella* consume readily available vitamin B12 and salvage exogenous corrinoids which are metabolized into vitamin B12. **C.** Species of *Lachnospiraceae*, *Ruminococcus* and *Succiniclasticum* produce vitamin B12 for their own benefit. **D.** Intrinsic factor binds vitamin B12 to be recognized by the cubilin receptor and mediated into the cow's anatomical systems.

CHAPTER 6. CONCLUSION

Most bacterial species were found to be consumers of vitamin B12, despite lacking any vitamin B12 biosynthetic genes. *Prevotella* encoded the greatest number of vitamin B12-dependent genes, indicating that they are likely major consumers of vitamin B12. It was discovered that *Prevotella* spp. are incapable of producing vitamin B12 via the *de novo* pathway but possibly have the ability to utilize exogenous corrinoids for their own benefit. Contrarily, species of *Methanobrevibacter* and *Methanosphaera* were found to be solely involved in the production of vitamin B12.

REFRENCES

Albers, S. V., Koning, S. M., Konings, W. N., & Driessen, A. J. M. (2004). Insights into ABC transport in archaea. *Journal of Bioenergetics and Biomembranes, 36*(1), 5-15.

Albers, S. V., & Meyer, B. H. (2011). The archaeal cell envelope. *Nat Rev Microbiol, 9*(6), 414-426. doi:10.1038/nrmicro2576

Albert, M. J., Mathan, V. I., & Baker, S. J. (1980). Vitamin B12 synthesis by human small intestinal bacteria. *Nature, 283*(5749), 781-782.

Allen, R. H., & Stabler, S. P. (2008). Identification and quantitation of cobalamin and cobalamin analogues in human feces. *The American journal of clinical nutrition, 87*(5), 1324-1335.

Arnstein, H. R. V., & White, A. M. (1961). The Function of Vitamin B12 in the Metabolism of Propionate by the Protozoan *Ochromonas malhamensis*. *Biochemical Journal, 83*, 264-270.

Barnes, R. H., & Fiala, G. (1958). Effects of the Prevention of Coprophagy in the Rat. *The Journal of Nutrition, 65*(1), 103-114. doi:10.1093/jn/65.1.103

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852-857.

Bushnell, B. (2021). BBTools. Retrieved from https://sourceforge.net/projects/bbmap/

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods, 13*(7), 581-583. doi:10.1038/nmeth.3869

Chaucheyras-Durand, F., & Ossa, F. (2014). REVIEW: The Rumen Microbiome: Composition, abundance, diversity, and new investigative tools. *Professional Animal Scientist, 30*, 1-12.

Chimento, D. P., Mohanty, A. K., Kadner, R. J., & Wiener, M. C. (2003). Substrate-induced transmembrane signaling in the cobalamin transporter BtuB. *Nature structural biology, 10*(5), 394-401.

Chong, J., Liu, P., Zhou, G., & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data.

Chuang, S. T., Ho, S. T., Tu, P. W., Li, K. Y., Kuo, Y. L., Shiu, J. S., . . . Chen, M. J. (2020). The Rumen Specific Bacteriome in Dry Dairy Cows and Its Possible Relationship with Phenotypes. *Animals (Basel), 10*(10). doi:10.3390/ani10101791

Creevey, C. J., Kelly, W. J., Henderson, G., & Leahy, S. C. (2014). Determining the culturability of the rumen bacterial microbiome. *Microbial biotechnology, 7*(5), 467-479. doi:10.1111/1751-7915.12141

Dali-Youcef, N., & Andrès, E. (2009). An update on cobalamin deficiency in adults. *QJM : monthly journal of the Association of Physicians, 102*(1), 17-28. doi:10.1093/qjmed/hcn138

Degnan, P. H., Barry, N. A., Mok, K. C., Taga, M. E., & Goodman, A. L. (2014). Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. *Cell host & microbe, 15*(1), 47-57. doi:10.1016/j.chom.2013.12.007

Degnan, P. H., Taga, M. E., & Goodman, A. L. (2014). Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab, 20*(5), 769-778. doi:10.1016/j.cmet.2014.10.002

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic acids research, 45*(W1), W180-W188. doi:10.1093/nar/gkx295

Doxey, A. C., Kurtz, D. A., Lynch, M. D., Sauder, L. A., & Neufeld, J. D. (2015). Aquatic metagenomes implicate Thaumarchaeota in global cobalamin production. *The ISME journal, 9*(2), 461-471. doi:10.1038/ismej.2014.142

Dukowicz, A. C., Lacy, B. E., & Levine, G. M. (2007). Small Intestinal Bacterial Overgrowth: A Comprehensive Review. *Gastroenterology & Hepatology, 3*(2).

Duplessis, M., Pellerin, D., Cue, R. I., & Girard, C. L. (2016). Short communication: Factors affecting vitamin B12 concentration in milk of commercial dairy herds: An exploratory study. *J Dairy Sci, 99*(6), 4886-4892. doi:10.3168/jds.2015-10416

Duplessis, M., Pellerin, D., Robichaud, R., Fadul-Pacheco, L., & Girard, C. L. (2019). Impact of diet management and composition on vitamin B12 concentration in milk of Holstein cows. *Animal : an international journal of animal bioscience, 13*(9), 2101-2109. doi:10.1017/S1751731119000211

Edwards, J. E., McEwan, N. R., Travis, A. J., & John Wallace, R. (2004). 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie van Leeuwenhoek : International Journal of General and Molecular Microbiology, 86*(3), 263-281. doi:10.1023/B:ANTO.0000047942.69033.24

Ellenbogen, L., & Cooper, B. A. (1991). Vitamin B12. In L. J. Machlin (Ed.), *Handbook of Vitamins* (pp. 491-536). New York: Marcel Dekker.

Escalante-Semerena, J. C. (2007). Conversion of Cobinamide into Adenosylcobamide in Bacteria and Archaea. *Journal of Bacteriology, 189*(13), 4555-4560. doi:10.1128/jb.00503-07

Eschenmoser, A., & Wintner, C. E. (1977). Natural product synthesis and vitamin B12. *Science (New York, N.Y.), 196*(4297), 1410-1420.

Fang, H., Kang, J., & Zhang, D. (2017). Microbial production of vitamin B12: a review and future perspectives. *Microb Cell Fact, 16*(1), 15. doi:10.1186/s12934-017-0631-y

Finlay, B. J., Esteban, G., Clarke, K. J., Williams, A. G., Embley, T. M., & Hirt, R. P. (1994). Some rumen ciliates have endosymbiotic methanogens. *FEMS microbiology letters, 117*(2), 157-161. doi:10.1111/j.1574-6968.1994.tb06758.x

Fouts, D. E., Szpakowski, S., Purushe, J., Torralba, M., Waterman, R. C., MacNeil, M. D., . . . Nelson, K. E. (2012). Next Generation Sequencing to Define Prokaryotic and Fungal Diversity in the Bovine Rumen. *PloS one, 7*(11), e48289. doi:10.1371/journal.pone.0048289

Franco-Lopez, J., Duplessis, M., Bui, A., Reymond, C., Poisson, W., Blais, L., . . . Ronholm, J. (2020). Correlations between the Composition of the Bovine Microbiota and Vitamin B12 Abundance. *mSystems, 5*(2). doi:10.1128/msystems.00107-20. (Accession No. 32127420)

Giannella, R. A., Broitman, S. A., & Zamcheck, N. (1971). Vitamin B12 uptake by intestinal microorganisms: mechanism and relevance to syndromes of intestinal bacterial overgrowth. *The Journal of clinical investigation, 50*(5), 1100-1107.

Gille, D., & Schmid, A. (2015). Vitamin B12 in meat and dairy products. *Nutrition Reviews, 73*(2), 106-115. doi:10.1093/nutrit/nuu011

Girard, C. L., Santschi, D. E., Stabler, S. P., & Allen, R. H. (2009). Apparent ruminal synthesis and intestinal disappearance of vitamin B12 and its analogs in dairy cows1. *Journal of Dairy Science, 92*(9), 4524-4529. doi:10.3168/jds.2009-2049

Goldstein, S. (1973). Zoosporic marine fungi (Thraustochytriaceae and Dermocystidiaceae). *Annual Reviews Microbiology, 27*, 13-25.

Green, R. (2010). Ins and outs of cellular cobalamin transport. *Blood, 115*(8), 1476-1477. doi:10.1182/blood-2009-12-254037

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., . . . Dijkstra, J. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports, 5*.

Hook, S. E., Steele, M. A., Northwood, K. S., Wright, A.-D. G., & McBride, B. W. (2011). Impact of High-Concentrate Feeding and Low Ruminal pH on Methanogens and Protozoa in the Rumen of Dairy Cows. *Microbial Ecology, 62*(1), 94-105. doi:10.1007/s00248-011-9881-0

Huerta-Cepas, J., Forslund, K., Coelho, L. P., Szklarczyk, D., Jensen, L. J., von Mering, C., & Bork, P. (2017). Fast Genome-Wide Functional Annotation through Orthology Assignment by eggNOG-Mapper. *Molecular Biology and Evolution, 34*(8), 2115-2122. doi:10.1093/molbev/msx148

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., . . . Bork, P. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology

resource based on 5090 organisms and 2502 viruses. *Nucleic acids research, 47*(D1), D309-D314. doi:10.1093/nar/gky1085
Jami, E., Israel, A., Kotser, A., & Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *The ISME journal, 7*(6), 1069-1079. doi:10.1038/ismej.2013.2
Jami, E., & Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. *PloS one, 7*(3), e33306. doi:10.1371/journal.pone.0033306
Janssen, P. H., & Kirs, M. (2008). Structure of the Archaeal Community of the Rumen. *Applied and environmental microbiology, 74*(12), 3619-3625. doi:10.1128/aem.02812-07
Johnson, M. A. (2008). If High Folic Acid Aggravates Vitamin B12 Deficiency What Should Be Done About It? *Nutrition Reviews, 65*(10), 451-458. doi:10.1111/j.1753-4887.2007.tb00270.x
Kang, D. D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., & Wang, Z. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ, 7*, e7359-e7359. doi:10.7717/peerj.7359
Keegan, K. P., Glass, E. M., & Meyer, F. (2016). MG-RAST, a Metagenomics Service for Analysis of Microbial Community Structure and Function. *Methods Mol Biol, 1399*, 207-233. doi:10.1007/978-1-4939-3369-3_13
Kim, M., Morrison, M., & Yu, Z. (2011). Phylogenetic diversity of bacterial communities in bovine rumen as affected by diets and microenvironments. *Folia microbiologica, 56*(5), 453-458. doi:10.1007/s12223-011-0066-5
Kittelmann, S., Pinares-Patiño, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C., & Janssen, P. H. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PloS one, 9*(7), e103171. doi:10.1371/journal.pone.0103171
Kumar, S., Indugu, N., Vecchiarelli, B., & Pitta, D. W. (2015). Associative patterns among anaerobic fungi, methanogenic archaea, and bacterial communities in response to changes in diet and age in the rumen of dairy cows. *Frontiers in Microbiology, 6*(781). doi:10.3389/fmicb.2015.00781
Lan, W., & Yang, C. (2019). Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Sci Total Environ, 654*, 1270-1283. doi:10.1016/j.scitotenv.2018.11.180
Li, D., Liu, C.-M., Luo, R., Sadakane, K., & Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics, 31*(10), 1674-1676. doi:10.1093/bioinformatics/btv033
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Subgroup, G. P. D. P. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics, 25*(16), 2078-2079. doi:10.1093/bioinformatics/btp352
Lin, C., Raskin, L., & Stahl, D. A. (1997). Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiology Ecology, 22*(4), 281-294. doi:10.1111/j.1574-6941.1997.tb00380.x
Lopez-Franco, J. (2019). *Correlations Between Concentration of Vitamin B12 in Milk and the Composition of the Bovine Microbiota.* McGill University,
Lu, X., Heal, K. R., Ingalls, A. E., Doxey, A. C., & Neufeld, J. D. (2020). Metagenomic and chemical characterization of soil cobalamin production. *The ISME journal, 14*(1), 53-66. doi:10.1038/s41396-019-0502-0
Lundrigan, M. D., Köster, W., & Kadner, R. J. (1991). Transcribed sequences of the Escherichia coli btuB gene control its expression and regulation by vitamin B12. *Proc Natl Acad Sci U S A, 88*(4), 1479-1483. doi:10.1073/pnas.88.4.1479
Ma, H., Wang, L., Zhang, C., & Yi, H. (2008). Biosynthesis, fermentation, application of vitamin B12 - a review. *Chinese Journal of Biotechnology, 24*(6), 927-932.
Magnúsdóttir, S., Ravcheev, D., de Crécy-Lagard, V., & Thiele, I. (2015). Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Frontiers in Genetics, 6*(148). doi:10.3389/fgene.2015.00148

Makarova, K. S., Aravind, L., Galperin, M. Y., Grishin, N. V., Tatusov, R. L., Wolf, Y. I., & Koonin, E. V. (1999). Comparative Genomics of the Archaea (Euryarchaeota): Evolution of Conserved Protein. *Genome Research, 9*, 608-628.

Martens, J. H., Barg, H., Warren, M. J., & Jahn, D. (2002). Microbial production of vitamin B12. *Appl Microbiol Biotechnol, 58*(3), 275-285. doi:10.1007/s00253-001-0902-7

Matte, J. J., Britten, M., & Girard, C. L. (2014). The importance of milk as a source of vitamin B12 for human nutrition. *Animal Frontiers, 4*(2), 32-37. doi:10.2527/af.2014-0012

Matte, J. J., Guay, F., & Girard, C. L. (2012). Bioavailability of vitamin B_{12} in cows' milk. *The British journal of nutrition, 107*(1), 61-66. doi:10.1017/S0007114511002364

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., & Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut microbes, 10*(2), 115-132. doi:10.1080/19490976.2018.1505176

McDowell, L. R. (1989). *Vitamins in animal nutrition: comparative aspects to human nutrition*.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., . . . Edwards, R. A. (2008). The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC bioinformatics, 9*, 386. doi:10.1186/1471-2105-9-386

Moore, S. J., Lawrence, A. D., Biedendieck, R., Deery, E., Frank, S., Howard, M. J., . . . Warren, M. J. (2013). Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B₁₂). *Proceedings of the National Academy of Sciences, 110*(37), 14906-14911. doi:10.1073/pnas.1308098110

Nahvi, A., Barrick, J. E., & Breaker, R. R. (2004). Coenzyme B12 riboswitches are widespread genetic control elements in prokaryotes. *Nucleic acids research, 32*(1), 143-150. doi:10.1093/nar/gkh167

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The Role of Ciliate Protozoa in the Rumen. *Front Microbiol, 6*, 1313. doi:10.3389/fmicb.2015.01313

Nielsen, M. J., Rasmussen, M. R., Andersen, C. B., Nexo, E., & Moestrup, S. K. (2012). Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol, 9*(6), 345-354. doi:10.1038/nrgastro.2012.76

Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a Web browser. *BMC bioinformatics, 12*, 385. doi:10.1186/1471-2105-12-385

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research, 25*(7), 1043-1055. doi:10.1101/gr.186072.114

Parks, D. H., Rinke, C., Chuvochina, M., Chaumeil, P.-A., Woodcroft, B. J., Evans, P. N., . . . Tyson, G. W. (2017). Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nature Microbiology, 2*(11), 1533-1542. doi:10.1038/s41564-017-0012-7

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A., & Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods, 14*(4), 417-419. doi:10.1038/nmeth.4197

Pérez-Cobas, A. E., Gomez-Valero, L., & Buchrieser, C. (2020). Metagenomic approaches in microbial ecology: an update on whole-genome and marker gene sequencing analyses. *Microbial genomics, 6*(8). doi:https://doi.org/10.1099/mgen.0.000409

Putnam, E. E., & Goodman, A. L. (2020). B vitamin acquisition by gut commensal bacteria. *PLoS pathogens, 16*(1), e1008208-e1008208. doi:10.1371/journal.ppat.1008208

Raux, E., Schubert, H. L., Roper, J. M., Wilson, K. S., & Warren, M. J. (1999). Vitamin B12: Insights into Biosynthesis's Mount Improbable. *Bioorganic Chemistry, 27*(2), 100-118. doi:https://doi.org/10.1006/bioo.1998.1125

Salfer, I. J., Staley, C., Johnson, H. E., Sadowsky, M. J., & Stern, M. D. (2018). Comparisons of bacterial and archaeal communities in the rumen and a dual-flow continuous culture fermentation system using amplicon sequencing. *Journal of Animal Science, 96*(3), 1059-1072. doi:10.1093/jas/skx056

Schjonsby, H., Halvorsen, J. F., Hofstad, T., & Hovdenak, N. (1977). Stagnant loop syndrome in patients with continent ileostomy (intra-abdominal ileal reservoir). *Gut, 18*(10), 795-799.
Scott, J. M., & Molloy, A. M. (2012). The discovery of vitamin B(12). *Ann Nutr Metab, 61*(3), 239-245. doi:10.1159/000343114
Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics, 30*(14), 2068-2069. doi:10.1093/bioinformatics/btu153
Sharp, R., Ziemer, C. J., Stern, M. D., & Stahl, D. A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiology Ecology, 26*(1), 71-78. doi:10.1016/S0168-6496(98)00024-5
Shelton, A. N., Seth, E. C., Mok, K. C., Han, A. W., Jackson, S. N., Haft, D. R., & Taga, M. E. (2019). Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. *The ISME journal, 13*(3), 789-804. doi:10.1038/s41396-018-0304-9
Shin, E. C., Choi, B. R., Lim, W. J., Hong, S. Y., An, C. L., Cho, K. M., . . . Yun, H. D. (2004). Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe, 10*(6), 313-319. doi:10.1016/j.anaerobe.2004.08.002
Skillman, L. C., Evans, P. N., Strömpl, C., & Joblin, K. N. (2006). 16S rDNA directed PCR primers and detection of methanogens in the bovine rumen. *Letters in Applied Microbiology, 42*(3), 222-228. doi:10.1111/j.1472-765X.2005.01833.x
Smith, R. M., & Marston, H. R. (1970). Production, absorption, distribution and excretion of vitamin B 12 in sheep. *Br J Nutr, 24*(4), 857-877. doi:10.1079/bjn19700092
Stabler, S. P. M. D. (2013). Vitamin B12 Deficiency. *The New England Journal of Medicine, 368*(2), 149-160. doi:10.1056/NEJMcp1113996
Stams, A. J., & Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol, 7*(8), 568-577. doi:10.1038/nrmicro2166
Stevenson, D. M., & Weimer, P. J. (2007). Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Applied Microbiology and Biotechnology, 75*(1), 165-174. doi:10.1007/s00253-006-0802-y
Su, Y., Bian, G., Zhu, Z., Smidt, H., & Zhu, W. (2014). Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. *Archaea (Vancouver, B.C.), 2014*, 547908. doi:10.1155/2014/547908
Tajima, K., Nagamine, T., Matsui, H., Nakamura, M., & Aminov, R. I. (2001). Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS microbiology letters, 200*(1), 67-72.
Thauer, R. K., Kaster, A. K., Seedorf, H., Buckel, W., & Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol, 6*(8), 579-591. doi:10.1038/nrmicro1931
Thomas, M. G., & Escalante-Semerena, J. C. (2000). Identification of an Alternative Nucleoside Triphosphate: 5′-Deoxyadenosylcobinamide Phosphate Nucleotidyltransferase in Methanobacterium thermoautotrophicum ΔH. *Journal of Bacteriology, 182*(15), 4227-4233. doi:10.1128/jb.182.15.4227-4233.2000
Tymensen, L. D., Beauchemin, K. A., & McAllister, T. A. (2012). Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. *Microbiology (Reading, England), 158*(Pt 7), 1808-1817. doi:10.1099/mic.0.057984-0
Vogels, G. D., Hoppe, W. F., & Stumm, C. K. (1980). Association of methanogenic bacteria with rumen ciliates. *Applied and environmental microbiology, 40*(3), 608-612. doi:10.1128/AEM.40.3.608-612.1980
Wang, L., Xu, Q., Kong, F., Yang, Y., Wu, D., Mishra, S., . . . Klieve, A. V. (2016). Exploring the Goat Rumen Microbiome from Seven Days to Two Years. *PloS one, 11*(5), e0154354. doi:10.1371/journal.pone.0154354

Warren, M. J., Raux, E., Schubert, H. L., & Escalante-Semerena, J. C. (2002). The biosynthesis of adenosylcobalamin (vitamin B12). *Nat Prod Rep, 19*(4), 390-412. doi:10.1039/b108967f

Watanabe, F., & Bito, T. (2018). Vitamin B12 sources and microbial interaction. *Exp Biol Med (Maywood), 243*(2), 148-158. doi:10.1177/1535370217746612

Wexler, A. G., Schofield, W. B., Degnan, P. H., Folta-Stogniew, E., Barry, N. A., & Goodman, A. L. (2018). Human gut Bacteroides capture vitamin B12 via cell surface-exposed lipoproteins. *eLife, 7*. doi:10.7554/eLife.37138

Wheatley, R. W., Ng, K. K. S., & Kapoor, M. (2016). Fungal cobalamin-independent methionine synthase: Insights from the model organism, Neurospora crassa. *Arch Biochem Biophys, 590*, 125-137. doi:10.1016/j.abb.2015.11.037

Whitford, M. F., Teather, R. M., & Forster, R. J. (2001). Phylogenetic analysis of methanogens from the bovine rumen. *Biomed Central Microbiology, 1*(5), 1-5.

Wilkens, S. (2015). Structure and mechanism of ABC transporters. *F1000prime reports, 7*, 14. doi:10.12703/P7-14

Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America, 87*, 4576-4579.

Woodson, J. D., Peck, R. F., Krebs, M. P., & Escalante-Semerena, J. C. (2003). The cobY Gene of the Archaeon Halobacterium sp. Strain NRC-1 Is Required for De Novo Cobamide Synthesis. *Journal of Bacteriology, 185*(1), 311-316. doi:10.1128/jb.185.1.311-316.2003

Woodson, J. D., Reynolds, A. A., & Escalante-Semerena, J. C. (2005). ABC transporter for corrinoids in Halobacterium sp. strain NRC-1. *J Bacteriol, 187*(17), 5901-5909. doi:10.1128/JB.187.17.5901-5909.2005

Woodson, J. D., Zayas, C. L., & Escalante-Semerena, J. C. (2003). A new pathway for salvaging the coenzyme B12 precursor cobinamide in archaea requires cobinamide-phosphate synthase (CbiB) enzyme activity. *J Bacteriol, 185*(24), 7193-7201. doi:10.1128/jb.185.24.7193-7201.2003

Wright, A. D., Auckland, C. H., & Lynn, D. H. (2007). Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. *Appl Environ Microbiol, 73*(13), 4206-4210. doi:10.1128/AEM.00103-07

Yoshii, K., Hosomi, K., Sawane, K., & Kunisawa, J. (2019). Metabolism of Dietary and Microbial Vitamin B Family in the Regulation of Host Immunity. *Frontiers in Nutrition, 6*(48). doi:10.3389/fnut.2019.00048

Zaidel, O., & Lin, H. C. (2003). Uninvited Guests: The Impact of Small Intestinal Bacterial Overgrowth on Nutritional Status. *Nutrition Issues in Gatroenterology, 7*, 27-34.

Zhang, Y., Rodionov, D. A., Gelfand, M. S., & Gladyshev, V. N. (2009). Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. *BMC Genomics, 10*(1), 78. doi:10.1186/1471-2164-10-78

Zhong, Y., Xue, M., & Liu, J. (2018). Composition of Rumen Bacterial Community in Dairy Cows With Different Levels of Somatic Cell Counts. *Frontiers in Microbiology, 9*, 3217-3217. doi:10.3389/fmicb.2018.03217

Zhu, Z., Kristensen, L., Difford, G. F., Poulsen, M., Noel, S. J., Abu Al-Soud, W., . . . Hojberg, O. (2018). Changes in rumen bacterial and archaeal communities over the transition period in primiparous Holstein dairy cows. *J Dairy Sci, 101*(11), 9847-9862. doi:10.3168/jds.2017-14366