INVESTIGATING THE ROLES OF B VITAMINS AND ONE-CARBON METABOLISM IN GUT HOMEOSTASIS

By

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ABSTRACT

Vitamins are the organic compounds essential for normal growth and metabolic processes in all living organisms. B vitamins, such as vitamin B9 and B12, act as cofactors in various metabolic reactions, including one-carbon (1C) metabolism, and typically are required in trace amounts. The gut harbors a diverse population of bacteria, some of which are capable of producing certain vitamins. These bacteria also require vitamins for their growth and proliferation, with requirements varying between vitamin-producing and non-producing strains. However, bacterially synthesized vitamins are limited, and dietary intake is vital for meeting the host's daily requirements. Despite the well-understood functional role of B vitamins, their precise impacts on gut ecology remains poorly understood. The current study aimed to investigate the impacts of vitamin B9 and B12 deficiencies on gut morphology, microbial composition, and their metabolites using a rodent model of vitamin deficiency. Our findings revealed that a deficiency in vitamin B9 or B12 altered mucosal height and decreased acidic mucin production in the colon. Of particular interest, we observed sex-dependent differences in the effects of vitamin deficiency on the gut microbial profile. The analysis of fecal metabolites showed notable alterations in metabolites associated with gut health in the vitamin-deficient groups compared to the control. This study provides evidence that deficiencies in B vitamins, particularly those involved in 1C metabolism, can disturb gut homeostasis in a sex-dependent manner.

CO-AUTHORSHIP STATEMENT

The work presented in Chapter three, which has been written in manuscript format, is currently in preparation to be submitted for publication in Journal of Nutrition. I, Khandkar Shaharina Hossain, was involved in the designing of the study, conducting experiments, analyzing, and interpreting all the data, and preparing the manuscript. Ava Rasouli and Sathya Amarasena were involved in animal termination and DNA extraction. Dr. Shyamchand Mayengbam designed the study, supervised all the experiments involved in the project, and has edited the manuscript. All the authors have read the manuscript and have agreed to publish it.

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ABBREVIATIONS

10-CHO-THF	10-Formyltetrahydrofolate
1C pathway	One carbon pathway
5,10-CH_2-THF	5,10-Methylenetetrahydrofolate
5-CH_3-THF	5-methyltetrahydrofolate
AB	Alcian blue
AD	Alzheimer disease
ASV	Amplicon sequence variant
BCAA	Branched chain amino acids
BHMT	Betaine homocysteine S methyltransferase
CD	Coeliac disease
CDC	Center for disease control and prevention
CHMS	Canadian Health Measures Survey
CVD	Cardiovascular disease
Cys	Cysteine
DFE	Dietary folate equivalents
DHF	Dihydrofolate
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
EMA	European Medicines Agency
FDA	Food & Drug Administration
GC-MS	Gas chromatography-mass spectrometry
GCP	Glutamate carboxypeptidase
GNMT	Glycine N-methyltransferase
H&E	Hematoxylin eosin
Hcy	Homocysteine
IBD	Inflammatory bowel disease
ICH	International council for harmonisation of technical requirements for pharmaceuticals for human use
IF	Intrinsic factor
LB12	Low B12
LB9	Low B9
LC-MS	Liquid chromatography-mass spectrometry
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
L-Met	L-methionine
MAP	Methionine aminopeptidase
MAT	Methionine adenosyl transferase
MMA	Methylmalonic acid
MRM	Multiple-reaction monitoring
MRP	Multidrug resistance protein
MS	Methionine synthase
MTHFR	Methyl tetrahydrofolate dehydrogenase reductase

NHANES	National health and nutrition examination survey
NMR	Nuclear magnetic resonance
NTD	Neural tube defects
PABA	Para-aminobenzoic acid
PAS	Periodic acid–Schiff
PCA	Principal component analysis
PCFT	Proton-coupled folate transporter
PLS-DA	Partial least squares-discriminant analysis
RBC	Red blood cell
RDA	Recommended dietary allowance
RFC	Reduced folate carrier
SAH	S- adenosylhomocysteine
SAH-H	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SCFA	Short chain fatty acid
SHMT	Serine hydroxymethyl transferase
TC	Transcobalamin
THF	Tetrahydrofolate
TYMS	Thymidylate synthase
VIP	Variable importance in projection

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CHAPTER ONE

Introduction and background

1.0 Introduction

Micronutrients, such as vitamins, play important roles in different metabolic processes [1]. Deficiency of these micronutrients is a major health concern and is mostly prevalent in developing countries [2]. While the roles of diet and dietary factors in regulating gut microbial composition is well-known, the impact of micronutrient deficiencies on gut homeostasis is less studied [3]. Among micronutrients, the water-soluble vitamins, B family is of particular significance as it is not synthesized by the host and thus, must be obtained from either the dietary intake or supplied by gut microbiota[4]. In the gut, almost 100 known bacterial species can produce B vitamins and symbiotically exchange them to survive [5]. Biosynthetic pathways for vitamins can differ among species, which highlights the existence of genetic variations [6]. Various taxa have evolved to fulfill specific roles in the synthesis and utilization of vitamins [7]. For instance, *Bacteroidetes* utilize a well-recognized thiamin biosynthesis pathway, whereas organisms like *Salmonella enterica*, which lack this pathway, depend on transporters to acquire thiamin [8]. Among all the vitamins synthesized in the gut, riboflavin and niacin are the most commonly produced vitamins [5]. However, despite the production of these vitamins by the gut microbiota, several factors such as site of production and competition between host and non-producing bacteria, result in an insufficient supply to meet the daily requirements of the host [5]. Consequently, individuals rely on dietary intake to meet their requirements of these micronutrients.

B vitamins, specifically vitamin B9 and B12, play vital roles in one-carbon (1C) metabolism, a shared biological pathway involved in various physiological activities. For example, it plays a critical role in DNA synthesis, production of methyl donors, amino acid homeostasis, and redox defense [9, 10]. Water-soluble vitamins i.e., B vitamins serves as co-enzymes as well as methyl

donors in 1C metabolism [11]. A slight alteration in this cycle may have association with several diseases including cardiovascular disease, Alzheimer's, cancer, and others [12].

Mutual symbiosis between the gut microbiota and the mammalian gut has shaped their interdependence, and the microbiota's ability to produce essential substances is heavily influenced by the host environment. The synthesis of these vitamins in the gut requires specific molecules and substrates, such as para-aminobenzoic acid (pABA) for folate synthesis and precorrin-2 for B12 production [5]. The synthesis of these precursors is also dependent on the availability of substrates in the gut. Thus, it is reasonable to assume that micronutrients, such as vitamin B9 and B12, could play a crucial role in the gut homeostasis. In the current study, we investigated the influence of dietary vitamin B9 and B12 on gut morphology, gut microbiota composition, and microbial metabolites using a B9 and B12 - deficient rat model.

1.1 Background

Vitamins are a group of organic compounds required for normal physiological function [13]. They are mainly divided into two groups: water-soluble and fat-soluble. Water-soluble vitamins such as B vitamins, (specifically vitamin B6, B9 and B12) play vital roles in several enzymatic reactions, including 1C metabolism. For example, folate and B12 play a critical role in the biosynthesis of DNA, lipids, proteins, methyl donors, and redox molecules [14]. Maternal 1C is also essential in controlling the methyl group for epigenetic functions and influencing gametogenesis and zygogenesis [15]. Perturbations of these metabolic pathways are associated with several diseases such as cardiovascular disease, Alzheimer's, neural tube defects, renal impairment, megaloblastic anaemia, congenital cardiac condition, non-alcoholic fatty liver disease, and cancer [12]. Our gut microbiota can produce a small amount of these vitamins, which is insufficient for the host's daily

requirements and therefore, requires dietary supplementation [16, 17]. As such adequate dietary intake of these vitamins is not only crucial for humans, but they are also essential for maintaining normal gut bacterial functions. Here, we discuss the details of vitamin B9 and B12 and their impact on gut microbiota.

1.1.1 Vitamin B9 or folate

1.1.1.1 Discovery of folate

The search for a cure for megaloblastic anaemia in the late 1870s and early 1880s resulted in the discovery of folate. Scientists found that incorporating animal liver in meals along with other vitamins improved the anaemic condition [18]. In 1941, Mitchell and his colleagues gave the term "Folic acid" to this vitamin. They isolated it from spinach and named it after the Latin word folium, which means leaf. Later, they discovered it's potential to promote the growth of bacteria such as *Streptococcus lactis* [19].

1.1.1.2 Structure of folate

Folate or pteroylglutamate is a generic term which refers to a larger family of compounds which contains a 2-amino-4-hydroxy-pteridine ring, a *p*-aminobenzoyl moiety, and either monoglutamate or poly-γ-glutamate moieties [14] The folate family can comprise as many as 150 compounds because of varying combinations of 1C unit, pteridine oxidation moiety, and different polyglutamate chain lengths [20]. This 1C unit can attach either at N5 or N10 position (marked red) or both (Figure 1.1). The 2-amino-4-hydroxy-pteridine ring is linked with *p*-aminobenzoyl moiety with a methylene (CH_2) group, and the *p*-aminobenzoyl moiety is further linked with an amide bond to the α-amino group of either monoglutamate or poly-γ-glutamate moiety. Though

this is a large family of compounds, however only one-third of them (<50) is found in plants and animals [21].

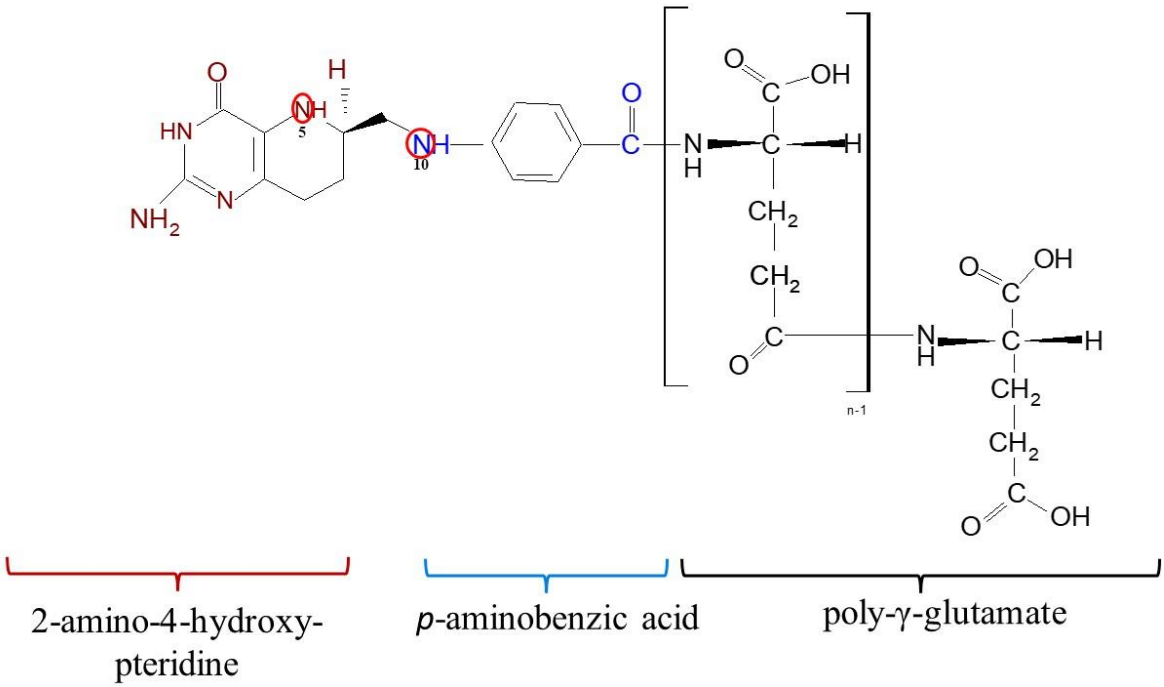

Figure 1.1. Chemical structure of folate. Three distinct chemical moieties are linked together to form the chemical structure of folate. The structure is divided into three parts, 2-amino-4-hydroxy-pteridine ring link with a methylene bridge to a *p*-aminobenzoyl linked via amide bond to poly-γ-glutamate. Figure is generated with ChemDraw.

1.1.1.3 Digestion, absorption, and metabolism of folate

Folate is highly bioavailable when consumed in its pure form on an empty stomach. For instance, folic acid, the supplemental form of folate, has an 85% bioavailability, whereas its bioavailability decreases when consumed as a part of food [62]. Dietary folate exists in both free and conjugated forms [22]. Free folic acid mostly exists as a ploy glutamate form in foods and must be transformed to monoglutamate before absorption. This conversion reaction is mediated by folate conjugase in the brush border of the proximal small intestine by glutamate carboxypeptidase II (GCPII, E.C. 3.4. 17.21) [23, 24]. Folate is primarily absorbed in the jejunum section of the small intestine and

poorly absorbed in the alkaline distal part of the intestine or colon [23, 25]. The absorption of folate is mediated by the pH-dependent carrier, proton-coupled folate transporter (PCFT, SLC46A1), Reduced Folate Carrier (RFC, SLC19A1), and three glycosylphosphatidylinositol-anchored receptors (FOLR1–3) [10, 26, 27]. Once absorbed, monoglutamate folate is converted into first 5-methyl tetrahydrofolate (THF), which occurs in the enterocyte of the jejunum brush border, and then it is transported into the portal vein by multidrug resistance-associated protein (MRP) [28, 29] (Figure 1.2). Folate circulates to the portal system and reaches targeted tissues by using either RFC or receptor-mediated endocytosis of the folate receptors (FRs), primarily the liver. In the liver, folate is stored temporarily as monoglutamate form before being released into the plasma when needed by converting monoglutamate into polyglutamate form [28]. The amount of folate excreted from the body ranges from 0.3 to 0.8% of daily folate intake [30, 31], primarily in the urine as *p*-aminobenzoylglutamate and its derivates [32]. Interestingly, as much as 90% of total body folate remains in circulation in the body fluid, while only 10% is stored in the tissues [33].

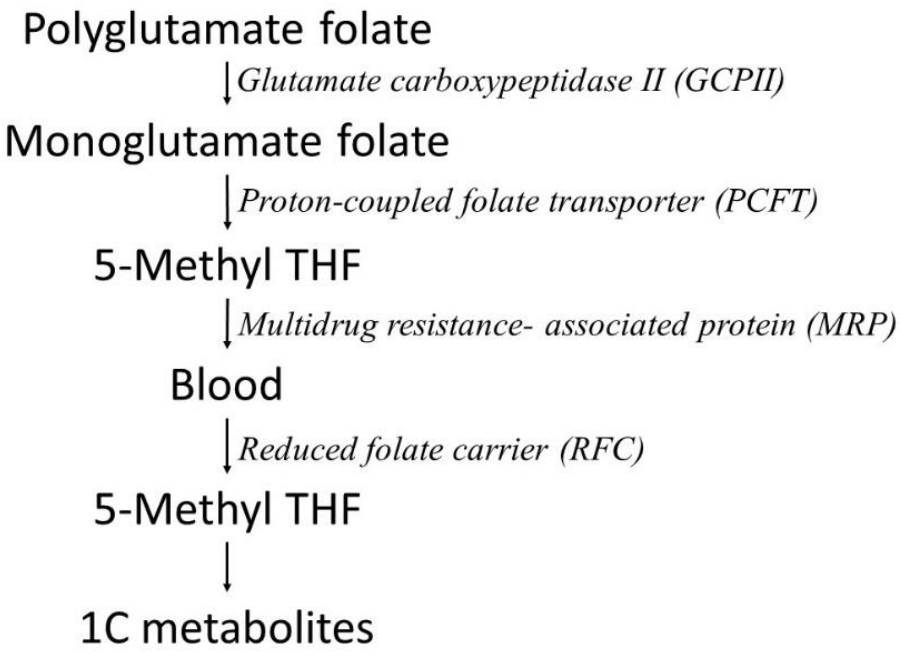

Figure 1.2: Absorption, transport, and cellular uptake of folate. GCPII; glutamate carboxypeptidase II, PCFT; proton-coupled folate transporter, MRP; multidrug resistance-associated protein, RFC; reduced folate carrier.

1.1.1.4 Assessment of folate status

Folate status can be assessed using dietary intake, blood biomarker concentration, or a combined method of these two [34]. According to Biomarkers on Nutrition and Development (BOND), serum and total red blood cell (RBC) folate levels are two of the main biomarkers for assessing folate status [35]. Assessment of folate status can be done through direct or indirect methods.

(i) Direct method for folate quantification

Folate concentrations can be directly measured using body fluids such as serum, RBC and urine. Serum folate concentration only reflects current dietary intake, but repeated measurement of serum folate periodically in one person can be used to identify folate deficiency [35]. On the other hand, RBC folate is used as a long-term indicator of folate status and represents approximately 40-50%

of total body folate [35]. It reflects the folate status in RBC during erythropoiesis, thus indicating the folate status over the last three months [14]. RBC folate status also indicates tissue folate status, such as liver folate content [36]. Urinary folate is also combined with serum and RBC folate to determine the daily average folate status [37].

(ii) Indirect method for folate quantification

Microbiological assay methods

Microbiological assays can be used to quantify the concentrations of B vitamins, as these vitamins are required for the growth of specific groups of bacteria. Several lactic acid bacteria, specifically *Lactobacillus casei*, are used for such assays. This assay can quantify the total folate present in a sample. However, the limitation of this assay is it can not distinguish between different forms of folate that co-exist in the sample [38]. Having said that, *L. casei* contains a vitamin B12-dependent methionine synthase (MS, also known as MTR, EC 2.1.1.13) which converts 5-methylene-THF to 5,10-methylene-THF [39]. This capability can be used to differentiate between polyglutamate and monoglutamate forms of folate based on the growth [40].

Deoxyuridine Suppression Test

Through 1C metabolism, folate plays a vital role in DNA synthesis by providing S-adenosyl methionine (SAM) [41]. SAM is essential for maintaining the integrity of specific parts of chromosomes via cytosine methylation [42, 43]. SAM deficiency results in cytosine hypomethylation and, consequently alterations in DNA [44]. Folate is required for deoxythymidine triphosphate (dTTP) synthesis via donating 1C to deoxyuridine monophosphate [35]. Uracil is removed from the DNA by two enzymes: uracil-DNA glycosylase and apyrimidinic endonuclease,

creating an unrepairable nick in the DNA strand [45]. This conversion of deoxyuridine monophosphate (dUMP) to dTTP and these resulting nicks are fully dependent on the availability of folate [41, 46]. Therefore, the amount of uracil content in DNA can be used as a genomic biomarker for folate status. Excessive uracil accumulation can lead to micronuclei formation and presence of micronuclei can also be an indicator of folate status [47]. However, micronuclei formation is not exclusively associated with folate deficiency but can also be triggered by deficiencies of other vitamins and minerals [14].

1.1.1.5 Folate Deficiency

Folate is primarily absorbed in the jejunum via several active and passive transporters. Therefore, patients with intestinal disorders of the small intestine, such as coeliac disease and amyloidosis, suffer from folate deficiency [48]. The clinical progression of folate deficiency involves changes in serum folate levels followed by a reduction in RBC folate and an elevated homocysteine (Hcy) level [49]. Folate deficiency can cause neural tube defects (NTD), megaloblastic anaemia, pancytopenia, glossitis, male infertility, angular stomatitis, and oral ulcers. Megaloblastic changes in bone and tissues are also observed in the second stage of folate deficiency [50]. These morphological changes are accompanied by cellular changes in neutrophils (hyper segmentation of nuclei) and lymphocytes (formation of micronuclei) [14]. Abnormal erythrocyte production and decreased oxygen-carrying capacity of the blood result in weakness and tiredness [51, 52]. Additionally, depression, irritability, insomnia, cognitive decline, fatigue, and psychosis can occur due to folate deficiency [14]. Several factors increase the risk of developing folate deficiency including pregnancy, medication, malabsorption, drinking, or other folate inhibitors [53]. Identifying folate deficiency at the earlier stage is crucial to prevent anaemia and other deficiency diseases.

1.1.1.6 Epidemiology of folate status

Epidemiological studies on folate status have been based on either nutrient intake from the food supply or plasma/serum folate concentration and have shown high variation between individuals [54]. The Recommended Dietary Allowance (RDA) is different at different stages of life. RDA of folate is 200 μg for men and 180 μg for women [38, 39]. However, according to the Center for Disease Control and Prevention (CDC), all women of reproductive age should take 400 mg of folic acid daily. This requirement increases to 600 mg during pregnancy and 500 mg during lactation [36, 37]. Among dietary sources, yeast and liver are the richest dietary sources of folate, containing 2 mg and 300 μg per 100 gm, respectively [41]. 75-80% of folate exists as polyglutamate; with 90% of folate in vegetables being either 5- or 10-formyl derivatives, while the remaining 10% or less is unreduced polyglutamate [42-44]. Regarding folate storage in the body, the liver contains approximately 50% of the total body folate [38]. The average serum/plasma folate concentration is 13.6 nmol/L, with levels lower than 6.8 nmol/L indicating folate deficiency, whereas 340 nmol/L is considered the optimum level of RBC folate [55].

The prevalence of folate deficiency is less than 20% in developing countries and is significantly lower in developed countries, at less than 5% [56]. Less than 1% of Canadians aged 6-79 years suffer from folate deficiency, as indicated by RBC folate concentration. Within this age group, approximately 40% of the population showed higher RBC folate, more than 1360 nmol/L, whereas the optimum level is 340 nmol/L [55, 57]. However, rates of folate insufficiency differ among childbearing-age women [57]. According to Canadian Community Health Survey (2015), 22% of childbearing-age women exhibited folate deficiency regardless of their age [57], even though 70% of women were knowledgeable about the consequences of folate deficiency [58].

1.1.1.7 Factors influencing folate status

Folate status can be influenced by several factors, ethnicity being one of them. Velez and his colleagues found a relationship between ethnicity and folate status. According to this study, non-Hispanic whites (NHW) are more prone to folate deficiency and its associated anemia compared to Asians [59]. Other than ethnicity, genetic factors such as the presence or absence of a specific allele can be an influencing factor for folate status. The section below describes how these factors influence the folate status.

Genetic factors

Several studies have identified a link between genetic polymorphism, the presence of two or more variants forms of a particular DNA sequence, and folate pathway through common C677T variant in the gene. MTHFR C677T encodes the enzyme methyltetrahydrofolate dehydrogenase reductase (MTHFR), which is required for converting 5,10-CH_2-THF into 5-CH_3-THF in the folate cycle of 1C metabolic pathway. Individuals who carry T allele tend to have lower MTHFR activity, leading to higher levels of Hcy in their blood [60-62]. Known polymorphisms that affect folate include MTHFR C677T and cystathionine β-synthase (CBS) [63].

Drugs

Compounds structurally similar to folic acid can affect the absorption of folate. Several drugs, such as methotrexate and aminopterin, can reduce the absorption of folate in the body by inhibiting the enzyme dihydrofolate reductase (Table 1.1) [64]. A known diamidine used for the treatment of pneumonia has been found to be associated with low serum folate levels [65]. Several antiepileptic

drugs (AEDs), such as carbamazepine, gabapentin, oxcarbazepine, phenytoin, primidone, or valproate, have been found to be associated with lower serum folate levels [66].

Table 1.1: Potential inhibitors of folate absorption (adapted from Waxman *et al.*, 1970) [65]

Folic acid inhibitor	Usage
a. Dihydrofolate reductase inhibitors	
4-amino-4-deoxyfolate	Chemotherapy, immunosuppression, proraisis
2,4-diaminopyrimidine	Malaria
Triamterene	Diuretic
Pentamidine	*Pneumosystis carinii*
Trimethiprim	Antibacterial
b. Impaired absorption or utilization of folic acid	
Diphenylhydantoin	Prevent and control seizures, antiepileptic
Primidone	Anticonvulsant
Barbiturates	Sedative
Oral contraceptive pill	Contraception

Physiological factors

Several physiological factors influence vitamin B9 status, including an individual's age, smoking, obesity, and others. Age contributes to an increase in the frequency of low or insufficient folate, ranging from 14% in persons in their 50s and 60s to 23% in those over 80, according to a study conducted in Ireland [67]. It is estimated that, among Irish people, one in eight persons over 50 had low to inadequate folate levels [68]. Apart from age, obese and overweight individuals

(BMI > 25 kg/m^2) have a 12% lower folate intake and an 8.5% lower serum folate concentration compared to healthy individuals (BMI < 25 kg/m^2) [69]. Smoking has also been associated with lower folate levels compared to healthy, non-smoking individuals [70]. Increased folic acid usage, urinary excretion, varying amounts in various tissues, and alteration to folate's endocrine roles all contribute to low folate status [71].

1.1.2 Vitamin B12 or cobalamin

1.1.2.1 History and discovery of vitamin B12

Vitamin B12, also known as cobalamin, acts as a co-enzyme for methyl unit transfer reactions. In the mid-19th century, pernicious anaemia led Thomas Addison to discover vitamin B12, which was later confirmed by Minot and Murphy [72]. Their attempts to treat pernicious anemia with either liver or liver extract subsequently prompted other scientists to unravel the chemistry of vitamin B12 [73].

1.1.2.2 Structure of vitamin B12

Vitamin B12 encompasses a group of compounds that share a complex structure known as corrinoid and are interconvertible. This corrinoid family includes all cobalamin compounds, characterized by a common feature containing a four-membered pyrrole ring called the corrin ring, which houses a central cobalt atom (Figure 1.3). The structure of vitamin B12 consists of two parts: the lower part and the upper part. Lower ligands have a base and sugar attached to a phosphate group [74]. The upper ligand exists in several forms, including cyano-, methyl-, deoxyadenosyl- and hydroxy-cobalamin [75]. Depending on these forms, their bioavailability varies. For instance, vitamin supplements are mostly in cyano form, which is more bioavailable, while regular foods contain vitamin B12 in five other forms, such as adenosylcobalamin,

methylcobalamin, hydroxocobalamin, sulphitocobalamin and cyanocobalamin form [76, 77], which are less bioavailable.

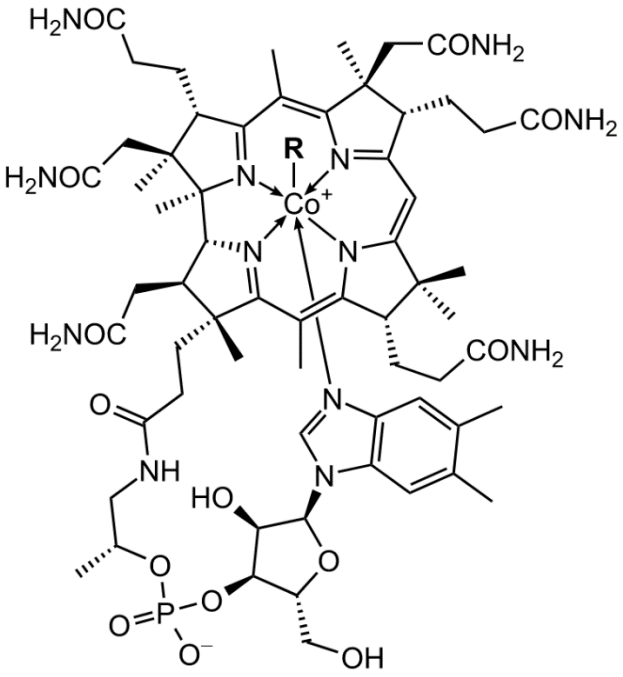

Figure 1.3: Chemical structure of vitamin B12 (Image source: Wikipedia)

1.1.2.3 Digestion, absorption, and metabolism of vitamin B12

The absorption of vitamin B12 takes place in the terminal ileum and varies depending on the type of food intake and increases with the intake of food containing a higher amount of B12 [78]. The absorption efficiency of the intestine of vitamin B12 ranges from 13 to 66%, depending on the type of food and the condition of gut health [79-82]. For example, vitamin B12 found in mutton meat is almost 83% bioavailable, whereas it is lower in liver meat, which is 4.5% [81, 83]. Vitamin B12, bound to protein in foods, needs to be cleaved from the protein using gastric hydrochloric acid to initiate the absorption. This process occurs in the stomach, and free vitamin B12 binds with R-protein, facilitating its transport to the duodenum. Upon reaching the duodenum, the R protein-bound vitamin B12 combines with intrinsic factor (IF). This IF-bound vitamin B12 is absorbed in

the ileum in the presence of calcium [84]. It is estimated that only 1.5 to 2 μg of vitamin B12 can occupy all the IF in the duodenum [76]. Excess vitamin B12 is excreted through feces, including unabsorbed vitamin B12 and bacterially synthesized vitamin B12 in the colon. Daily excretion of vitamin B12 is estimated to be around 0.1-0.5% of the body's stored amount, maintaining a consistent ratio between losses and stores [81]. This vitamin is absorbed in distal ileum where IF bound B12 is recognized by the receptor and this internalize the B12 into the cell by a receptor mediated endocytotic process. At this stage, endosome is generally incorporated with the lysosome and the IF are degraded while B12 is released into cytosol [85]. From cytosol, vitamin B12 is transported by transcobalamins (TC), such as TC-I, TC-II and TC-III [86, 87]. Transporters, specifically TC-II, are clinically significant as TC-II bound vitamin B12 shows the biologically active vitamin B12 that is used in DNA synthesis [88]. The excretion of excess vitamin B12 occurs through urine or feces, with feces being the primary route for excreting the most substantial amount of vitamin B12 [81, 89].

1.1.2.4 Vitamin B12 deficiency

Several factors, including certain drugs, gastritis, and ageing, can increase the risk of developing B12 deficiency [90]. Vitamin B12 deficiency can cause low haemoglobin, hypersegmented neutrophils, neutropenia, macrocytic anaemia, peripheral neuropathy, spinal cord degeneration, and cognitive diseases [91, 92]. The most common symptom of vitamin B12 deficiency is signs of anaemia, including fatigue and pallor. Another prominent sign of B12 deficiency could be jaundice, as a deficiency of vitamin B12 leads towards impaired red blood cell formation [93]. Maternal vitamin B12 deficiency is associated with NTD as well as offspring's brain development, possibly through demethylation [94]. One such example is Wnt signalling in the developing cerebellum in rats, which has been linked with enduring behavioural and memory impairments

due to a deficiency in methyl donors [194, 195]. Maternal vitamin B12 levels during pregnancy can also affect the offspring. In this case, children born to mothers with low levels of B12 during pregnancy have been found to have higher adiposity in the torso and increased insulin resistance, thereby increasing the risk of type 2 diabetes and cardiovascular diseases [196-198].

1.1.2.5 Assessment of vitamin B12 status

Traditionally, the assessment of vitamin B12 status involves measuring its concentration in the serum. However, concerns have been raised regarding the reliability of relying solely on serum measurements to determine vitamin B12 status [95]. Therefore, additional tests, such as transcobalamin-cobalamin complex (Holo) -TC, methylmalonic acid (MMA), and Hcy assays, are often employed to further confirm vitamin B12 deficiency. Evaluating Holo-TC levels is particularly useful when determining the level of vitamin B12 deficiency as they indicate the available B12 for the cells [96]. While MMA only indicates the status of cobalamin metabolism, an increase in Hcy indicates potential deficiencies in vitamin B6, B9 and B12, all of which are essential for the 1C metabolic reaction. Having said that these biomarkers can be influenced by several factors, such as compromised renal function, specific medication, and genomic changes in MTHFR. For instance, polymorphism in MTHFR can elevate Hcy, while impaired renal function can cause increased MMA concentrations in the blood [95, 97], which may or may not be associated with vitamin B12 deficiency. Therefore, the combined assessment of serum vitamin B12 along with subsequent examination of Hcy and MMA values, can provide an accurate approach to determine vitamin B12 status [98, 99].

1.1.2.6. Epidemiology of vitamin B12 status

The epidemiology of vitamin B12 deficiency varies depending on its etiology. Studies have revealed that approximately 1% to 2% of individuals with anaemia suffer from vitamin B12 deficiency. In contrast, among patients with clinical macrocytosis (MCV) less than 100 femtoliters (fL), the percentage of cases associated with vitamin B12 deficiency is higher [14, 100]. It has been established that vitamin B12 level below 0.22 nmol/L, an MMA level greater than 260 nmol/L and a Hcy concentration higher than 12 μmol/L are indicative of vitamin B12 deficiency [101, 102]. B12 deficiency resulting in pernicious anaemia is more prevalent among individuals with Northern European heritage. Conversely, people of African ancestry or those from other areas of Europe have a lower incidence of the disease [103, 104]. The National Health and Nutrition Examination Survey (NHANES) conducted in 2015–2016 revealed that individuals belonging to low-income status, women, and non-Hispanic blacks have the highest likelihood of having inadequate vitamin B12 intake [105]. Approximately 6% of adults under 60 in the United States and the United Kingdom have vitamin B12 deficiency, whereas the prevalence rises to around 20% in individuals over 60 [106]. According to the Canadian Health Measures Survey (CHMS), from 2009 to 2011, only 4% of the population did not have sufficient serum levels of vitamin B12 [107].

1.1.2.7 Factors influencing vitamin B12 status

Several factors including, age, certain drugs, dietary habits, diseases associated with gastrointestinal disorders, and a limited number of genetic defects influence one's vitamin B12 status. Individuals over 65 are often associated with vitamin B12 deficiency, most likely due to reduced gastric acids [108-110]. Certain drugs, including proton pump inhibitors (PPIs), metformin, nitrous oxide anaesthesia, specific antiepileptic drugs, and colchicine, are believed to

disrupt the absorption or metabolism of vitamin B12. PPIs, which are generally used by older people mainly for gastro-oesophageal reflux diseases, reduces gastric acid and ultimately decreases the absorption of vitamin B12 [111-114]. Metformin, a well-known drug for diabetes, has been found to cause megaloblastic anaemia, a disease related to a deficiency of vitamin B12 [115, 116].

Additionally, inadequate dietary intake, alcohol intake, malabsorption of food-bound cobalamin, and damaged intestine due to any disease or condition such as celiac disease, Crohn's disease, and others can also influence the vitamin B12 status. Individuals who follow a vegan or vegetarian diet, frequently suffer from vitamin B12 deficiency if not taking any supplement [107]. As this vitamin is primarily absorbed in the terminal ileum, patients with a history of gastric bypass surgery, damage in small intestine, pancreatic disease, *Diphyllobothrium latum* infection, and celiac disease have a higher risk of vitamin B12 deficiency [91, 117, 118]. A genetic defect disease called Imerslund-Gräsbeck, is known to cause vitamin B12 deficiency due to malabsorption. Moreover, mutations in IF, gene-producing TC-transporter and genes involved in vitamin B12 metabolism C677T gene are also linked to vitamin B12 deficiency [119-121]. Folate, vitamin B12 and 1C metabolic pathway

1.1.3 Folate, vitamin B_{12} and 1C metabolic pathway

The 1C metabolism is a pathway nestled with folate and methionine metabolism, where the 1C unit is produced. This universal reaction serves to activate and transfer of 1C units for biosynthetic processes. Vitamins such as folate and vitamin B12 play a crucial role in transferring 1C units from one intermediate to another, facilitating the synthesis of purines and thymidine, as well as other enzymatic reactions such as transmethylation and remethylation [12]. These integrated

metabolic reactions can be categorized into three major pathways: the folate cycle, transmethylation, and remethylation.

In the **folate cycle**, folate facilitates the binding of 1C units to nitrogen atoms at the 5-position of the pterdine ring and the 10-position of the para-aminobenzoic acid (PABA) moiety within tetrahydrofolate (THF). Since 1C-loaded folates do not move across intracellular membranes, producing 5,10-methylene-THF is necessary in the mitochondria and cytosol [122]. This process begins with the reduction of folate into dihydrofolate (DHF) and subsequently into THF by DHF reductase using NADPH as an electron donor (Figure 1.4). THF is then transformed into 5,10-CH_2-THF by acquiring the 1C unit from the conversion of glycine into serine [123]. The conversion occurs based on the demand for these amino acids in cytosol or mitochondria [124], facilitated by vitamin B6-dependent enzyme serine hydroxymethyltransferase (SHMT) [12]. Further along the cycle, 5,10-CH_2-THF is converted into either 10-CHO-THF by methylenetetrahydrofolate dehydrogenase (MTHFD, EC 1.5.1.5) or 5-CH_3-THF by enzyme methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20). This conversion occurs in conjunction with the enzyme thymidylate synthase (TYMS, EC 2.1.1.45), which converts dUMP into deoxythymidine monophosphate (dTMP) using 5,10-CH_2-THF [12]. The conversion of 5,10-CH_2-THF from THF is a reversible process, involving the addition or removal of the methyl group. The final step in the folate cycle is the removal of methyl group from 5-CH_3-THF [125], which requires vitamin B12 as a co-enzyme for MS (Figure 1.4) [126].

In the **transmethylation pathway**, S-adenosylhethionine (SAM) is synthesized from methionine by methionine adenosyl transferase (MAT, EC 2.5.1.6) [127]. In the next step, SAM reacts with glycine in the presence of the enzyme glycine N-methyltransferase (EC 2.1.1.8) to produce S-adenosylhomocystein (SAH). This reaction releases one methyl group and sarcosine. At the same

time, S-adenosylhomocysteine hydrolase (EC 3.3.1.1) initiates a reversible reaction resulting in the production of Hcy and release of an adenosyl group [128-130]. Sarcosine further converts back to glycine catalyzed by mitochondrial sarcosine dehydrogenase (EC 1.5.8.3), releasing one partially oxidized methyl group that enters the folate cycle [131].

The **remethylation pathway** occurs by converting Hcy back into methionine [131]. This pathway is controlled by two enzymes, MS and betaine–homocysteine *S*-methyltransferase (BHMT, EC 2.1.1.5) [132, 133]. These two enzymes utilize methyl groups from folate co-enzyme pool or betaine [131]. BHMT uses betaine as a substrate for donating methyl group, converting Hcy into methionine, and producing dimethylglycine as a byproduct [134]. Conversely, MS, coupled with vitamin B12, produces methionine by utilizing the methyl group from 5-CH_3-THF from the folate cycle of the 1C metabolic reaction [131]

As such, the 1C metabolic cycle is also involved in maintaining sulphur-containing amino acids such as methionine and Hcy. In the case of inadequate vitamin B12 supply, folate becomes trapped in the form of 5-methyltetrahydrofolate, obstructing the restoration of methionine. This phenomenon is referred to as a ‘methyl folate trap’ and consequently increases the levels of Hcy and its byproducts, which are toxic for the cells [135]. Impairment in the 1C pathway and sulphur amino acid metabolism may contribute to the onset of various diseases, including cardiovascular disease, cancer, and neurodegenerative diseases such as Alzheimer’s disease [134].

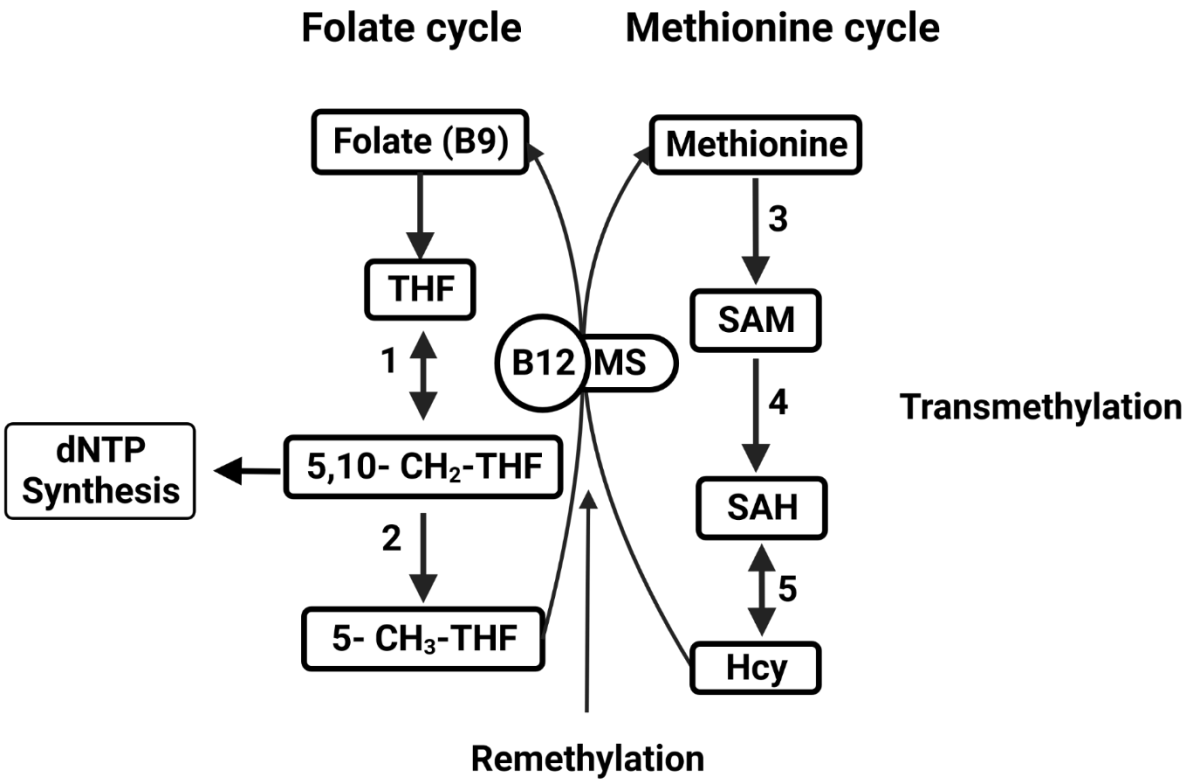

Figure 1.4: Role of folate and vitamin B12 in 1C metabolic reaction. Enzymes involved in this reaction are as follows:

1. Serine hydroxymethyl transferase (coenzyme vitamin B6, EC 2.1.2.1): Folate accepts the 1C unit from serine residue, converts it into glycine, and produces 5,10-CH_2-THF.
2. Methylene-THF reductase: Using $FADH_2$ and NADPH, 5,10-CH_2 is converted into 5-CH_3-THF by reducing FAD and NADP.
3. Methionine adenosyl transferase: Using ATP as an energy source, this enzyme converts methionine into SAM with the expense of pPi+Pi.
4. Glycine N-methyl transferase: This enzyme converts glycine into methylated glycine or sarcosine.
5. S-adenosyl homocysteine hydrolase: Initiates a reversible hydration reaction of SAH, converting it into adenosine and Hcy.

1.1.4 The 1C metabolism in nucleotide synthesis

The 1C metabolism is involved in DNA synthesis through three distinct pathways (Figure 1.5). In the first pathway, 10-formyl-THF donates two carbons to purine by converting folate into 5,10-methylene-THF (Figure 1.5 A) [136]. In the second pathway, 5,10-methylene-THF donates a methyl group, which is then used in the formation of uracil. Subsequently, uracil is converted into thymidine for DNA synthesis (Figure 1.5 B) [137]. In the third pathway, 5-methyl-THF provides the methyl group to methionine, which converts methionine into SAM. SAM contributes a methyl group to cytosine, thereby regulating gene expression (Figure 1.5 C) [138]. Hcy can be either utilized in trans-sulphuration pathway to produce cysteine or be converted back to methionine by MS with vitamin B12 as coenzyme [139].

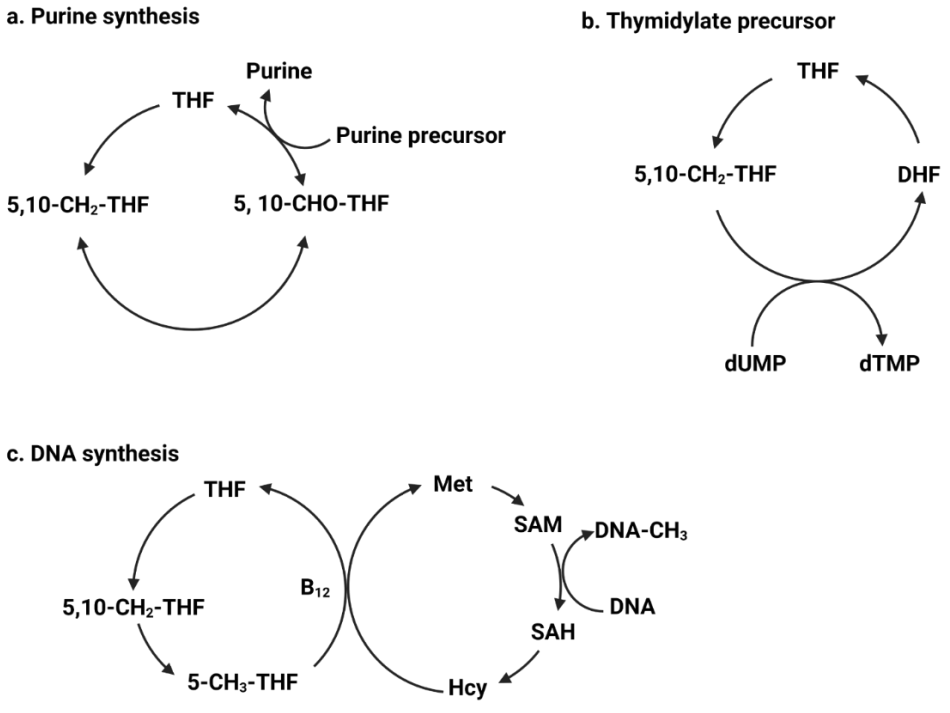

Figure 1.5: Involvement of folate and vitamin B12 in DNA synthesis. THF, tetrahydrofolate; DHF, dihydrofolate; 5,10-CH_2-THF, methylenetetrahydrofolate; 10-CHO-THF, formyltetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; DNA-CH3, methylated DNA; dUMP, deoxyuridylate; dTMP, thymidylate; Met, Methionine; and Hcy, Homocysteine.

1.1.4 Gut Microbiota

The human gastrointestinal (GI) tract serves as a significant interface spanning 250 to 400 square meters, connecting to its host and various surrounding environments within the body [140]. Approximately 100 trillion microbes, encompassing bacteria, fungi, archaea and protozoa, reside in the human gastrointestinal tract [141, 142]. These microbes play an integral role in human health by bolstering the immune system, maintaining metabolic homeostasis, and modulating neurological functions [143-146]. Gut bacteria ferment indigestible substances, such as dietary fibers, to produce short-chain fatty acids (SCFA), while also synthesizing B vitamins, neurotransmitters, and antimicrobial compounds [147]. Maintaining bacterial homeostasis is crucial, as impairment in the composition of these gut bacteria, known as gut dysbiosis, disturbs the production of these vital molecules. In fact, gut dysbiosis has been shown to be associated with several chronic diseases, including obesity, cardiovascular disease, inflammatory bowel disease (IBD), diabetes, coeliac disease (CD), and Alzheimer's disease [148].

1.1.4.1 Role of gut microbiota

The gut microbiota maintains a symbiotic relationship with its host. It extracts nutrients from the diet and can modulate our metabolism, including nutrient absorption, energy production, as well as the synthesis of several vitamins, neurotransmitters, and bioactive compounds. As a result, it operates as a distinct organ that possesses a wide range of metabolic capabilities and excellent adaptability [149, 150]. The section below provides an overview of functions performed by gut microbiota.

Nutrient metabolism

The primary source of energy for gut microbiota is dietary carbohydrates. Fermentation of indigestible carbohydrates produces SCFA, such as acetate, butyrate, and propionate [151]. Several

bacteria, such as *Bacteroides, Roseburia,* and *Bifidobacterium,* perform this fermentation process [152, 153]. These SCFAs are essential for host metabolism, providing energy to the colon, skeletal muscle, heart, and brain [154, 155]. In addition to producing SCFA, gut microbes aid in amino acid conversion [156]. Gut bacteria not only help with nutrient metabolism but also synthesized micronutrients like vitamins. Approximately 40-60% of the human gut microbiota can produce eight B vitamins in the gut [5, 157]. Several genera of bacteria, including *Bifidobacterium*, *Bacteroides*, and *Enterococcus,* are commonly known to produce vitamin K and B vitamins in the gut [147, 158, 159].

Antimicrobial protection

The gut microbiota consists of both beneficial and pathogenic bacteria, and maintaining its homeostasis is essential for a healthy gut. The mechanism of antimicrobial action differs between the small and large intestines. In the large intestine, two layers of mucus prevent damage of the luminal cells from pathogenic bacteria [160]. The inner layer is generally germ-free, whereas the outer layer hosts beneficial microbes and provides nutrients to the gut microbiota [161]. On the other hand, the small intestine has a much thinner mucus layer, resulting in lesser physical protection of the gut from pathogenic bacteria. Instead, antimicrobial proteins produced by the beneficial bacteria could play a significant role in protecting against pathogens [162, 163]. Several microbes can synthesize antimicrobial proteins, such as cathelicidins, C-type lectins, inside the intestine, thus protecting it from pathogenic microbes [164, 165]. Additionally, certain gut microbes, like *Lactobacillus,* can produce lactic acid inside the intestine, which acts as an antimicrobial agent [166].

1.1.4.2 Vitamin B9 and gut microbiota

The human body cannot synthesize vitamins on its own; therefore, dietary intake is the primary source of such micronutrients. That being said, gut microbiota can contribute to a certain amount of vitamins, as a selected number of bacteria can produce specific vitamins including, vitamin B9 [147]. Approximately 13.3% of human gastrointestinal bacteria can synthesize B9 *de novo*, whereas 39% can produce this vitamin if pABA, a structural component of folate, is provided [167]. Magnúsdóttir *et al.*, predicted that 26% Actinobacteria, 71% Proteobacteria, 79% Fusobacteria, and 92% Bacteroidetes can produce vitamin B9 in the gut [5].

Gut microbiota not only helps us produce vitamins in the gut, but the non-producers also require these vitamins for their metabolic reactions [16, 167]. These bacterial cells also use folate to perform a variety of biosynthetic processes. Although such cellular reactions are universal, their certain biosynthesis pathways may differ from host to host. Organisms such as fungi, plants, bacteria, and some specific archaea use a similar folate biosynthetic pathway with slight modifications [168]. Several bacteria can produce folate in the gut, including *Bacteroides fragilis*, *Prevotella copri*, *Clostridium difficile, Lactobacillus plantarum, L. reuteri, L. delbrueckii* spp. *Bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium* spp (some species), *Fusobacterium varium,* and *Salmonella enterica*. Bacterial species such as *Bifidobacterium* are well studied and have been categorized based on their folate-producing ability as: high folate producers such as *Bifidobacterium bifidum* and *B. longum* subsp. *Infantis* and low folate producers such as *B. breve, B. longum* subsp. *Longum* and *B. adolescentis* [169]. Folate synthesis requires pABA and 4-amino-4-deoxychorismate lyase (EC 4.1.3.38). This enzyme can be found only in the genome of *B. adolescentis* and *B. dentium* Bd1 as these two can *de novo* biosynthesis of pABA [170]. Another common group of folate-producing bacteria is *Lactobacilli*. Like *Bifidobacterium, Lactobacillus*

also requires the pABA for folate production. Several *Lactobacillus* species, for example, *L. plantarum*, *L. sakei*, *L. delbrueckii*, *L. reuteri*, *L. helveticus*, and *L. fermentum* can produce 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and can synthesize folate in the presence of pABA [171]. However, this is not required for other folate-producing genus such as *Lactococcus* and *Streptococcus* [172].

1.1.4.3 Vitamin B12 and gut microbiota

Vitamin B12 is a unique vitamin not found in plants but extensively synthesized by bacteria and archaea [173]. Bacteria use either bacterially synthesized corrinoids or from diets to synthesize this vitamin in the gut; however, a limited amount of bacterially synthesized vitamin B12 is available to the host. This is because the site of production, mostly in colon where microbial diversity is highest, differs from the primary site of absorption i.e. the small intestine. [174]. This vitamin requires IF to be absorbed and this is found primarily in the small intestine [175]. Some gut microbes use the bacterially produced vitamin B12, and ~80% of unused B12 are converted to vitamin B_{12} analogs (mostly cobamides) by transforming them into benzimidazole derivatives [89, 176]. This gives the gut bacteria the advantage of using vitamin B12 or vitamin B12 analogs for their metabolic reactions [174, 177, 178].

The biosynthesis of vitamin B12 by microorganism is made possible due to the presence of almost 30 genes that can function in either aerobic or anaerobic pathways. [179]. Interestingly, *Lactobacillus* spp. does not have the *de novo* biosynthetic pathway of vitamin B12. However, when it converts glycerol into propanediol, it utilizes a pathway that can produce this vitamin [157]. Some other microbes produce vitamin B12 from *precorrin*-2, which is converted into adenosylcobalamin but it can not be absorbed into the body at the large intestine [5]. Some vitamin B12 producing bacteria include *Bacteroides fragilis, Prevotella copri, Clostridium difficile,*

Faecalibacterium prausnitzii, Ruminococcus lactaris, Bifidobacterium animalis, B.infantis, B.longum, Fusobacterium varium [157].

1.1.4.4 Bacterial 1C metabolic pathway

Bacteria also possess the universal 1C metabolic pathway; however, some of the downstream metabolic reactions may differ from those of humans. In bacteria, amino acids play an essential role in the aminoacylation of tRNA [180, 181], where methionine is used for regulating steps. During this process, N-terminal methionine is removed by methionine-aminopeptidase (MAP, EC 3.4.11.18) [182, 183]. The level of methionine is indirectly regulated by the Cys level and thus affects the changes in tRNA [184, 185]. Additionally, 10-CHO-THF, a methyl donor of 1C metabolism, is involved in the formylation of methionine-tRNA in the presence of the deformylase enzyme (EC 3.5. 1.31) [186].

1.1.5 Metabolomics: a tool to study nutritional and microbial metabolites

Modern nutritional research focuses on enhancing our understanding of the impact of nutrients and food ingredients on human health [187]. Exploring new biomarkers and pathways represents an encouraging step forward in nutritional biochemistry [188]. Existing biomarkers provide valuable insights into the consequences of vitamin deficiencies on metabolic processes [189]. However, innovative techniques and approaches may be required to gain a deeper understanding of the potential pathways affected by nutrient deficiencies. Metabolomics presents an opportunity for such exploration [190].

1.1.5.1 Metabolomics

Metabolomics is the study of small molecule compounds of biological origin, known as metabolites. It aims to identify and quantify metabolites present in a complex biological sample,

such as blood, serum, plasma, tissues, and others [191]. It can be regarded as an approach for visualizing the biochemical activities within the body [192]. Studying metabolites is highly useful as it uncovers valuable biological insights into molecules linked to biological outcomes and their potential involvement in various human diseases [193]. Metabolomics is also instrumental in identifying important biomarkers that can pave the way towards the discovery of treatment of diseases [194].

1.1.5.2 Methodology in metabolomics

Metabolomics can be performed on different samples in different approaches and analytical platforms [193]. The method can be categorized into targeted and non-targeted approaches. The targeted approach is employed to quantify the known metabolites present in the specific sample, while untargeted approach is used to identify and quantify all the metabolites present in the sample, including the unknown metabolites. Common platforms used in metabolomics studies include gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic Resonance (NMR) spectroscopy [195]. One of the most popular platforms for untargeted analysis is the use of high-resolution LC-MS-based technology. In this platform, all possible metabolites or features are collected in the form of mass-to-charge ratios (m/z). The spectrum is then analyzed based on the interest of the specific metabolite/feature, enabling the identification of the compound. Characterization and identification can also be done by searching through the existing databases [196-198].

1.1.5.3 Metabolomics in the study of vitamin B9 and B12

Metabolomics is being used to study the effect of dietary molecules on human health and disease [191]. Studying nutritional metabolomics, sometimes also known as nutrimetabolomics, has been proposed to study personalized nutrition [199]. A targeted metabolomics approach has recently

been employed to simultaneously detect nine metabolites of the 1C metabolic reaction, providing crucial information on how these metabolites change in biological samples subjected to vitamin B9 and B12 deficiencies [200].

The human gut consists of 10^{15} microbial cells, producing several metabolites in the gut [201]. These microbes can also produce various enzymes primarily to support the fermentation of various indigestible substrates, such as dietary fibre [202]. This capability results in the production of a wide range of products that can be broadly divided into following groups: 1) metabolites produced from dietary fibre, and 2) metabolites produced from bile acid [203]. The most extensively studied microbial metabolites are SCFAs. The major SCFAs produced by gut microbiota are acetate, butyrate and propionate, constituting around 95% of total SCFAs [151, 204]. Acetate is primarily produced by enteric microbes such as *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Prevotella* spp., *Ruminococcus* spp., and *Streptococcus* spp. Common propionate producing bacteria are *Bacteroides* spp., *Dialister* spp., *Salmonella* spp., *Veillonella* spp. and, butyrate producing bacteria are *Roseburia* spp., *Clostridium leptum, Coprococcus catus* [205-207]. Most SCFAs are produced from indigestible carbohydrates, primary dietary fibre and a small amount from certain proteins and peptides that contain branched-chain amino acids (BCAAs). Protein and peptide-derived BCAA are generally converted to fatty acids such as 2-methylbutyrate and iso-valerate [208]. The absorption of SCFAs takes place in the colon of the large intestine using either hydrogen-dependent or sodium-dependent monocarboxylate transporters [209]. Once absorbed, they are used as an energy source in the colon; the rest goes into blood circulation [210].

1.3 Conclusion

Micronutrients, especially water-soluble vitamin such as vitamin B9 and B12 play critical role in various metabolic processes in human body. 1C metabolism is one of these processes and plays a

pivotal role in the interplay between folate and methionine metabolism, where 1C units are produced and transferred to support essential biosynthetic pathways. The 1C metabolic pathway and its intricate network of enzymatic reactions are crucial for DNA synthesis, gene expression regulation, and the maintenance of sulfur-containing amino acids. B vitamins, specifically B9 and B12, are an integral part of 1C metabolism and are of particular interest because humans do not have the capacity for de novo synthesis. Thus, they must be obtained through dietary intake or microbial activity. However, vitamins produced by gut microbiota are not sufficient to meet daily requirements of the host, making the diet the primary source of these vitamins. Consequently, deficiencies in these micronutrients in the diet will alter the 1C pathway, leading towards metabolic diseases. Although deficiencies of these two vitamins are not very common in developed countries, they appear to be a global concern, specifically in developing countries or in association with certain health conditions and in older people. Even though the interaction between diet and host is well established, the impact of micronutrient deficiency on the host is poorly understood. As such, more research is needed to shed the light on how micronutrient deficiency affects gut health and its ecology.

CHAPTER TWO

Rational, hypothesis and objective

2.0 Study rationale

B vitamins, specifically vitamin B9 and B12, are involved as a cofactor in 1C metabolism. Folates provide the crucial intermediate THF required for the folate cycle. This metabolic process is vital as it provides 1C groups for numerous enzymatic reactions, including the synthesis of purines and pyrimidines as well as oxidative reactions. [12]. Specifically, these micronutrients are essential for providing the formyl group required for DNA synthesis and for the conversion of methionine to SAM by MAT enzyme in the methionine cycle [14]. However, the precise impact of dietary deficiency of these crucial vitamins on the growth and ecology of gut microbes remains unknown. Most of the human gut microbiota requires corrinoids, the core nutrient for vitamin B12 synthesis, for their growth [178]. Similar to humans, microbial 1C metabolism is also folate and vitamin B12 dependent [211]. Therefore, we hypothesized that deficiency of these vitamins will alter the 1C metabolic pathway, which is critical not only for various metabolic reactions in the host but also for the bacteria colonizing in the gut. Consequently, these deficiencies will affect the growth and proliferation of gut microbiota and, as a result, its composition. This imbalance in the gut ecosystem will lead to changes in the production of various gut metabolites, ultimately affecting gut homeostasis.

2.1 Hypothesis

A deficiency in either vitamin B9 or B12 will impair the composition of intestinal microbiota and gut metabolites, potentially disturbing the balance of gut homeostasis.

2.2 Overall aim and specific objective

The overall aim of the work presented in this thesis was to investigate the impacts of dietary deficiencies in B vitamins, specifically B9 and B12, on 1C metabolic reactions, intestinal morphology, gut microbiota, and gut metabolites. The study focused on the following objectives.

2.2.1 Objective 1

To investigate the effects of dietary vitamin B9 and B12 deficiencies on physical parameters, such as body weight, organ weights, intestinal length, colon morphology, and mucin production.

2.2.2 Objective 2

To investigate the effects of dietary vitamin B9 and B12 deficiencies on the composition of gut microbiota.

2.2.3 Objective 3

To investigate the effects of dietary vitamin B9 and B12 deficiencies on serum metabolites linked to 1C metabolic pathway using targeted and untargeted metabolomics.

CHAPTER THREE

Investigating the roles of B vitamins and one-carbon metabolism in gut homeostasis

3.0 Abstract

Vitamins are the organic compounds essential for normal growth and metabolic processes in all living organisms. B vitamins, such as vitamin B9 and B12, act as cofactors in various metabolic reactions, including one-carbon metabolism (1C), and are typically required in trace amounts. A diverse array of bacteria reside in the gut, and some of these microbes have the capacity to synthesis specific vitamins. These bacteria also require certain vitamins for their growth, with requirements varying between vitamin-producing and non-producing strains. Despite the well-understood functional role of B vitamins, their impacts on gut microbial growth and the production of microbial metabolites remain poorly understood. The current study aimed to investigate the impacts of vitamin B9 and B12 deficiencies on gut morphology, microbial composition and their metabolites. A total of 48 Sprague-Dawley rats were fed either a control diet (n=15), a low B9 diet (LB9, n=16), or a low B12 (LB12, n=16) diet for six weeks. Body weights were measured weekly, and at the end of the study, blood, tissue, and fecal samples were collected for various biochemical analyses. Our results revealed significant sex effects on body weight, liver weight and brain weight. Upon separating the sex, we did not find any significant treatment effects. Serum vitamin levels were significantly lower ($p<0.001$) in the respective deficient groups compared to the control group, as expected. However, the serum metabolites of 1C metabolic reactions did not show significant differences in the deficient groups compared with the control. Histological analysis showed a significant longer in mucosal height in the LB12 group ($p=0.006$) compared to the control group. Although there were no significant differences in the total mucin content in the colon, we observed a notably lower percentage of acidic mucin ($p = 0.02$) in both LB9 and LB12 rats when compared to the control rats. Of particular interest, we observed sex-dependent differences in the impacts of vitamin deficiencies on the gut microbial profile. Vitamin B9

deficiency altered the abundance of various bacterial genera in males, including *Colidextribacter, Dorea, Lactobacillus,* and *Streptococcus,* while *Blautia* was altered in females. Similarly, vitamin B12 deficiency altered *Dorea*, *Streptococcus* was altered in males, and *Blautia, Lachnoclostridium* and *Colidextribacter* in females. Furthermore, fecal metabolite analysis revealed that concentrations of tryptophan and histidine were lower in LB9 compared to the control, whereas hydrocinnamic acid, ethylmethylacetic acid, N,N-diacetylsperodine contents were higher in LB9 group, while N-acetylneuraminic acid was higher in LB12 group. This study provides evidence that deficiencies of vitamin B9 and B12, which are critical for 1C metabolism, may exert sex-dependent effects on gut morphology, gut microbial composition, and associated metabolites.

Keywords: vitamin B9, B12, deficiency, gut, microbiota, metabolites

3.1 Introduction

Micronutrients such as vitamins play crucial roles in diverse metabolic processes [1]. B vitamins, specifically vitamin B9 and B12, function as co-enzymes and methyl donors in one-carbon (1C) metabolism [11]. This shared biological pathway is integral to various physiological reactions, including nucleotide synthesis, DNA methylation, and redox balance [9, 10]. Disruption to this pathway and its associated metabolites are linked to the development of numerous diseases, including cancer, cardiovascular disease, Alzheimer's disease and others [12, 212].

While the roles of diet and dietary factors in regulating gut microbial composition are well-known, the impact of micronutrient deficiencies on gut ecology remains understudied [3]. In the gut, nearly 100 bacterial species can produce some of the B vitamins and exchange them symbiotically to ensure survival [5]. Biosynthetic pathways for vitamins can vary among species, highlighting the existence of genetic variations [157]. However, mutual symbiosis between the gut microbiota and the mammalian gut has shaped interdependence between gut and gut microbes. The microbiota's ability to produce essential substances, including vitamins, is significantly influenced by the host environment, including availability of substrates. For instance, folate can be produced by several bacterial species, including *Bacteroides fragilis*, *Lactobascillus plantarun*, and *Bifidobacterium spp* by utilizing para-aminobenzoic acid (pABA) [5, 157]. Whereas bacteria like *Pseudomonas denitrificans* and *Bacillus megaterium* use bacterially synthesized precorrin-2 for vitamin B12 synthesis [5]. Furthermore, various taxa have evolved to fulfill specific roles in relation to the synthesis and utilization of vitamins [7]. Among all the vitamins synthesized in the gut, riboflavin and niacin are the most commonly produced vitamins [5]. Despite the gut microbiota's ability to produce these vitamins, several reasons, such as the production site and competition between host

and non-producing bacteria, render the vitamin unavailable or less available to the host [5]. This results in dependency on the dietary source to meet daily micronutrient requirements [13].

Severe deficiencies of these micronutrients are rare in developed countries, but moderate deficiencies are still observed [2]. The prevalence of folate deficiency is under 5% in developed countries but much higher in developing countries (closer to 20%) [56]. Although the folate deficiency is limited in healthy individuals, Canadian Health Measures Survey (CHMS) indicated that 22% of child bearing women showed folate deficiency regardless of their age [57]. On the other hand, the epidemiology of vitamin B12 deficiency varies depending on its etiology. Studies revealed that approximately 1-2% of individuals with anemia suffer from B12 deficiency. Around 5% of adults under the age of 60 in Canada, the United States, and the United Kingdom have vitamin B12 deficiency, while the prevalence rises to around 20% in individuals over the age of 60 [106, 107].

Although severe deficiencies of B9 and B12 are not common, when specific health conditions such as pregnancy, physical and metabolic stress, or certain diseases is involved, even moderate deficiencies can become problematic. These health conditions increase the requirements of B9 and B12, particularly folate, compared to normal conditions [213-216]. Moreover, such conditions are always associated with changes in gut microbiota [217]. Therefore, it becomes important to study how varying levels of vitamin B9 and B12 affect our metabolic processes in the body, particularly gut health. It is highly likely that micronutrients, such as vitamin B9 and B12, play a crucial role in modulating the composition and metabolic capacity of the microbiota. Thus, in the current study, we investigated the influence of dietary vitamin B9 and B12 on gut homeostasis, focussing on gut morphology, microbiota composition, and microbial metabolites using a B9 and B12 deficient rat model.

3.2 Material and method

3.2.1 Animals and diet

A total of forty-eight Sprague–Dawley rats of both sexes (24 females and 24 males) were obtained from Charles River Laboratories. The animal protocol was approved by the Institutional Animal Care Committee at Memorial University of Newfoundland (MUN) and adhered to the guidelines of the Canadian Council on Animal Care. The 4-week-old animals were housed in pairs of the same sexes in a humidity-controlled room maintained at 22˚ C with a reverse 12h light-dark cycles. After a one-week acclimatization period fed with a standard chow diet, they were randomly assigned in a pair of same sexes to AIN-93G-based three diet groups: control group, vitamin B9 deficient group (LB9) or vitamin B12 deficient group (LB12). The diets were purchased from Dyets Inc. (Bethlehem, PA, USA). The control diet is the AIN-93G diet, the LB9 diet is a modified AIN-93G-based diet without added vitamin B9 in Vitamin Mix # 318258, and the LB12 diet is modified AIN-93G-based diet without added vitamin B12 in Vitamin Mix # 318258. Both the LB9 and LB12 diets contains traces of vitamin B12 (0.004mg/kg) and vitamin B9 (0.04mg/kg) from casein. The diet composition is displayed in Table 3.1.

At baseline, 4th, and 6th week, fecal samples were collected using autoclaved cages and immediately transferred to dry ice before being stored it at -70˚C. The animals were fed *ad libitum* for six weeks with their respective diets. Food intake was measured every three days, and body weight was measured weekly. After six weeks, the rats were fasted for 8 hours and euthanized under 1-1.2 L oxygen /min mixed with 4-5% of isoflurane. Blood was collected via cardiac puncture and centrifuged at 4000 × g for 10 minutes to separate the serum, which was subsequently stored at -80˚ C until further analyses. All tissues, including brain, liver, kidney, adipose, gut, and

muscle, were collected using the snap-freezing method with liquid nitrogen and stored at -80˚ C for further use. Cecal contents were also collected and stored it at -80˚ C for further analyses.

One male in control group died unexpectedly, thus all statistical analyses were conducted on samples collected from forty-seven samples. Some cecal sample were lost during collection, and this is indicated whenever appropriate.

Table 3.1: AIN-93G diet composition

Ingredients	Control diet (g/kg)	B9 deficient diet (g/kg)	B12 deficient diet (g/kg)
Casein	200	200	200
L-Cystine	3	3	3
Sucrose	100	100	100
Cornstarch	395.4	395.4	395.4
Dyetrose	132	132	132
Soybean Oil	70	70	70
t-Butylhydroquinone	0.01	0.01	0.01
Cellulose	50	50	50
Mineral Mix #210025	35	35	35
Choline Bitartrate	2.5	2.5	2.5
Vitamin Mix # 318258 (no B12 or Folate)	10	10	10
Folic Acid Premix 1mg/gm	2	--	2
Vitamin B12 (0.1%)	2.5	2.5	--

3.2.2 Vitamin B9 and B12 analysis

Serum samples were shipped on dry ice to the Nutritional Biomarker Laboratory, University of British Columbia (UBC), for folate and B12 analyses. Serum total vitamin B12 concentration were quantified on fully automated Abbott Architect Immunoanalyzers. Forty-seven samples were measured on the Architect i1000SL (Abbott Laboratories, USA) at BCCHRI Analytical Core for

Metabolomics and Nutrition. Serum B9 concentration was measured using a microbiological folate assay, employing chloramphenicol-resistant *L. casei* as a test microorganism, as previously described [218, 219].

3.2.3 Histological analysis

3.2.3.1 Hematoxylin & eosin (H&E) Staining

A 0.5 mm-thick section of proximal colon was excised and washed with PBS to remove any unwanted fecal matter. Subsequently, it was placed in 10% neutral buffered formalin and stored for histological analysis. The samples were prepared by the Histology Laboratory, Faculty of Medicine, MUN and visualized using a Zeiss Axiostar Plus Microscope with a OMAX A35180U3 18MP camera. Muscle thickness, mucosal height, and other histological parameters were measured using OMAX ToupView (ver 4.11) software by two individuals who were blinded to the treatment groups. The average reading of the two individuals have been used for data analysis. The detailed histological protocols are given in Appendix I.

3.2.3.2 Periodic acid–Schiff and Alcian Blue staining

Periodic acid-Schiff (PAS) and Alcian blue (AB) staining were conducted with and without diastase on the colon section to stain to distinguish different types of mucins. PAS-AB with diastase staining used were as a control for proper staining. Slides were prepared by Histology Laboratory, Faculty of Medicine, MUN. Acidic mucin stained blue, neutral mucin stained magenta and a combination of both mucins stained as deep purple. The slides were analyzed using Olympus BX50WI upright biological microscope with a SC50 Olympus 5-megapixel digital color camera and an Olympus uPlanSApo 40x/0.95na lens. Images were captured using CellSens Standard software. Mucin quantification was performed using ImageJ software and color deconvulation2

plugin. Mucin contents were expressed in the percentage of total mucin, acidic and neutral mucin. The detailed histological protocols are given in Appendix I.

3.2.4 Gut microbiota sequencing

Cecal samples were homogenized utilizing a bead-beating homogenizer (MP Biomedicals, Lachine, QC, Canada), and genomic DNA was extracted using a FastDNA spin kit (MP Biomedicals, Lachine, QC, Canada). The microbial composition was determined by using Ilumina's 16S rRNA gene sequencing of the V3-V4 regions. DNA quantification was performed on a Thermo Nanodrop Spectrophotometer. For samples with low DNA content and high salt content, de-salting was carried out until a minimum of 10 ng/uL of DNA sample was obtained. The samples were stored at -80˚ C before being sent on dry ice for sequencing at Integrated Microbiome Resource (IMR), Dalhousie University, NS. Sequencing primers and low quality reads were removed using Trimmonatic version 0.39 [220]. A table of amplicon sequence variants (ASVs) was generated using DADA2 version 1.28.0 and Silva V138 database using R platform version 4.2.1 [221, 222]. Statistical analysis for the microbial data was performed using MicrobiomeAnalyst version 2.0. Raw sequence data is available upon request. The detailed bioinformatics procedure is given in Appendix II.

3.2.5 Metabolomics analysis

3.2.5.1 Targeted serum 1C metabolites analysis.

Serum targeted 1C metabolites were measured using an LC-MS/MS-based analytical platform with the use of isotope-labeled internal standards at the Metabolomics Innovation Centre, Edmonton. The analysis also included one blank, three zero samples, six standards (serine, glycine, glutamic acid, homocysteine, methionine and betaine), and three quality control samples. Samples

were thawed on ice, vortexed and centrifuged at 13,000x g for 10 mins. Subsequently, 10 µL of each sample was dried in a stream of nitrogen, and phenyl-isothiocyanate was added for derivatization. After incubation, the filtered spots were dried again using an evaporator. The derivatized metabolites were extracted by adding 300 µL of extraction solvent. The extracts were centrifuged and, if necessary diluted. Mass spectrometric analysis (MS/MS) was conducted using an ABSciex 4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1290 series UHPLC system (Agilent Technologies, Palo Alto, CA).

3.2.5.2. Untargeted fecal metabolomics

Fecal metabolomics was performed at The Britz-McKibbin Laboratory, McMaster University. All chemical standards and calibrants were purchased from Sigma-Aldrich (St. Louis, MO, USA), including analytical grade amino acids, acylcarnitines, ammonium acetate, ammonium bicarbonate, ammonium hydroxide, formic acid, organic acids, sodium hydroxide and recovery/internal standards: 4-aminobutyric acid-2,2,3,3,4,4-d6 (GABA-d6), choline-d9, 4-fluoro-*L*-phenylalanine (F-Phe), 3-chloro-*L*-tyrosine (Cl-Tyr), fluoro-tyrosine (F-Tyr), trimethylamine-d9 N-oxide (TMAO-d9), creatinine-d3, $^{15}N_2$-ornithine, ^{15}N-histidine, carnitine-d3, methylhistidine-d3, naphthalene monosulfonic acid (NMS) and D-glucose-$^{13}C_6$ (^{13}C-glucose). All LC-MS grade solvents, including acetonitrile, isopropanol, methanol, and water, were obtained from Caledon Laboratories Ltd (Georgetown, ON, Canada). Instrumental conditions and data pre-processing steps were described previously [223, 224]. Dry stool pellets (10-15 mg), including a pooled quality control (QC) sample, were homogenized. A 200 µL of solution containing 4 µM of recovery standards in 70% MeOH was added to sample vials. After vortexing, the supernatant was dried using nitrogen gas and reconstituted in 40 µL of 70% MeOH containing internal standards (IS).

Untargeted analysis was conducted using an Agilent 6230B time-of-flight (TOF) mass spectrometer with an electrospray ionization (ESI) source equipped to an Agilent G7100A capillary electrophoresis (CE) unit was used for all experiments (Agilent Technologies Inc., Mississauga, ON, Canada). The detailed procedures are given in Appendix III.

3.2.6 Statistical analysis

All statistical analyses were performed on IBM SPSS statistics (ver 28.0.1.0(142)) and GraphPad Prism (9.5.1). Data were normalized with log transformed, and missing data were replaced with mean. The data were analyzed using a two-way ANOVA to examine any potential effects of sex, treatment, and their interaction. In the case of significant sex effect, data for males and females were analyzed separately and if no significant sex effect is observed, then data is presented as one-way ANOVA. The least significant difference (LSD) post hoc test was used to analyze the treatment differences. Microbial data with a minimum of 4 counts were included in the data analysis and bacterial species having zero abundance with a minimum of 20% of samples were removed from the analysis. Statistics for microbial composition, such as alpha and beta-diversity was analysed to study the evenness and richness of the gut bacteria using Microbiomanalyst version 2.0.. We analyzed the fecal metabolite data using MetaboAnalyst version 5.0. Metabolite data were normalized with quality control samples, followed by square root, and mean centering methods. Partial least square discriminant analysis (PLS-DA) was performed, and model validation was conducted using R2 and Q2 test with the fivefold cross-validation method at $p < 0.05$. For all other analyses, $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 B9 and B12 deficiency exerted sex differences in their physical parameters

We found a significant sex effect on the body weight ($p<0.001$), liver ($p=0.034$), and brain weight ($p<0.001$), but no treatment and interaction effects (>0.05, Appendix IV). Similarly, we also found significant sex differences in body weight adjusted duodeno-jejunal length ($p<0.001$) but did not find any significant treatment effects after separating males and females. Duodeno-jejunal length (34.07±4.35) of females was significantly ($p<0.001$) longer than males (20.52±1.63). We also we did not find any significant sex and/or treatment effects on ileum ($p= 0.67$) or colon length ($p= 0.96$) (Appendix IV).

3.3.2 Serum vitamin B9 and B12 concentration differs among the treatment groups

Serum B9 concentration was significantly lower in LB9 group (15.4±11.0 nmol/L) compared to control (103.0±17.6 nmol/L) ($p< 0.001$) (Figure 3.1 A). Similarly, serum B12 level was significantly lower in LB12 group (187.7±36.73 pmol/L) compared to control (478.9±119.99 pmol/L) ($p< 0.001$) (Figure 3.1 B). However, we did not find any sex and interaction effects (>0.05) on serum B9 and B12 level.

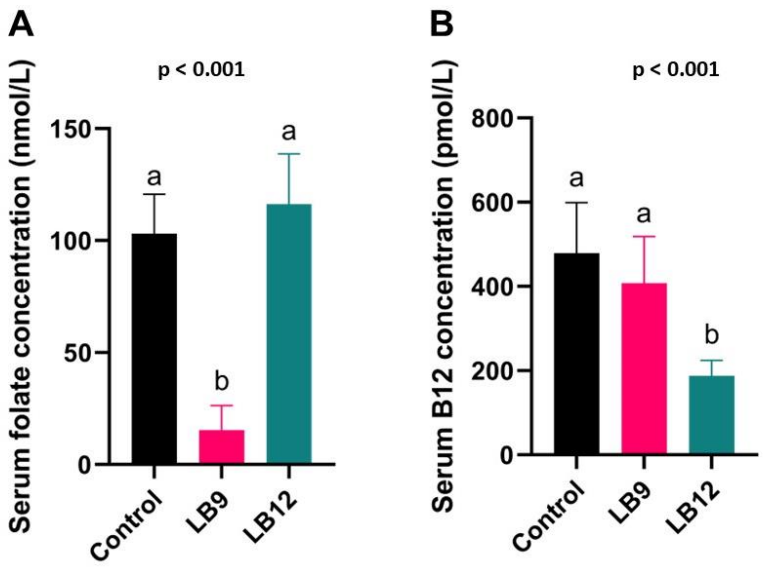

Figure 3.1: Serum vitamin B9 (A) and B12 (B) concentrations after six weeks of exposure to vitamin-deficient diets. [ab]Groups with different letters are significantly different by one way ANOVA-test. Values are expressed as mean ± SD, n= 16 in LB9 and LB12 and n= 15 in control.

3.3.3 Serum 1C metabolites were not affected by B9 and B12 deficiency.

Serum 1C metabolites were analyzed using targeted approach by using LC-MS/MS method. No significant differences were measured for the six metabolites associated with 1C metabolism when animals were exposed to vitamin B9 and B12 deficient diets for six weeks (Table 3.2). However, serum glycine level (p=0.002) and methionine level (p=0.004) have sex effects but no treatment and interaction effects (>0.05) on any of the metabolites.

Table 3.2: Serum 1C metabolites concentrations by treatment groups.

Metabolites (µM)	Control	LB9	LB12	P-value
Betaine	105.14±33.65	98.7±39.55	101.06±33.15	0.61
Glutamic acid	123.37±69.40	113.1±35.91	112.03±39.87	0.79
Glycine	236.76±97.97	186.69±45.34	229.11±83.31	0.16
Homocysteine	5.79±0.57	6.05±0.68	6.1±0.87	0.35
Methionine	58.26±12.22	51.68±10.99	59±15.83	0.24
Serine	324.11±60.33	315.56±46.81	299.25±49.40	0.28

Values are expressed as mean ± SD, n= 16 in LB9 and LB12 and n= 15 in control.

3.3.4 Histological analyses

3.3.4.1 Vitamin B12 deficiency increased colon mucosal height

There was significantly greater (p = 0.006) mucosal height in the colon in the LB12 group compared to the control group (Figure 3.2). However, we have seen a trend in being significant in wall thickness (p= 0.07) and muscle thickness (p= 0.08); we did not find any significant differences in submucosal thickness (p= 0.11) and crypt height (p= 0.44). The gut physical parameters with their mean values are listed in Table 3.3.

Table 3.3: Histological analysis using H&E staining showing mucosal height is significantly different in LB12 compared to the control.

Parameters (mm)	Control	LB9	LB12	p-value
Wall Thickness	1.65±0.43	1.92±0.29	1.96±0.28	0.07
Muscle Thickness	0.87±0.3	1.09±0.21	0.99±0.19	0.08
Submucosal Thickness	0.22±0.1	0.27±0.09	0.3±0.1	0.11
Mucosal Height	0.18±0.03[a]	0.18±0.03[a]	0.2±0.02[b]	**0.006**
Crypt Height	0.54±0.13	0.5±0.11	0.56±0.07	0.44

[ab]Groups with different letters in the row are significantly different by one way ANOVA-test.

Values are expressed as mean ± SD, n= 16 in LB9 and LB12 and n= 15 in control.

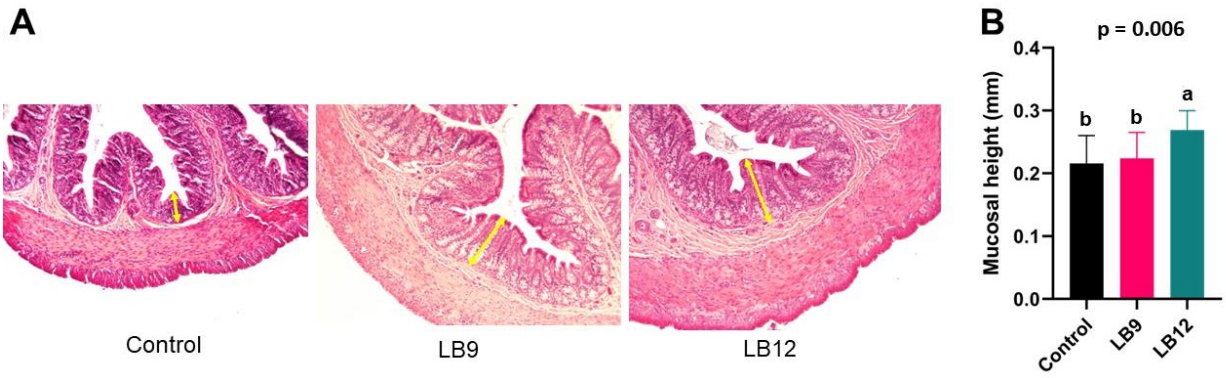

Figure 3.2: Representative examples of histology image showing the difference in mucosal height (A) with corresponding bar graph (B). The yellow arrows show how measurement were taken in the colon H&E section. The length was measured from two mucosal folding and from the top of the submucosal layer to the top of mucosal area using OMAX ToupView (ver 4.11). All the

measurement were taken in mm. The mucosal height of the LB12 was significantly higher (p=0.006) (0.22±0.02 mm) compared to control (0.18±0.03 mm), and LB9 (0.18±0.03 mm) group. [ab] Groups with different letters are significantly different by one way ANOVA-test. Values are expressed as mean ± SD, n= 16 in LB9 and LB12 and n= 15 in control.

3.3.4.2 LB9 and LB12 significantly decreased acidic mucin in the colon

There were no significant differences in percent area for content of total mucin content (p=0.83) or neutral mucin (p= 0.94). However, interestingly, we found a significantly lower (p = 0.02) in the percentage of content of acidic mucin in both LB9 and LB12 groups compared to the control group (Figure 3.3)

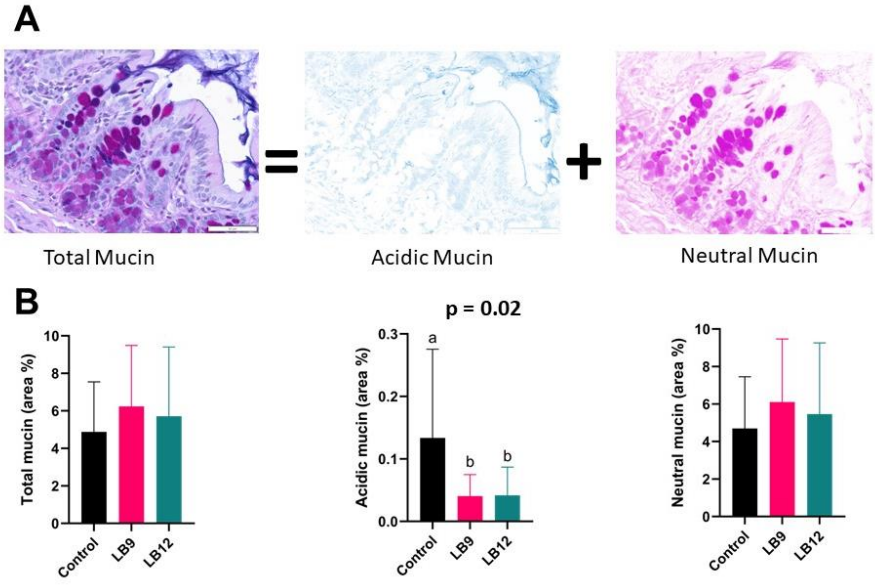

Figure 3.3: Representative PAS-AB-stained gut histology images showing different mucins (A) with their corresponding bar graphs (B) (p= 0.02). The figures are represented as % of the area of the slides. The ImageJ software was used to quantify total (purple), acidic (blue) and neutral (magenta) mucins using color deconvulation2 plugin. [ab]Groups with different letters are significantly different by one way ANOVA-test. Values are expressed as mean ± SD, n= 16 in LB9 and LB12 and n= 15 in control.

3.3.5 Vitamin B9 and B12 deficiency altered gut microbial composition in sex-dependent manner

We detected 1562 ASVs in our sample. After screening the data manually and removing ASVs which had 0 abundances in 70% of the total samples, we had 94 ASVs that we considered for data analysis. Alpha and beta diversity was examined to study the richness and composition of the gut bacteria, but we did not find any significant changes in terms of Shannon index (Figure 3.4 A, p=0.69), and PCoA analysis (Figure 3.4 B, PERMANOVA p=0.27). Although the relative abundances of Verucombimicrobiota in LB12 seemed to be different from the control and LB9 group at phylum level (Figure 3.4 C, p=0.2) but at genus level (Figure 3.4 D, p=0.06), there were no significant differences among the treatment groups.

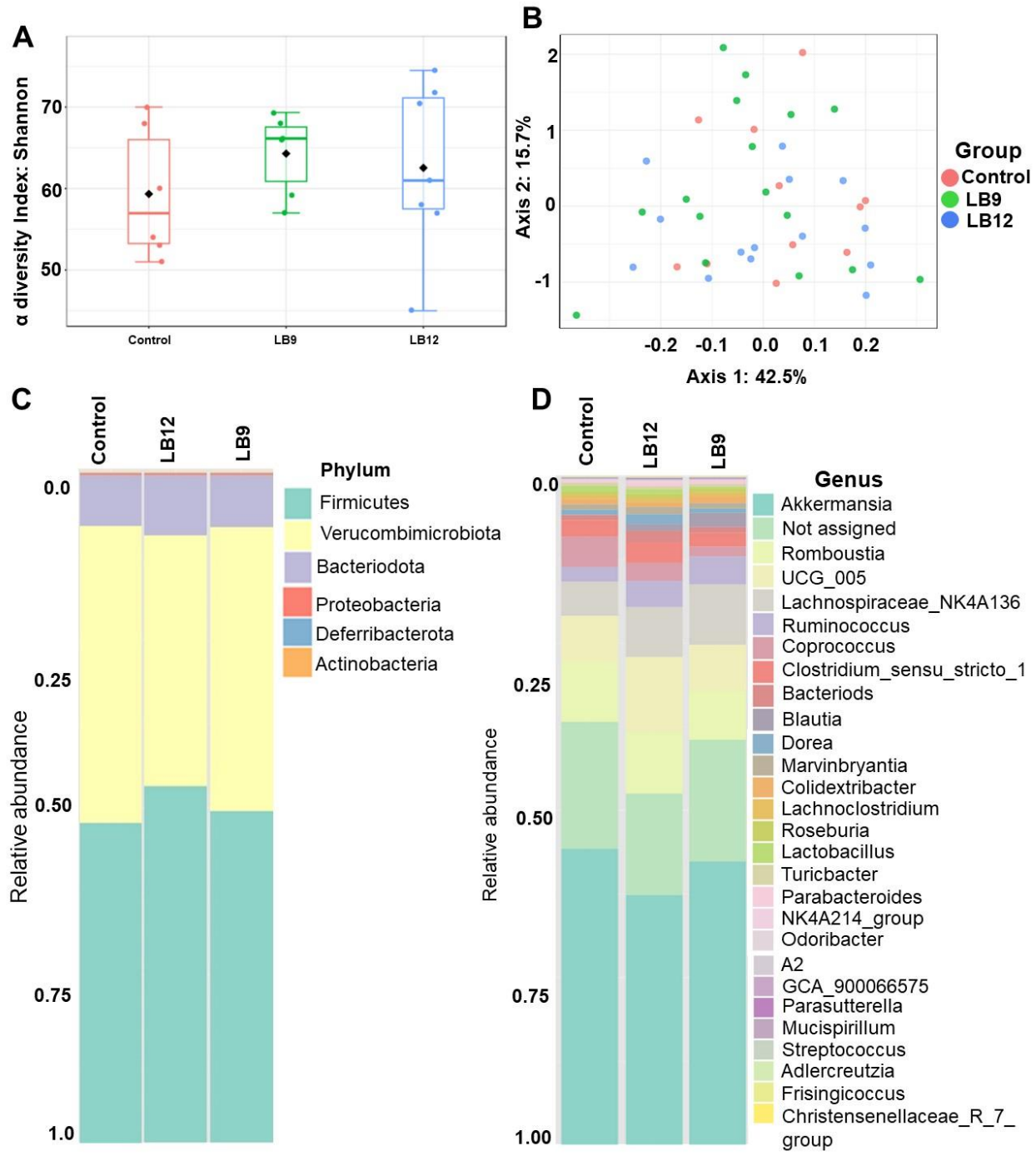

Figure 3.4: Cecal microbiota showing Shannon index alpha-diversity (A), PCoA plot (B), Relative abundance of the bacteria at phylum level (C) and genus level (D). There were no significant differences in alpha diversity, no significant separations of the bacteria in different treatment groups and no significant changes in the relative abundances of the bacteria at phylum and genus levels. Values are expressed as mean ± SD, n= 11 in control, n=14 in LB9, and n=15 in LB12.

Interestingly, there was a significant sex effect on the richness and evenness (Shannon index, p=0.05, Figure 3.5 A), as well as compositional differences (PCoA beta diversity, PERMANOVA p=0.001, Figure 3.5 B) of the gut microbial profile. Figure 3.5 C shows the microbes responsible for the separation between the sexes, which include *Akkermansia, Balutia, Bacteroides, Parasutterella, Adlercreutzia, Christensenellaceae_R_7_group, Marvinbryantia, Dorea, Romboutsia.* Thus, we further analyzed the gut microbial data to investigate treatment effects by segregating the data based on sex.

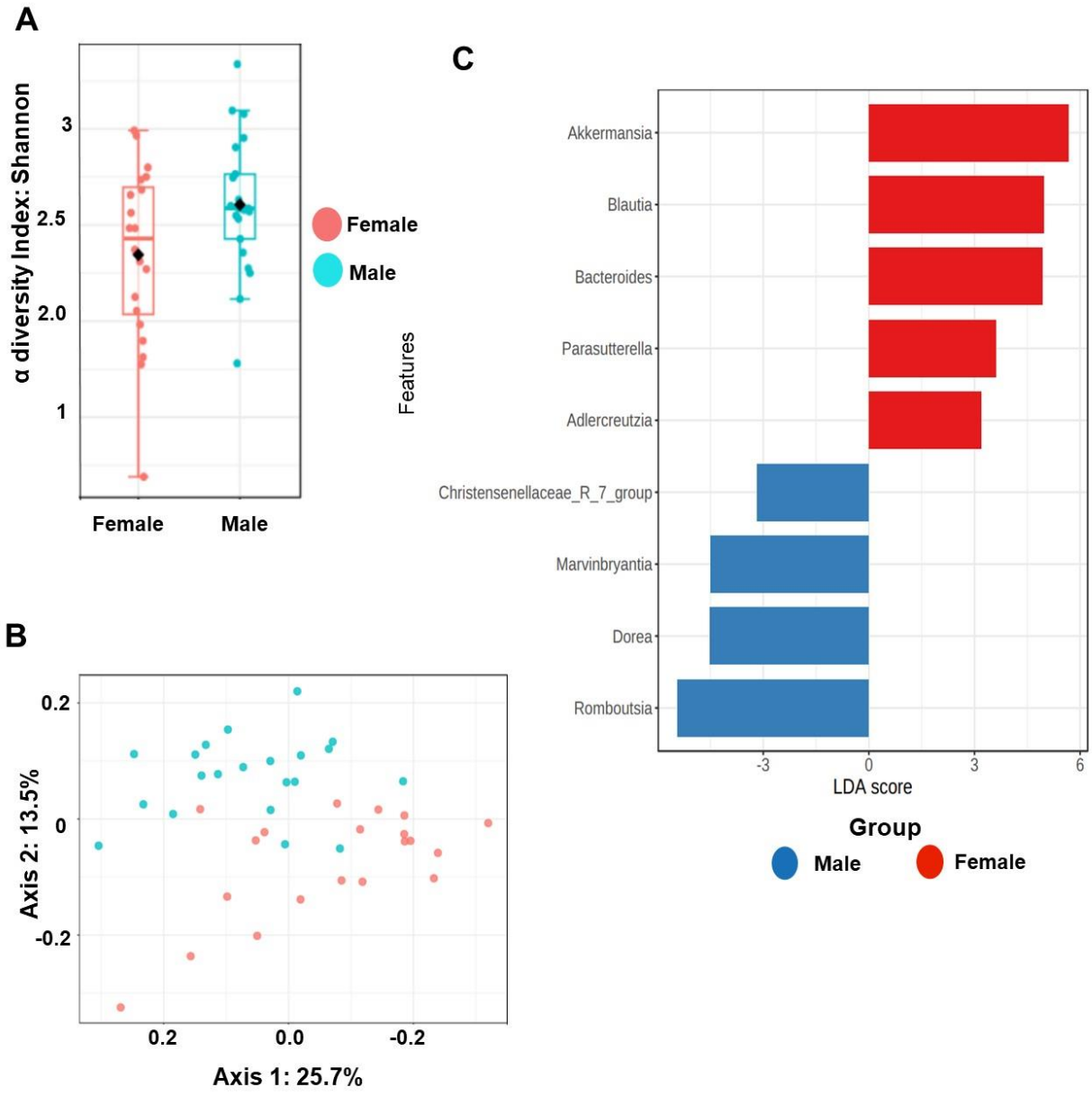

Figure 3.5: Gut microbial profile showing alpha diversity by Shannon's index (A) and beta diversity as PCoA (B) showing distinct microbial composition between males and females of microbial data based on sex. Gut microbiota was significantly different between the males and females when measured in Shannon's index ($p<0.05$) and PCoA analysis (PERMANOVA $p=0.001$). n=20/group for females and n=21/group for males. Major bacteria responsible for separating between the sexes have been identified by their Linear Discriminant Analysis (LDA) score (C).

Impact of vitamin B9 and B12 deficiencies on microbial profile of male rats

In the male rats, the microbial diversity, based on Shannon index (α diversity) and PCoA analysis (β diversity), did not show significant separation between the treatment groups (Figure 3.6 A and B). To examine significant bacteria, we conducted an additional analysis using linear discriminant analysis (LDA) effect size (LEfSe), comparing each treatment groups with the control. Significant bacteria were identified based on the LDA scores and their corresponding p-values. The bacterial profiles did not differ at the phylum level (Figure 3.6 C); however, at family level, the abundances of Lactobacillaceae ($p=0.003$) and Streptococcaceae ($p =0.02$) were comparatively lower, whereas Ruminococcaceae showed an increase ($p=0.02$) in LB9 group compared to the control. Conversely, the abundances of both Micrococcaceae ($p =0.04$) and Streptococcaceae ($p=0.05$) were decreased in LB12 compared to the control. At the genus level, we observed a significant reduction in the abundance of *Dorea* and *Lactobacillus* in LB9 group, while the abundance of *Strepcococcus* was significantly reduced in both LB9 and LB12 groups compared to the control (Figure 3.7: A-C).

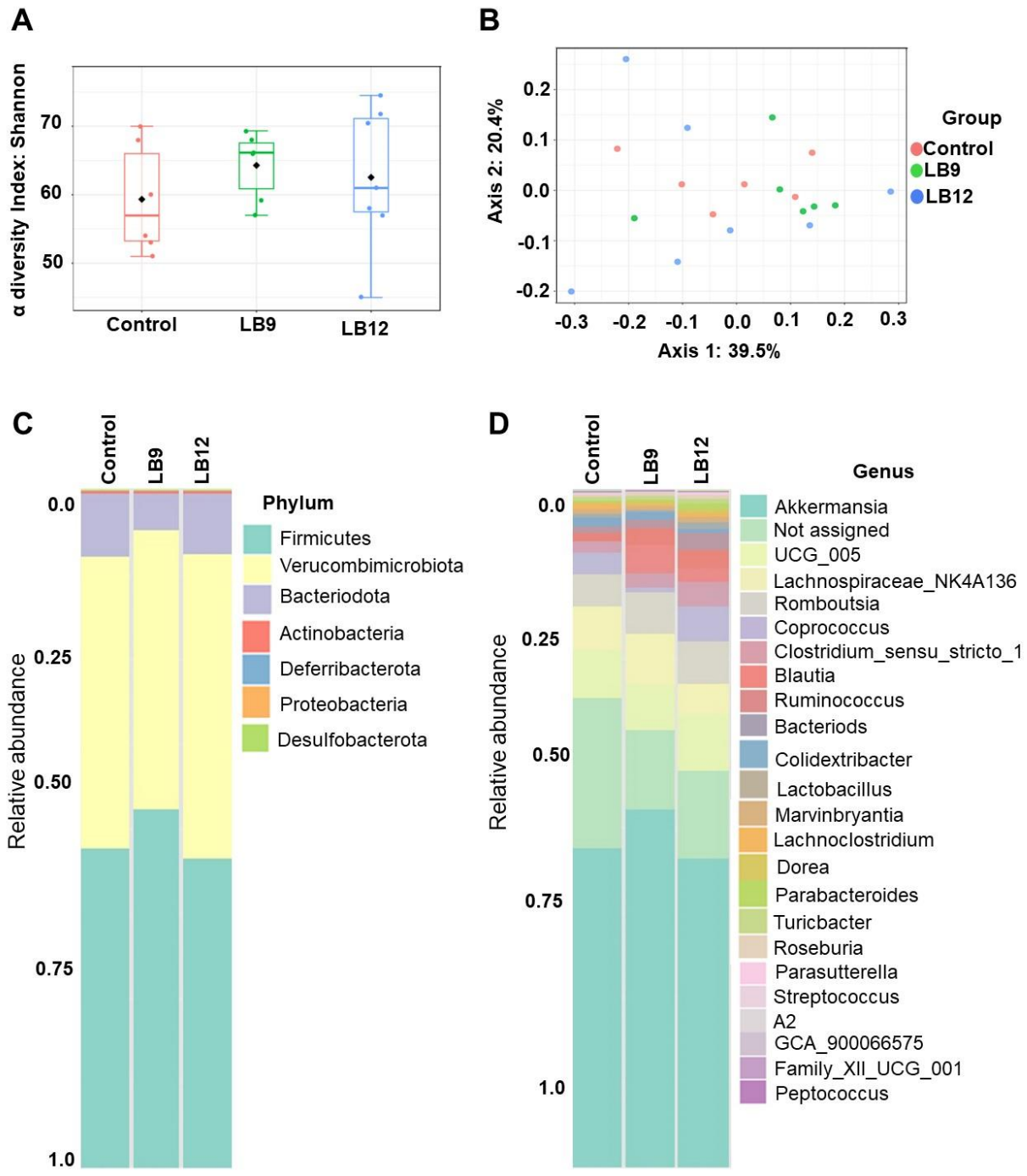

Figure 3.6: Cecal microbiota does not show any significant differences between alpha diversity (Shannon index, p=0.23) (A), PCoA plot (B), and relative abundance of the bacteria at phylum level (C). There were no significant differences in alpha diversity, no significant separations of the bacteria in different treatment groups and no significant changes in the relative abundances of the bacteria at phylum levels. At genus levels, abundance of *Colidextribacter*, *Lactobacillus*,

Streptococcus and *Dorea* were significantly altered (D). Values are expressed as mean ± SD, n= 5 in control, n= 8 in LB9 and LB12.

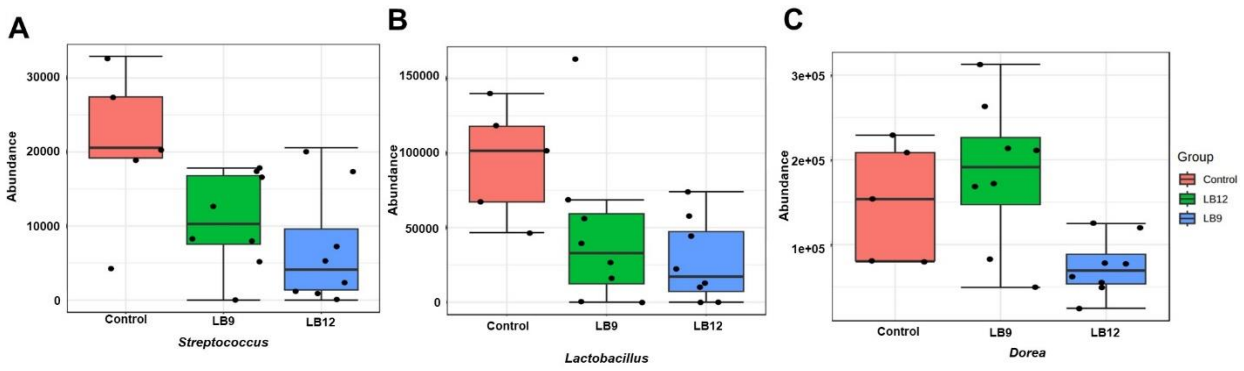

Figure 3.7: Abundance of altered bacterial genera using 16S rRNA gene sequencing in males. Three genera i.e *Lactobacillus*, *Streptococcus* and *Dorea* were significantly altered in males. *Streptococcus* showed a significant decrease in both the treatment group (A), *Lactobacillus* showed a significant decrease in LB9 (B), and *Dorea* showed a decrease in LB9 group (C). Values are expressed as mean ± SD, in males, n= 5 in control, n= 8 in LB9 and LB12 and in females, n= 6 in control, n= 6 in LB9 and n= 7 in LB12.

Impact of vitamin B9 and B12 deficiencies on microbial profile of female rats

Similar to the male rats, there were no significant separation of microbial diversity between the treatment groups in the female rats (Figure 3.8 A and B). The LEfSe analysis revealed that the abundance of Bacteroidota was significantly lower in LB9 group compared to the control group (p=0.05), but no significant difference was found for LB12 group at the phylum level (Figure 3.8 C). There were no changes observed for LB9 group at family level, but Anaerovoracaceae was significantly lower (p=0.004) in the LB12 group compared to control. At genus level, *Lachnoclostridium* was significantly decreased in LB9 group compared to control, whereas *Blautia* was significantly increase in both the LB9 and LB12 groups compared to the control (Figure 3.9: A & B).

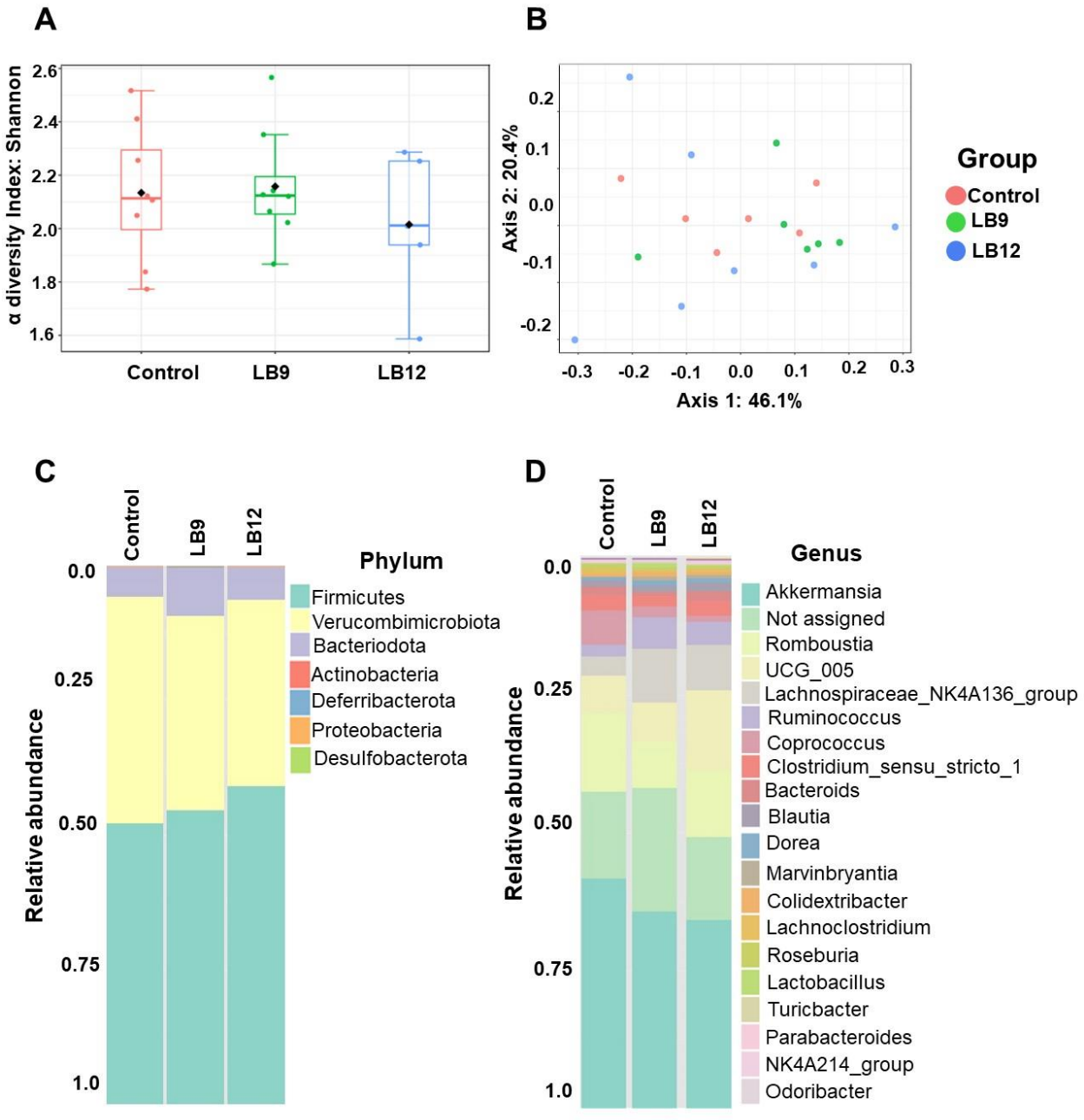

Figure 3.8: Females gut microbiota did not show significant separation between the treatment groups based on alpha diversity (Shannon index, p=0.68) (A), PCoA analysis (B), and relative abundance of the bacteria at phylum level (C). At genus levels abundance of *Colidextribacter*, *Lachnoclostridium* and *Blautia* was significantly altered (D). Values are expressed as mean ± SD, n= 6 in control, n= 6 in LB9 and n= 7 in LB12.

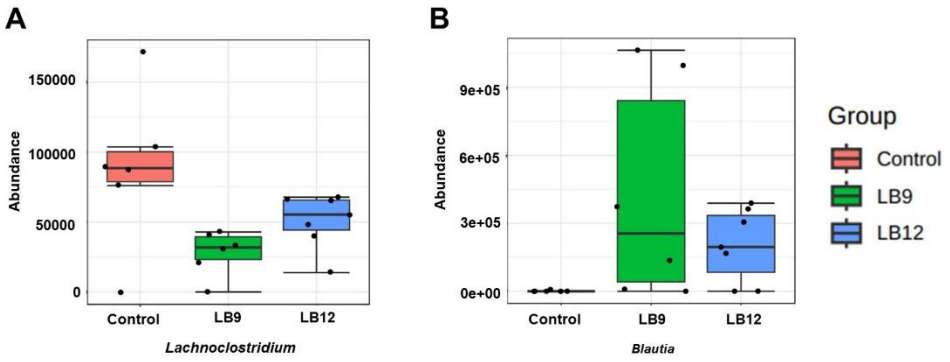

Figure 3.9: Abundance of altered bacterial genera using 16S rRNA gene sequencing in females. Two genera i.e *Lachnoclostridoum* (A) showed significant decrease in LB9 group where as *Blautia* (B), were significantly increased in LB9 group in females. Values are expressed as mean ± SD, n= 6 in control, n= 6 in LB9 and n= 7 in LB12.

We identified one significant bacterial genus, *Colidextribacter,* which was found to be common in both males and females. In males, the abundance of *Colidextribacter* significantly increased in LB12 compared to the control (Figure 3.10 A). In females, the abundance of *Colidextribacter* showed a significant decrease in LB12 group compared to the control (Figure 3.10 B).

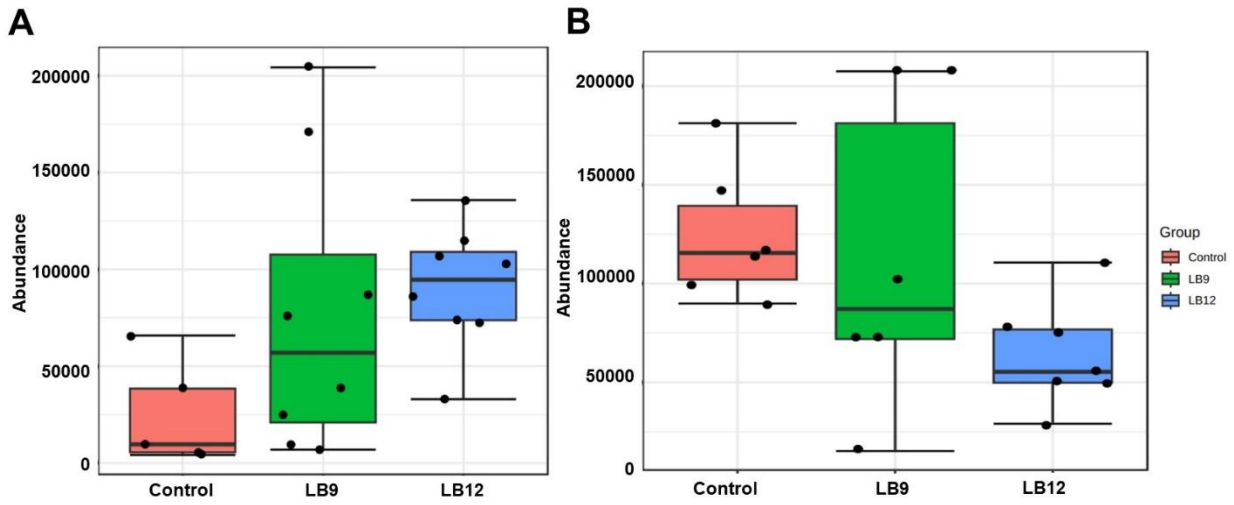

Figure 3.10: *Colidextribacter* showed significant alteration in both the sexes, but degree of alteration differs between the groups and genera. In males, abundance of *Colidextribacter* is significantly higher in LB12 group when compared with control (A). Whereas in females, it showed a significant decrease in LB12 group when compared with control (B). Values are expressed as mean ± SD, in males, n= 5 in control, n= 8 in LB9 and LB12, and in females, n= 6 in control, n= 6 in LB9 and n= 7 in LB12.

3.3.6 B9 and B12 deficiencies altered fecal metabolites in a sex dependent manner

A total of 86 fecal metabolites were identified, along with their peak intensities, using targeted and untargeted metabolomics. The data were further screened using MetaboAnalyst version 5.0, and metabolic features containing more than 20% of '0' values of the total samples were removed. The data were normalized using quality control (QC) samples and autoscaling. Figure 3.11 shows the score plot of the principal component analysis (PCA) analysis and its biplot. The PCA represents a total of 45.9% variations, with PCA 1 accounting for 30.2% and PC2 for 15.7%. We did not find any significant separation among the treatment groups (Figure 3.11). We also conduced Partial leased Square-discriminant analysis (PLS-DA), but the cross-validation of the model was not significant, indicating that the metabolites abundances were not different.

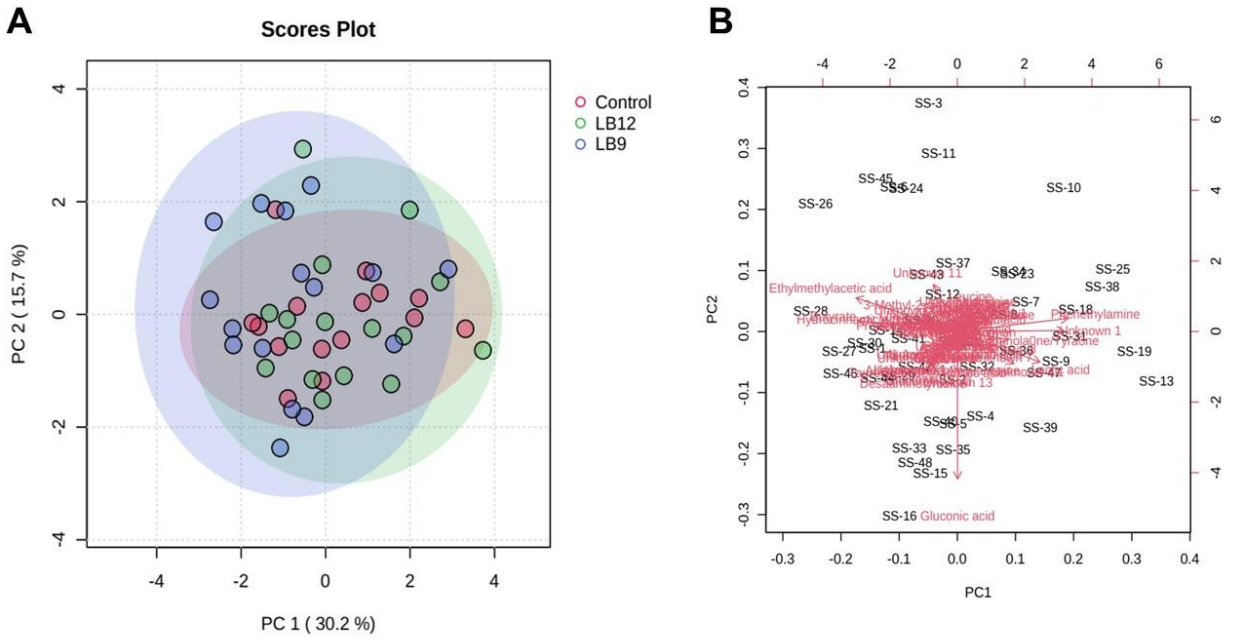

Figure 3.11: PCA score plot (A) and its biplot (B) of fecal metabolites identified by targeted and untargeted approach. Ellipses represent 95 % confidence intervals for each individual group on PCA plots. n= 15 in control, n= 16 in LB9 and LB12.

We further investigated sex effect on the fecal metabolite profile. Figure 3.13 illustrates the PLS-DA score plot colored by sex (Figure 3.12 A) and variable importance of projection (VIP) score plot (Figure 3.12 B). PC 1 represents 29.2%, and PC 2 represents 11.3% of the variation, accounting for a total of 40.5% of variation. Interestingly, the treatment groups were separated based on the sex, indicating a difference in the metabolite concentration between the sexes. Important metabolite identified by VIP scores that drives the separation includes phenethylamine, lactic acid, gluconic acid, ethylmethyl acetate, desaaminotyrosine and glucuronic acid.

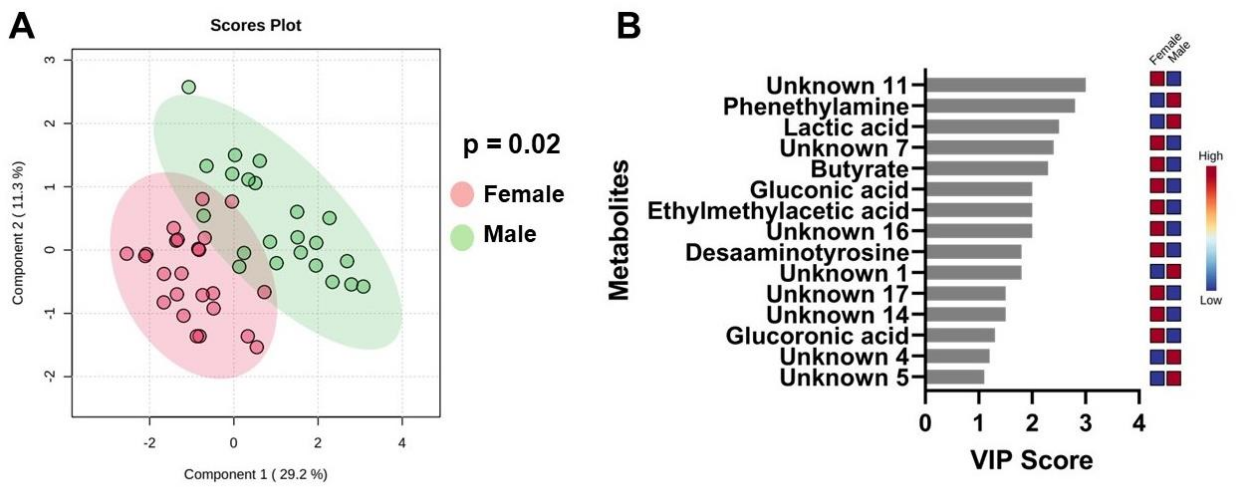

Figure 3.12: Fecal metabolite shows significant separation between sex in PLS-DA plot (A). Ellipses represent 95 % confidence intervals for each individual group on PLS-DA plots with Q^2 =0.42 and R^2 =0.52. VIP scores reflect the degree of importance of a metabolite that might be responsible for this significant separation between males and females (p = 0.02), with values >1.0 is considered as important metabolites responsible behind the separation (B). n=24 in females and n=23 in males.

As we observed a significant separation of fecal metabolites between the sexes (Figure 3.12), we further analysed the treatment effects by separating the data based on sex (Figure 3.13). However, there was no significant separation between treatment groups in the PCA score plot for both males (A) and females (C). The PCA analysis in males represent a total of 55.4% variations, with PCA1 accounting for 39.6% and PCA 2 accounts for 15.8% in males (Figure 3.13 A). In females, the PCA analysis represents a total of 40.2% variations, with PCA1 accounting for 20.6% and PCA 2 accounts for 19.6% in females (Figure 3.13 C). Figure 3.13 B, D illustrate the biplot of the PCA analysis.

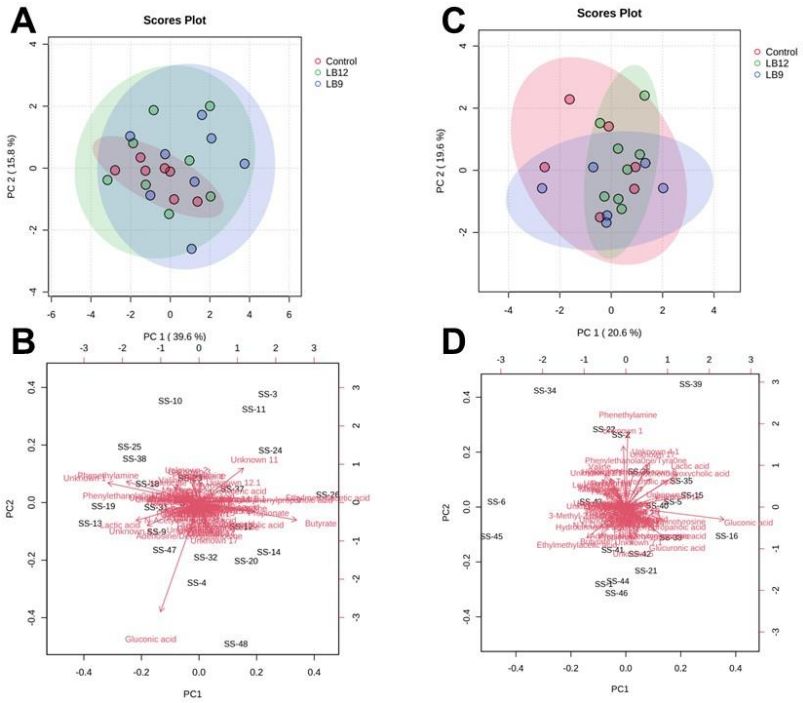

Figure 3.13: PCA plot in males (A) and in females (C) showed no significant separation between treatment. Biplot showing possible metabolite that might be contributing towards the separation between males (B) and females (D).

Next, we performed a volcano test to examine whether the metabolites identified for each treatment group were different from control. Interestingly, we found that tryptophan and histidine were lower where as hydrocinamic acid, ethylmethylacetic acid, and N,N-diacetylsperodine were higher in LB9 group compared to the control group (Figure 3.14 A). Similarly, N-acetylneuraminic acid and three other unidentified compounds were higher in LB12 group when compared with the control (Figure 3.14 B).

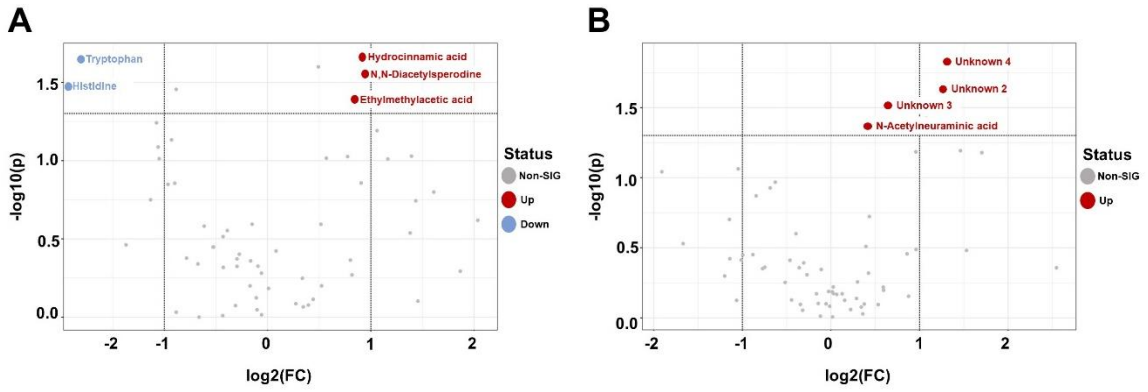

Figure 3.14 Volcano plots showing the significant metabolites in LB9 (A) and LB12 (B) groups. Metabolites with log2 fold change with $p<0.05$ were considered significant. Significant metabolites higher in LB9 groups are hydrocinnamic acid, N,N-diacetylsperodine and ethylmethylacetic acid whereas tryptophan and histidine is lower. On the other hand, N-acetylnuraminic acid and some unknown metabolites were higher in LB12 group. n=15 in control and n= 16 in LB9 and LB12.

3.4 Discussion

B vitamins, including B9 and B12 play important roles in human metabolism as co-factors for biochemical reactions, primarily linked to 1C metabolism [126]. Essential B vitamins can be obtained through dietary intake or synthesized by bacteria residing in the gut. Nevertheless, only a limited number of gut bacteria have the ability to produce B vitamins [3, 225]. Thus, these microbes along with host heavily rely on diet sources of these vitamins. However, there is limited knowledge regarding how diet-derived vitamin B9 and B12 interact with the gut morphology, microbiota and metabolites. Thus, the primary focus of the current investigation was to analyze the potential effects of insufficient dietary vitamin B9 and B12 on gut health, specifically, indices of gut morphology, microbial profile and the metabolites produced by the host and the gut microbiota. The findings from our study provide evidence that a deficiency in dietary vitamin B9 and B12 led to an alteration in gut health.

There were significant sex differences in the body weight among groups, but there was no treatment effect, which is contrary with a previous B6 study [226]. We also found significant sex differences in brain and liver weight after adjusting with body weight. However, there was no treatment effect on males and females separately. Alterations of intestinal morphology due to disease are well documented, for example with such as short bowel syndrome (SBS), deeper colon crypt were observed [227]. While there is less information on the impact of dietary vitamin deficiency on intestinal morphology, Berg and colleagues reported that patients suffering from vitamin B12 and B9 deficiency had shorter but broader villi in duodeno-jejunal area [228]. To our knowledge, morphological changes and specifically colon mucosal height differences due to dietary vitamin deficiency has not been reported yet. However, there is evidence that, in the event of B12 deficiency under diseased conditions, colon length was shortened when compared with a healthy mouse model [229]. In rats, B vitamin deficiencies led to an almost 50% reduction in colonic area (length X width) [230]. Overall, these studies suggest that intestinal morphology changes with intestinal disease and vitamin deficiency conditions.

Mucin, a glycoprotein secreted mainly by epithelial and goblet cells plays a crucial role in protecting and lubricating intestinal membranes [231]. It also facilitates communication between external environment and epithelial cells through cellular signaling [232]. Mucins can be acidic or neutral based on constituents of the glycoproteins. Acidic mucin, primarily found in the large intestine, is believed to defend against bacterial invasion due to its less degradable sulfated constituent [233]. Diet composition may play an important role in mucin production. For instance, heteroxenic rats on an inulin enriched diet showed reduced colonic acidic mucin compared to control rats [234]. Another study provided evidence that, butyrate level up to 5mM can increase mucin production. But over 5mM concentration can cause a gradual decrease the mucin

production in the gut [235]. In our study, we found decreased acidic mucin in both LB9 and LB12 groups. Colon mucin production is influenced by various factors, including diet, microbes, and microbial metabolites [236]. As such, low levels of B9 and B12, altered bacterial composition, and lower levels of serum glycine likely played significant roles in reducing acidic mucin levels in LB9 and LB12 rats.

Deficiency in vitamin B9 or B12 resulted in sex-specific alterations in the gut microbiota and associated metabolites; this was an unexpected outcome. One of the interesting findings of our current study was the alteration of *Colidextribacter* in both the sexes but it showed a fully opposite treatment effect in males and females. *Colidextribacter* is a recently characterized bacterial genus that belongs to phylum Firmicutes and family Oscillospiraceae [237]. It is predominantly found in males and is heavily influenced by diet [238]. In addition, we found that the abundance of *Lachnoclostridium* decreased in LB9 while the abundance of *Blautia* increased in both the LB9 and LB12 group in females. *Lachnoclostridium* is a known butyrate producer [239]. Thus, decreased abundance of *Lachnoclostridium* might have led to lower butyrate production in the current study. *Lachnoclostridium* is a B9 producer and synthesizes vital enzymes required in the folate metabolism according to Kyoto Encyclopedia of Genes and Genomes (KEGG) (pathway number: 00790 and 00670 for folate biosynthesis and one carbon pool by folate respectively) [240-242]. On the other hand, *Blautia* which earlier was thought to be a member of *Ruminococcus,* is also a putative propionate and butyrate producer and a higher abundance of *Blautia* is associated with obesity which includes inflammation and insulin resistance [243-245]. Previous studies also found a relation between *Blautia*, and a micronutrient, vitamin D [246]. This implies that, *Blautia* can be affected in response to micronutrient deficiency. In males, *Lactobacillus, Streptococcus,* and *Dorea* were altered in response to the B9 and B12 deficiencies. *Lactobacillus* is a vitamin B9,

B12 and butyrate producer, while *Dorea* is also a putative butyrate producer [247-249]. Another bacterial genus, *Streptococcus* that was decreased in both the group is also a butyrate producer [250]. Even though we found several butyrate producers were decreased in the gut, there could be other genera utilizing diet constituents to regulate secretory function of colonic goblet cells.

Hydrocinnamic acid is an organic compound, also known as phenylpropanoic acid, and is generally found in kidney, liver, and several microorganisms [198]. Hydrocinnamic acid is thought to be a product of phenylalanine which is produced by gut microbiota and mostly found in feces [251]. In our study, we found that, in LB9 group, hydrocinnamic acid was higher compared to control which suggests that phenylalanine utilization capabilities of gut microbiota or abundance of microbes that were utilizing phenylalanine, might have been increased. Another metabolite found in feces at higher level in LB9 group was N,N-Diacetylsperodine. N,N-Diacetylsperodine is a kind of polyamine that it found in all organisms and is known to have several functions related to cell growth and regulation, nucleic acid regulation and others [252-254]. Recent studies have found that concentration of this polyamine is higher in the blood and liver of patients with colorectal, and breast cancer and in inflammatory bowel disease [255-257]. These polyamines are synthesized by S-adenosyl-methionine decarboxylase from SAM, and SAM is a crucial intermediary metabolite of 1C pathway [258, 259]. Interestingly, *Lactobacillus* can produce this polyamine in the gut [260, 261]. This polyamine is positively related to folic acid but it is inversely related to dietary folate intake, which is consistent with previous findings B9 [262] and our findings of higher N,N-diacetylsperodine in LB9 group. Even though folate is an essential nutrient to synthesize polyamide, surprisingly their concentration is not decreased significantly in a folate deficient environment, which might be a result of a compensatory response [263]. Ethylmethylacetic acid or 2-Methybutyric acid (HMDB0002176) has been found in higher

concentrations in LB9 group compared to control. Ethylmethylacetic acid is a metabolite found in *Lactobacillus lactis* and generally found during carbohydrate starvation [264]. In our study, we found in LB9 group, *Lactobacillus* was significantly lower in comparison with control. It might be possible that carbohydrate utilization in the event B9 deficiency has been disrupted, but this needs further clarification. On the other hand, lower metabolites in LB9 are tryptophan and histidine. Tryptophan metabolism is solely dependent on several B vitamins which includes B6, B9 and B12 [265]. Interestingly, gut tryptophan concentration was significantly higher in germ free mice compared to conventional ones most likely due to the lack of tryptophan degrading microbiota [266]. This intricate relation of tryptophan, folate and tryptophan degrading microbiota might be the reason for lower level of tryptophan in our study. On the other hand, histidine catabolism is folate dependent, and folate deficiency leads towards an increased urinary secretion of histidine [267, 268]. This might be the reason why we found lower levels of histidine in folate deficient rats.

We found an upregulation of N-acetylneuraminic acid in LB12 compared to the control. N-acetylneuraminic acid, a member of sialic acid group, is one of the components of mucin [269]. Kiran *et al.*, 2023 reported that, an increase in total serum sialic acid and lipid bound sialic acid in B12 deficient children which was associated with elevated oxidative stress and inflammation [270]. There are other members of sialic acid, and N- acetylneuraminic acid is one of them [271]. It is possible that the increase in N-acetylneuraminic may lead to the failure to activate and incorporate it into the mucin synthesis in B12-deficient animals. Figure 3.15 illustrates the overall effects of B9 and B12 deficiency on 1C metabolic reaction and gut homeostasis.

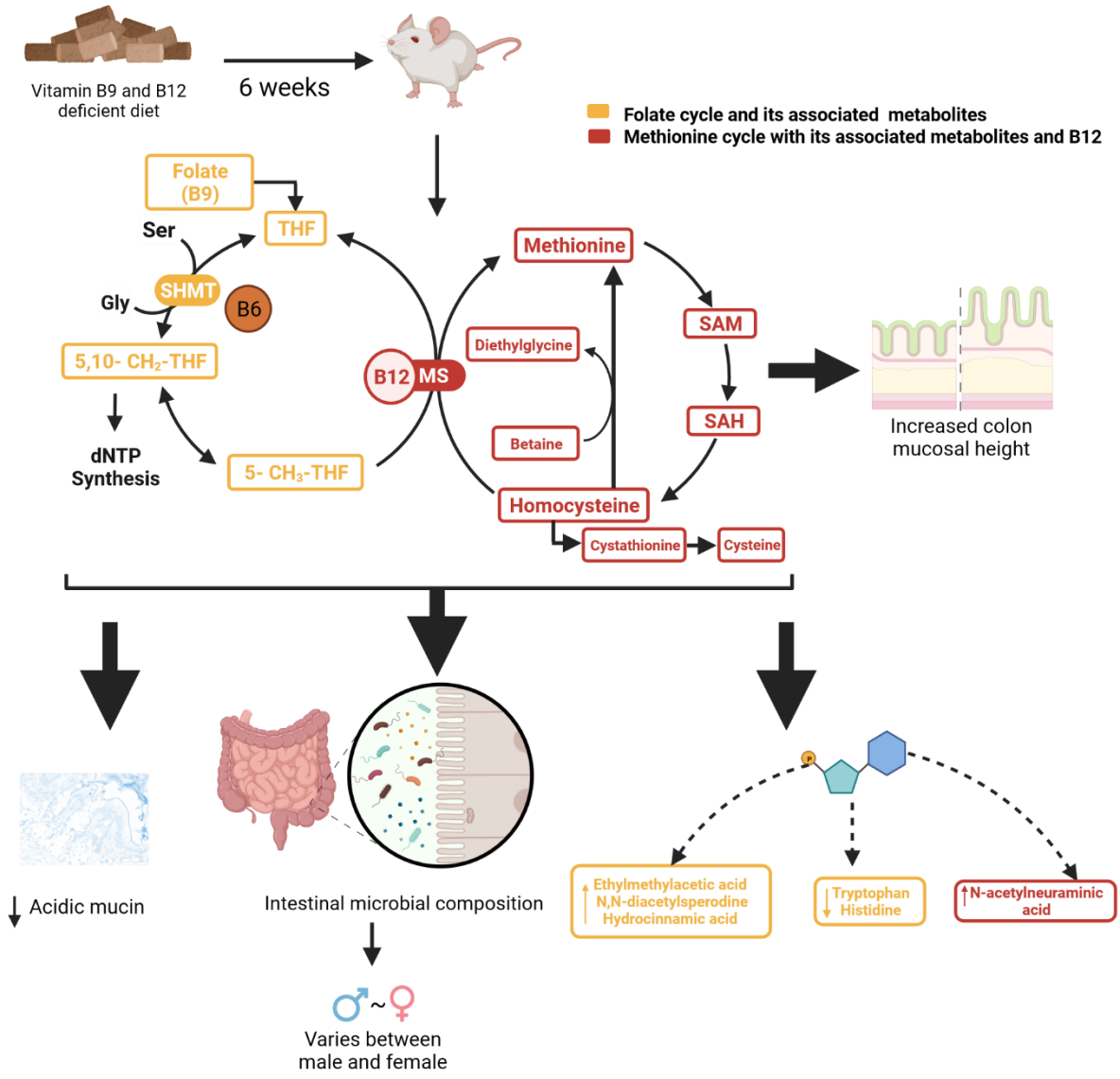

Figure 3.15: Overview of the impacts of B-vitamin deficiency on one-carbon (1C) metabolism and gut homeostasis: Deficiencies in vitamins B9 or B12 led to reduced acidic mucin content and increased mucosal height in the colon. This was associated with a reduced glycine level, a 1C metabolites, and alterations in specific gut microbiota and metabolites in a sex-dependent manner. Figure is generated with BioRender.

3.5 Conclusion

Microbially produced vitamins are not enough for the host to meet its daily requirements thus, dietary supply is essential. We observed that deficiencies of B9 and B12 resulted in a sex dependent

alteration in the intestinal length, with females being more susceptible to both the deficiencies. Additionally, B12 deficiency increased colon mucosal height, but it decreased acidic mucin production in both sexes. In our study, we identified changes in the gut microbiota composition, with sex-specific variations. *Colidextribacter*, a genus associated with diet, showed opposing treatment effects in males and females. Hydrocinnamic acid, N,N-Diacetylsperodine, and other metabolites changed as a result of the treatment, revealing information about the capacity of protein fermentation and polyamine synthesis. N-acetylneuraminic acid, a component of mucin, was elevated in response to B12 deprivation, whereas tryptophan and histidine levels were decreased.

Our work demonstrates the complex interactions between B9 and B12, the gut microbiota, and gut health. Dietary deficits of certain B vitamins can affect metabolite profiles and gut microbiota composition differently depending on sex. Understanding these interactions might play a crucial role in dietary interventions to maintain and improve gut health in future.

CHAPTER FOUR

Summary, and key findings

4.0 Summary

This thesis focused on exploring novel roles of dietary micronutrients on gut morphology, microbes, and metabolites and how they interact to maintain gut homeostasis. B vitamin, specifically B9 and B12 acts a co-factor for 1C metabolic pathway [14]. Humans cannot synthesize these water-soluble vitamins and must obtain them primarily through dietary intake, although a limited amount is produced by the gut bacteria [272]. Thus, we hypothesized that dietary deficiency of these two vitamins will impair the 1C metabolic pathway and alter the host gut ecosystem through a change in gut microbiota and production of their metabolites.

Contrary to our previous findings on the effect of vitamin B6 deficiency, we did not find any significant differences in terms of body growth and organ weights between the groups. This might be the result of the inability to induce vitamin deficiency at the host physiological level. It is supported by our findings that, although serum B9 and B12 levels are lower, most of the metabolites linked to 1C pathway did not change. One of the most significant findings of our study was the reduction in acidic mucin content of colon in vitamin-deficient groups. Acidic mucin is a type of mucin that acts as a protective layer against pathogens [273]. Interestingly, we also found an alteration of serum glycine and fecal N-acetylneuraminic acid levels, which are known to be involved in mucin synthesis.

The next important finding was the sex-dependent alteration of the gut microbial profile in vitamin-deficient groups. Cecal microbiota analysis revealed that the microbes were altered in a sex-dependent manner. For instance, in males, genera such as *Dorea*, *Lactobacillus* and *Streptococcus* were altered, but in females *Blautia* and *Lachnoclostridium* were altered in folate-deficient animals. One genus, *Colidextribacter*, was common in both sexes, but the degree of change was completely opposite between the sexes. Fecal metabolomics revealed that tryptophan

and histidine were lower, whereas hydrocinamic acid, ethylmethylacetic acid, N,N-diacetylsperodine is higher compared to control in folate-deficient animals. On the other hand, N-acetylneuraminic acid was higher in vitamin B12-deificient animals. Thus, our data provide evidence that vitamin B9 and B12 affect gut morphology, microbiota, and their metabolites differentially in a sex-dependent manner.

4.1 Key findings

1. Body and organ weight: Vitamin B9 and B12 deficient diet did not cause any significant difference in body weight and organ weight.
2. Intestinal morphology: Duodeno-jejunal length adjusted with body weights showed a significant sex difference. Rats in LB12 group had significantly higher colon mucosal height, but not those in the LB9 group.
3. Mucin production: Total mucin and neutral mucin in % area were not significantly altered, but acidic mucin was significantly decreased in both groups.
4. Microbiota: Microbes were altered in a sex-dependent manner. In males, genera such as *Dorea*, *Lactobacillus* and *Streptococcus* were altered, while in females, *Blautia* and *Lachnoclostridium* were altered. One genus, *Colidextribacter*, was commonly affected in both sexes, but the degree of change was completely opposite between the sexes.
5. Metabolites: In LB9 group, tryptophan and histidine were lower, whereas hydrocinamic acid/phenylpropanoic acid, ethylmethylacetic acid, N,N-diacetylsperodine were higher compared to control. Similarly, in LB12 group, N-acetylneuraminic acid was higher.

4.2 Limitations

In this study, the dietary vitamin deficiency treatments result in a marginal level of vitamin deficiencies. Even though animals will have a negligible amount of the two vitamins in their deficient diets, previously stored vitamins, specifically vitamin B12, which can be stored in the body for up to six months, might have worked to buffer the deficiency at the physiological level. The coprophagy nature of animals is one way to obtain these vitamins. A study by Sukemori *et al.* 2006 revealed that B12 intake through coprophagy could provide a similar vitamin B12 level as that of a B12-supplemented diet [274]. Preventing coprophagy is a stressful event for the animals but could be something to consider in such experimental designs.

4.3 Conclusion

This study sheds light on the effects of B9 and B12 deficiency on gut health. Our finding revealed that vitamin deficiencies altered gut morphology, including increased mucosal height and decreased acidic mucin production. Sex-dependent alterations in microbial composition revealed changes in the abundance of certain vitamin and SCFA producing bacteria, including *Lachnoclostridium, Lactobacillus, Colidextibactor,* and *Blautia.* Although serum metabolites of 1C pathway were not affected by the vitamin deficient diets, changes are observed in their fecal metabolites. Overall, this study provides crucial insights into the significant role that B vitamins play in maintaining gut homeostasis.

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CHAPTER FIVE

Appendix

APPENDIX I

Histological analysis

H&E staining

For H&E staining, we have used Mayer's haematoxylin and eosin method.

Procedure:

1. De-paraffin slides in xylene for 3-5 min.
2. Rehydrate by placing in decreasing concentrations, Absolute, 95%, 80%, 70%, for 2 min. each.
3. Rinse in water 1 min.
4. Stain in Mayer's haematoxylin for 20-30 min.
5. Rinse in water
6. Place in STWS for a 3min. until sections are blue.
7. Rinse in water for 5 minutes.
8. Stain in Eosin 15 sec.-2 min.
9. Dehydrate quickly in 95%, and absolute alcohol.
10. Clear in xylene and mount.

Periodic acid–Schiff (PAS) and Alcian Blue (AB) staining

PAS-AB staining was performed on the colon section to stain the mucin in the colon. Slides were prepared by Histology department, Faculty of Medicine, MUN. Acidic mucin was stained blue, neutral mucin were stained magenta and combination of both mucin was stained as blue/deep purple. Slides were analyzed using Olympus BX50WI upright biological microscope with a SC50 Olympus 5-megapixel digital color camera and an Olympus UPlanSApo 40x/0.95na lens and pictures were taken using CellSens Standard software. To quantify the mucin, the first step is to set the scale. To set the scale, we need to open Image J and go to *Analyze>Set Scale.* In the length of the line selection (219 pixels) is automatically entered as the "Distance in Pixels". Click the line on the panel and use this to measure the distance of scale bar on the picture. ". Enter the scale bar

length (50 µm) as the "Known Distance" and "um" as the "Unit of Length". ImageJ will automatically convert the "u" to the micrometer symbol (µ). Click "OK".

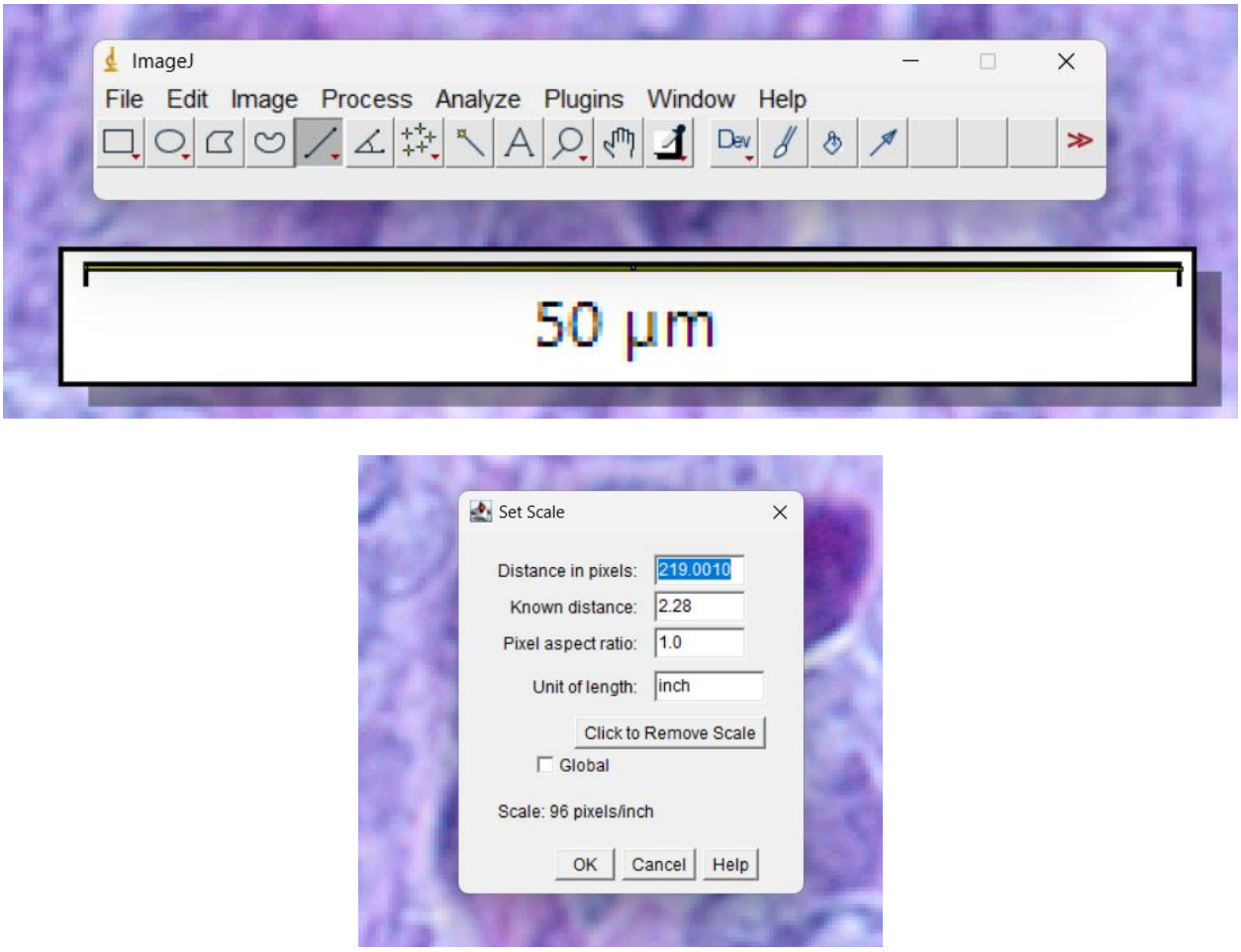
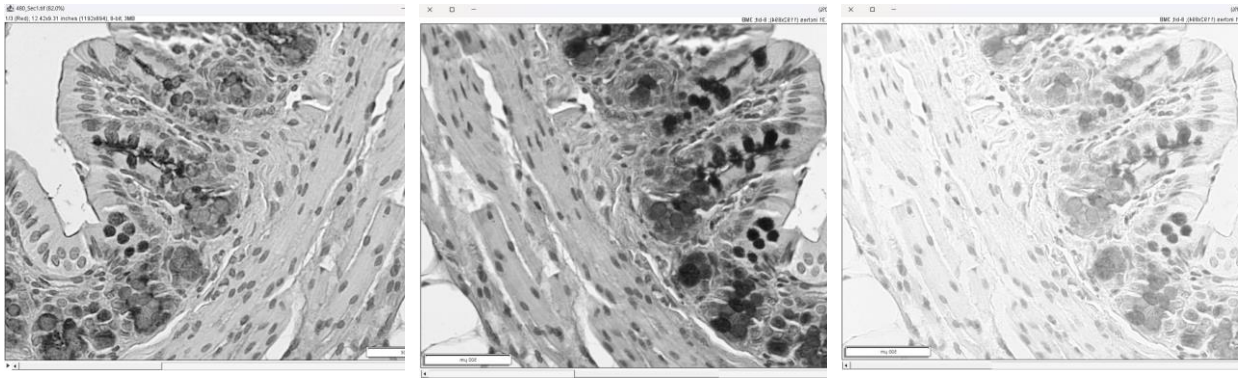


Figure 5.1: Setting scale for measurement of mucin.

Then next step is to set the RGB. For that, open *Image>Type>RGB Stack* command to split the image into red, green and blue channels. Use green channel for this quantification. Then go to *Image>Adjust>Threshold* tool only works with grayscale images. Try to threshold an RGB image and you will get a lot of beeps and the status bar message "RGB images cannot be thresholded". Move the slider to view each of the channels. Notice that the green channel has the best separation. Use the *Image>Stacks>Make Montage* command to view all three channels at the same time (not necessary unless you want to compare between three types). Select the RGB stack (with the Green

channel selected) and press shift-t (*Image>Adjust>Threshold*). The "Threshold" tool opens and the the green channel is automatically thresholded. Unfortunately, ImageJ is not able to correctly threshold this image so the threshold must be manually adjusted. Do this by moving the the lower slider until the stained collagen is highlighted in red.



Red Green Blue

Figure 5.2: Using RGB stack of ImageJ to measure mucin

A crucial step is to erase scale bar. To do that, use the rectangular selection tool to select the scale bar, then press Backspace (Delete on the Mac). If the current background is not white, use the color picker tool (*Image>Color>Color Picker*) to change it to white. Note that the current background color is indicated by the color, which is on bottom, in this case, its white. If you select with rectangle, pressing backspace will result in a white color background.

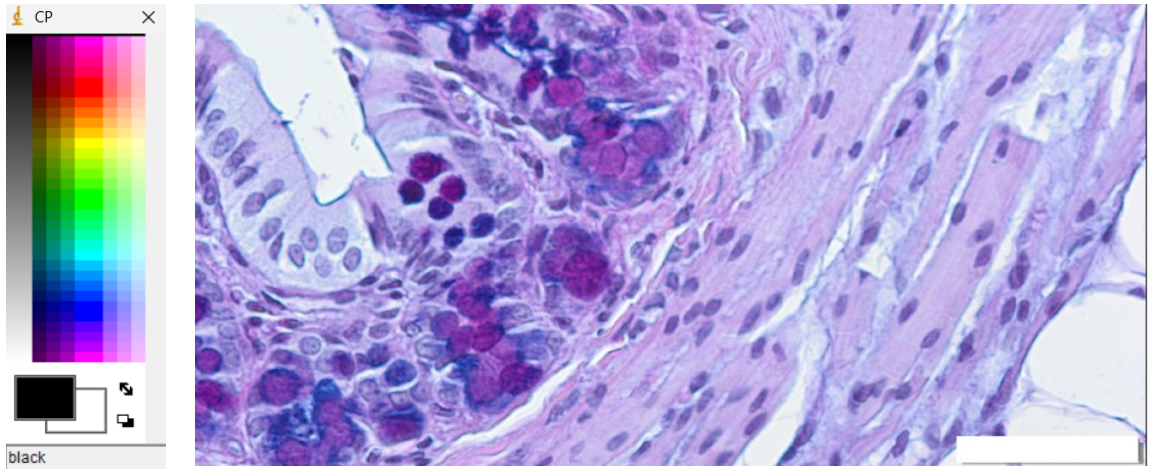


Figure 5.3: Preparation of image to remove artifacts from the picture

To quantify the mucin, go to *Analyze>Set Measurements* dialog and checking "Area", "Area Fraction", "Limit to Threshold" and "Display Label". Then press "m" (*Analyse>Measure*) and the area and percent area will be displayed in the "Results" window. Right click in the "Results" window and select "Save As" to save the results as a tab-delimited text file or select "Copy" to copy the results to the Clipboard. Once you set the measurement, check the global setting. When you open a new image, it will ask you to disable the global calibration, uncheck the box. In this way, all your image will be measured under same calibration and you don't need to set the scale every time.

Figure 5.4: Setting the option for uniform usage of color to quantify mucin

To quantify the acidic and neutral mucin, we need Color deconvolution plugin. Now this plugin has been replaced by Color convulation2 plugin. Download the jar/java file and extract it to the plugin folder of ImageJ. ImageJ>File>Open. Then once your image is opened in the ImageJ, go to color deconvulation2.

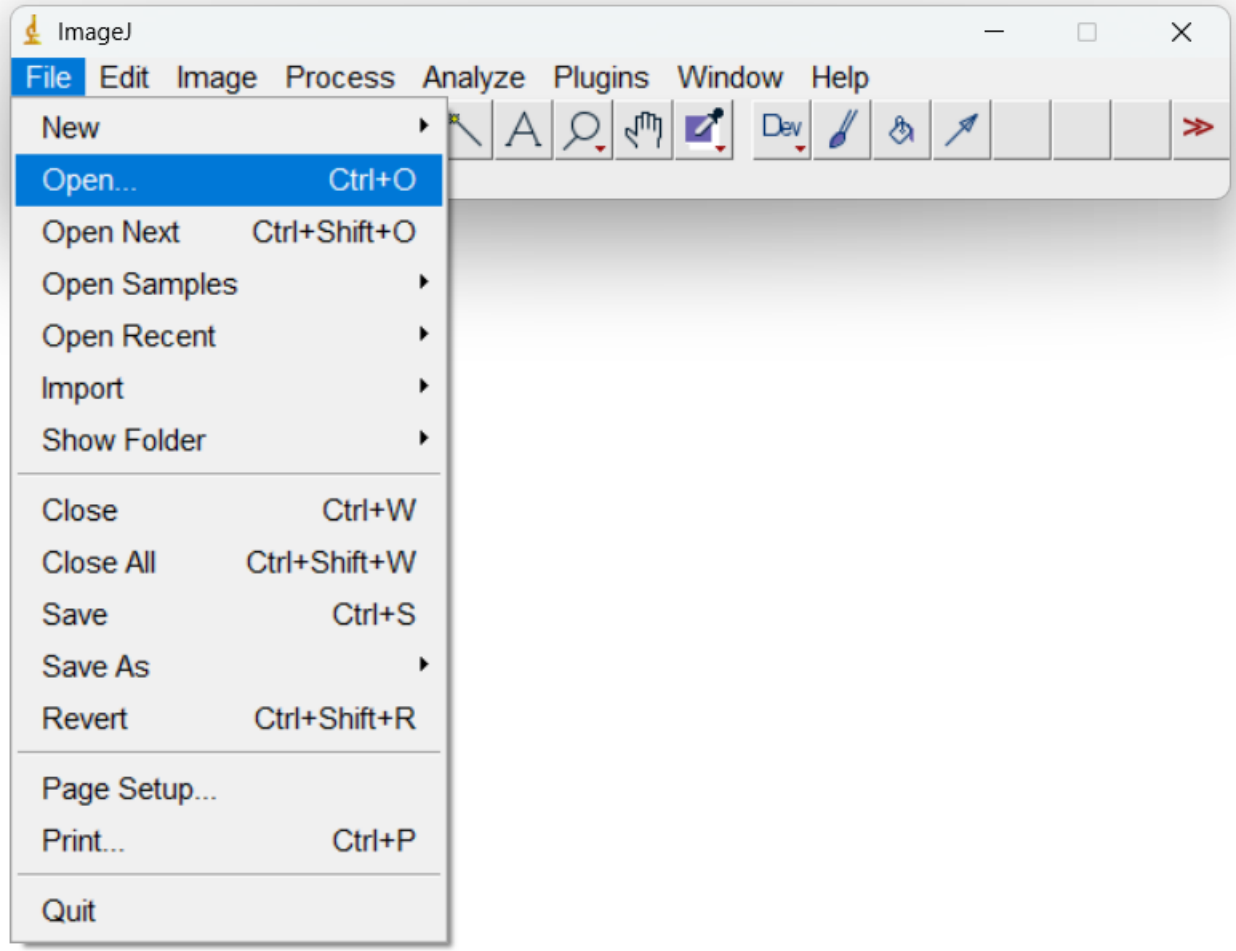


Figure 5.5: Opening image for quantification

In this case, this plugin is shown under plugin dropdown menu.

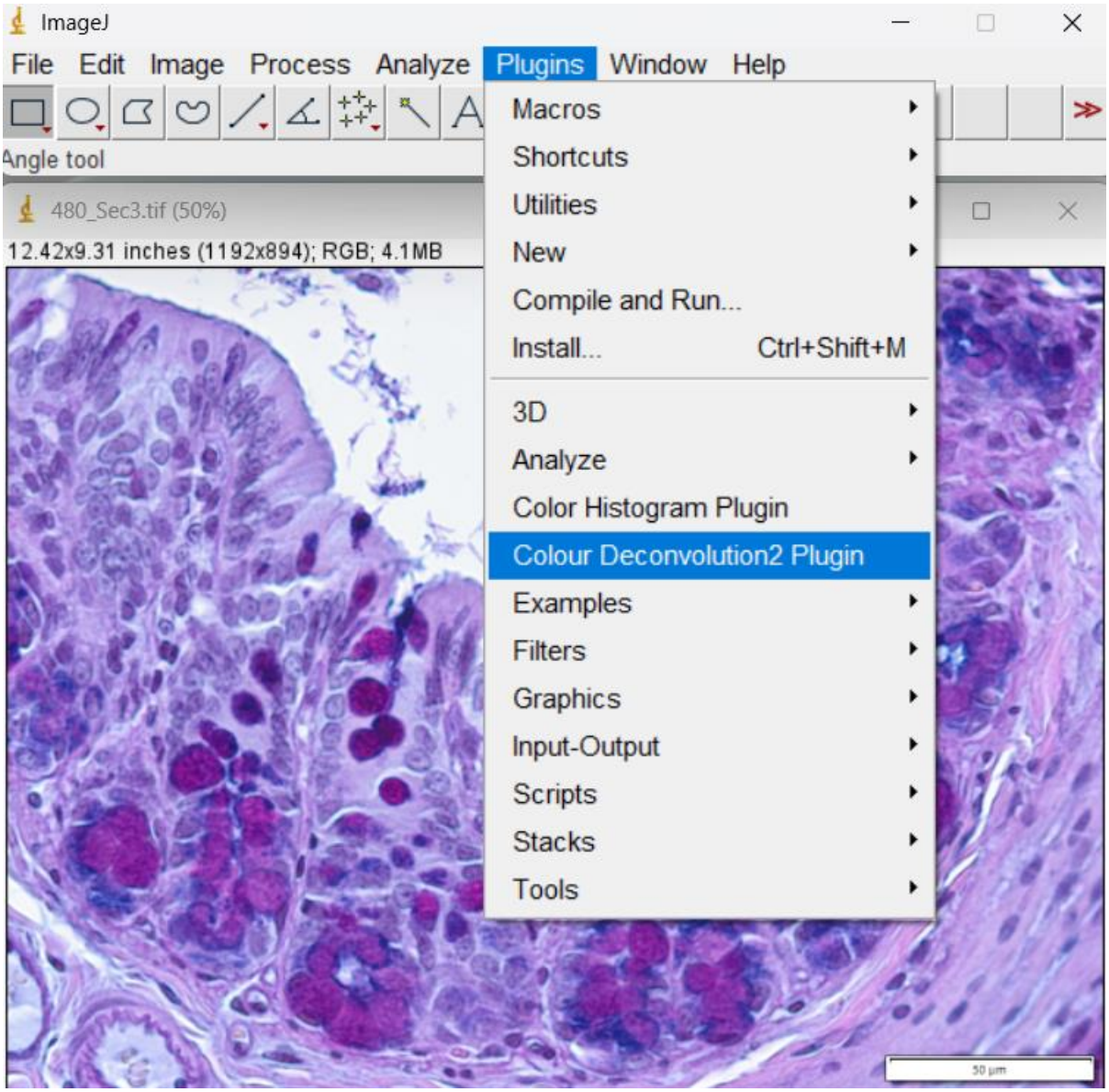


Figure 5.6: Opening color denconvulation option for mucin quantification

Once you do this, it will ask you the type of staining.

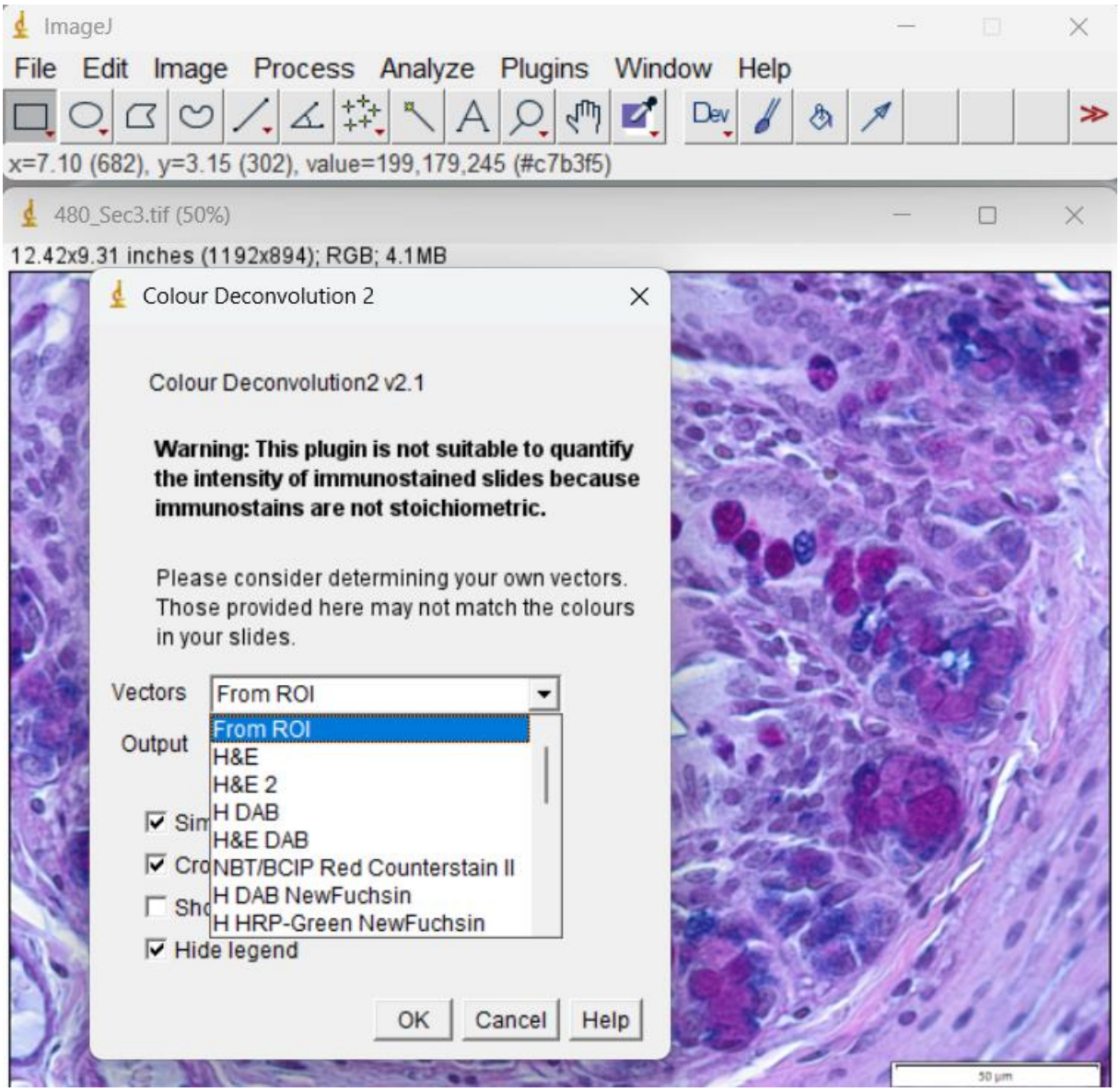


Figure 5.7: Selecting appropriate stain for quantification of mucin

In this case, its Alcian blue.

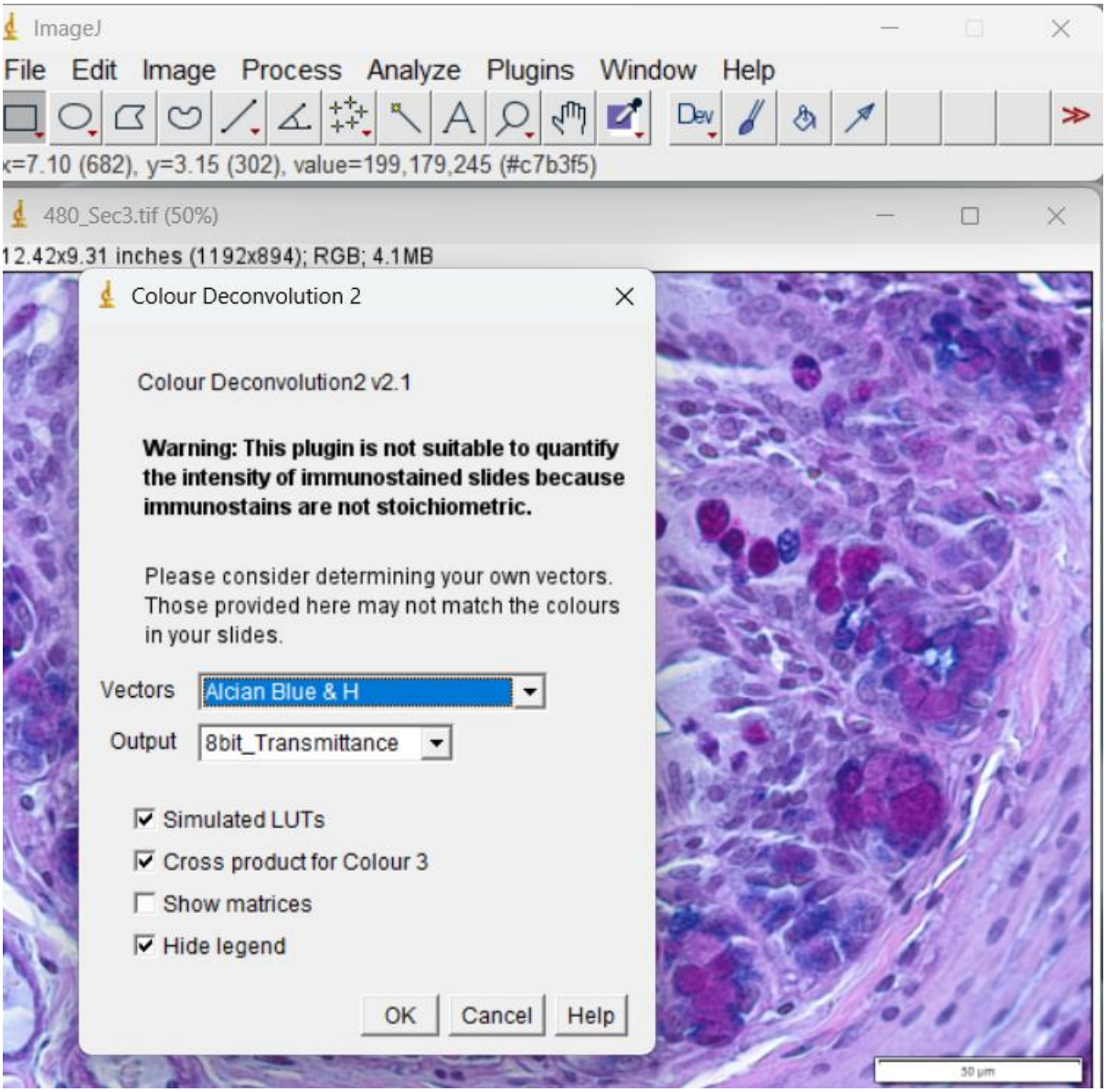


Figure 5.8: Selecting the appropriate staining

You want to separate the blue from magenta/purple. Click OK and it will open 3 different windows with 3 different colors. Here the first one is purple color, second picture is our sample, third one is blue color and fourth one is white. We need to measure the ratio of purple and blue.

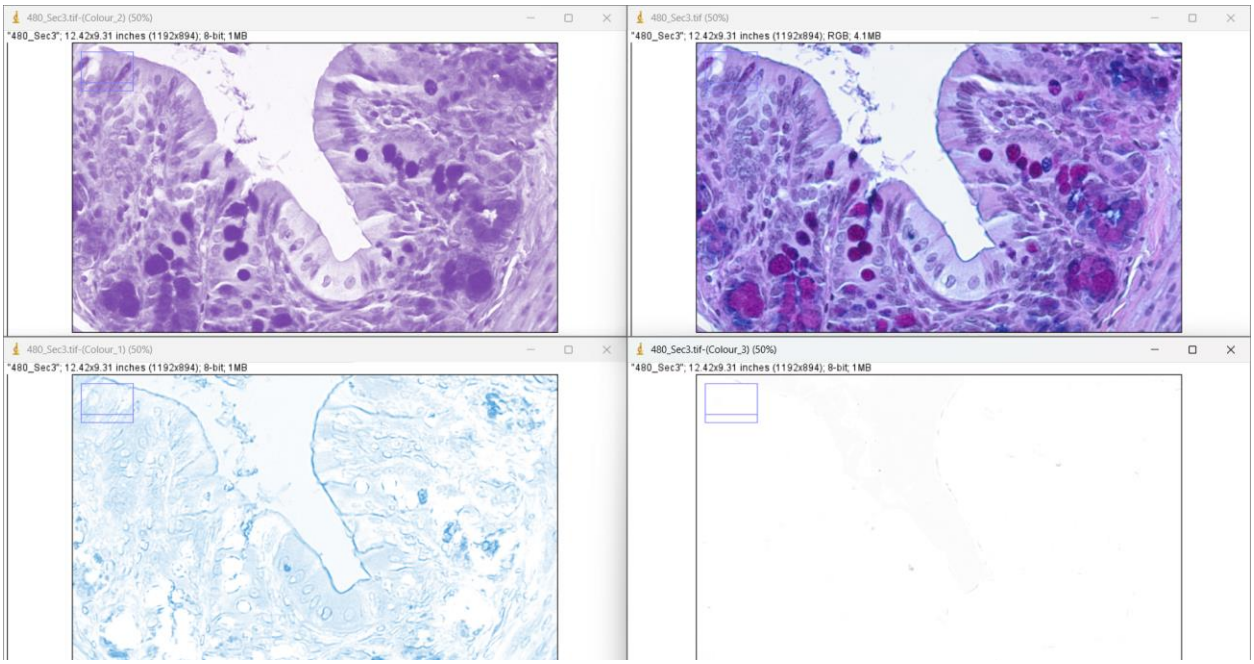


Figure 5.9: Pannel to select the image for quantifying mucin

Select one picture, let's say blue color, go to Image>Adjust>Threshold. Adjust the threshold according as per need.

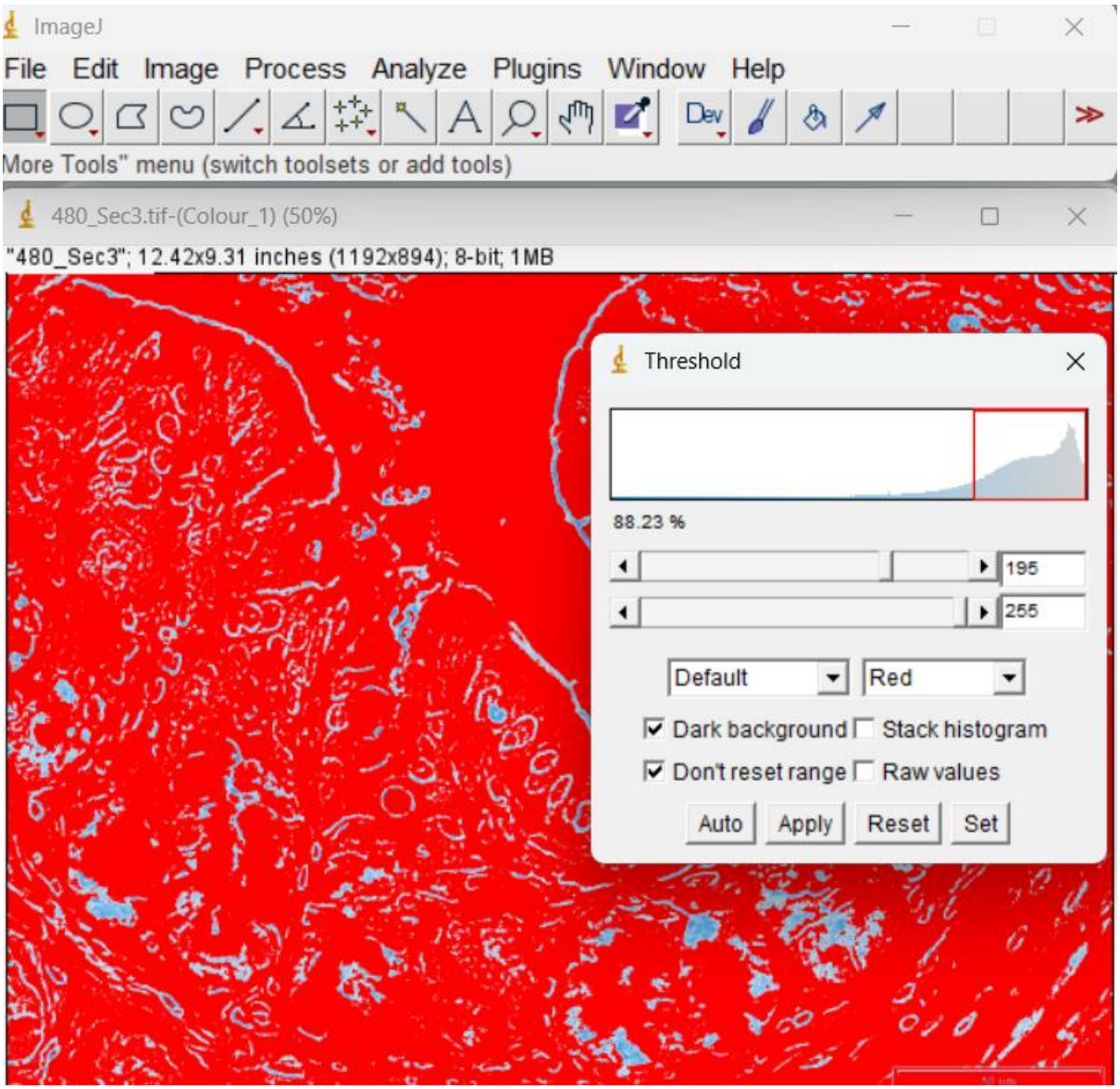


Figure 5.10: Adjusting the threshold for quantification of mucin

After adjusting, click Analyze>Analyze particles. Once you do that, this window will appear. Press OK.

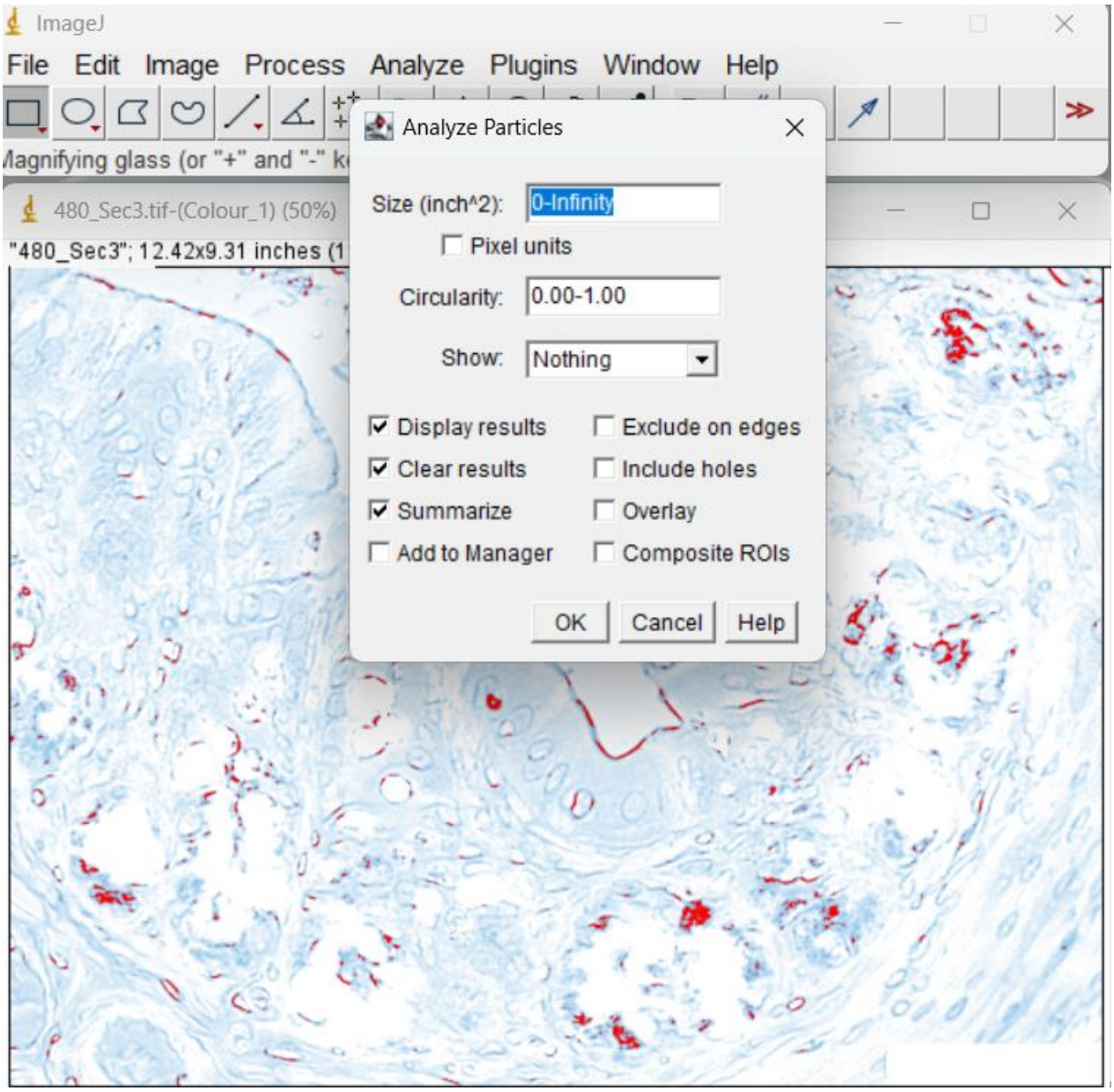


Figure 5.11: Measuring red particles considered as acidic mucin

Once you click OK, you will find this dialogue. Repeat the same for the purple slide.

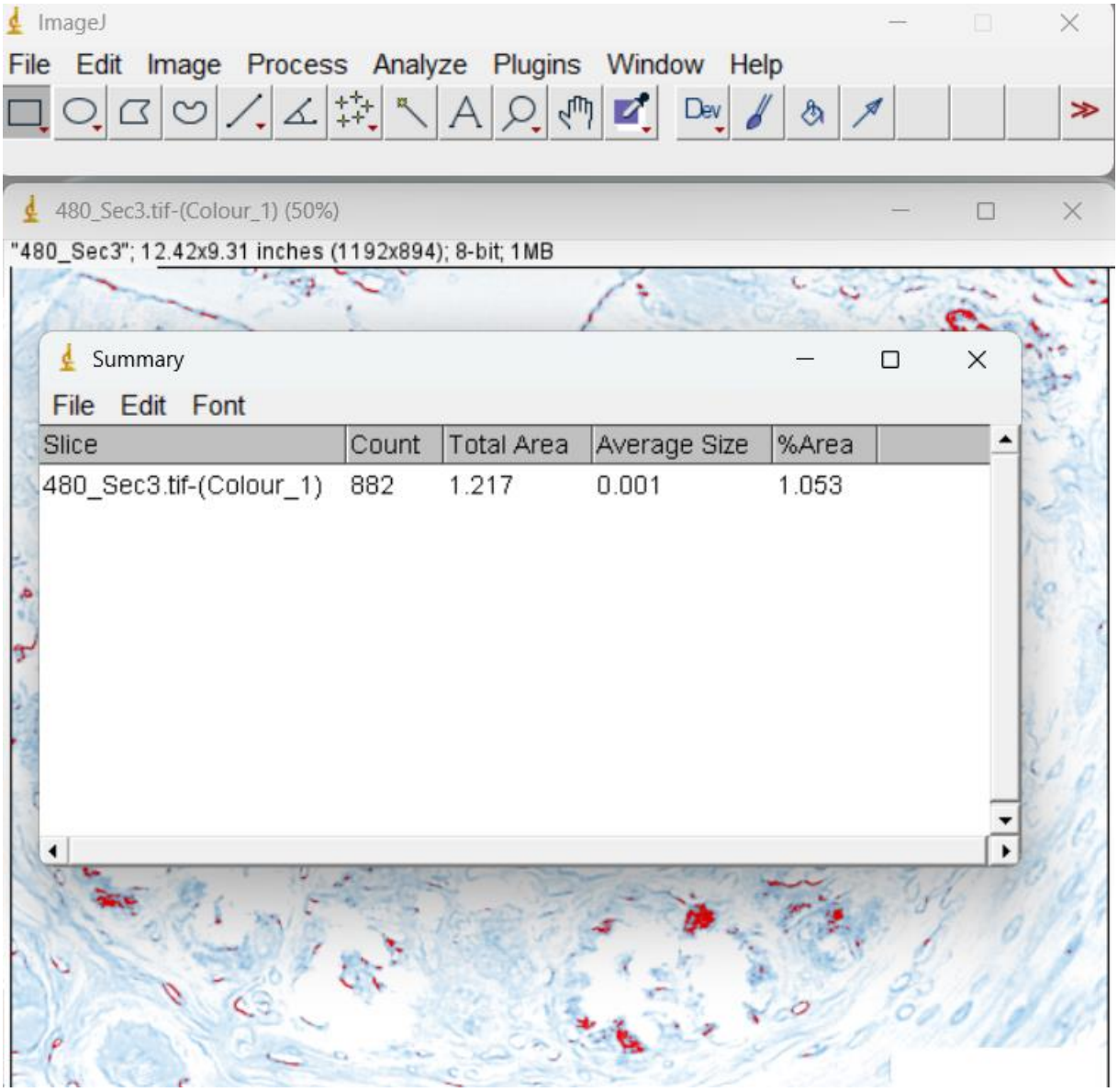


Figure 5.12: Measuring window of mucin quantification (Acidic)

Now its time to measure neutral mucin. Repeat the same but select PAS to identify the magenta/neutral mucin and select the Magenta picture (see below).

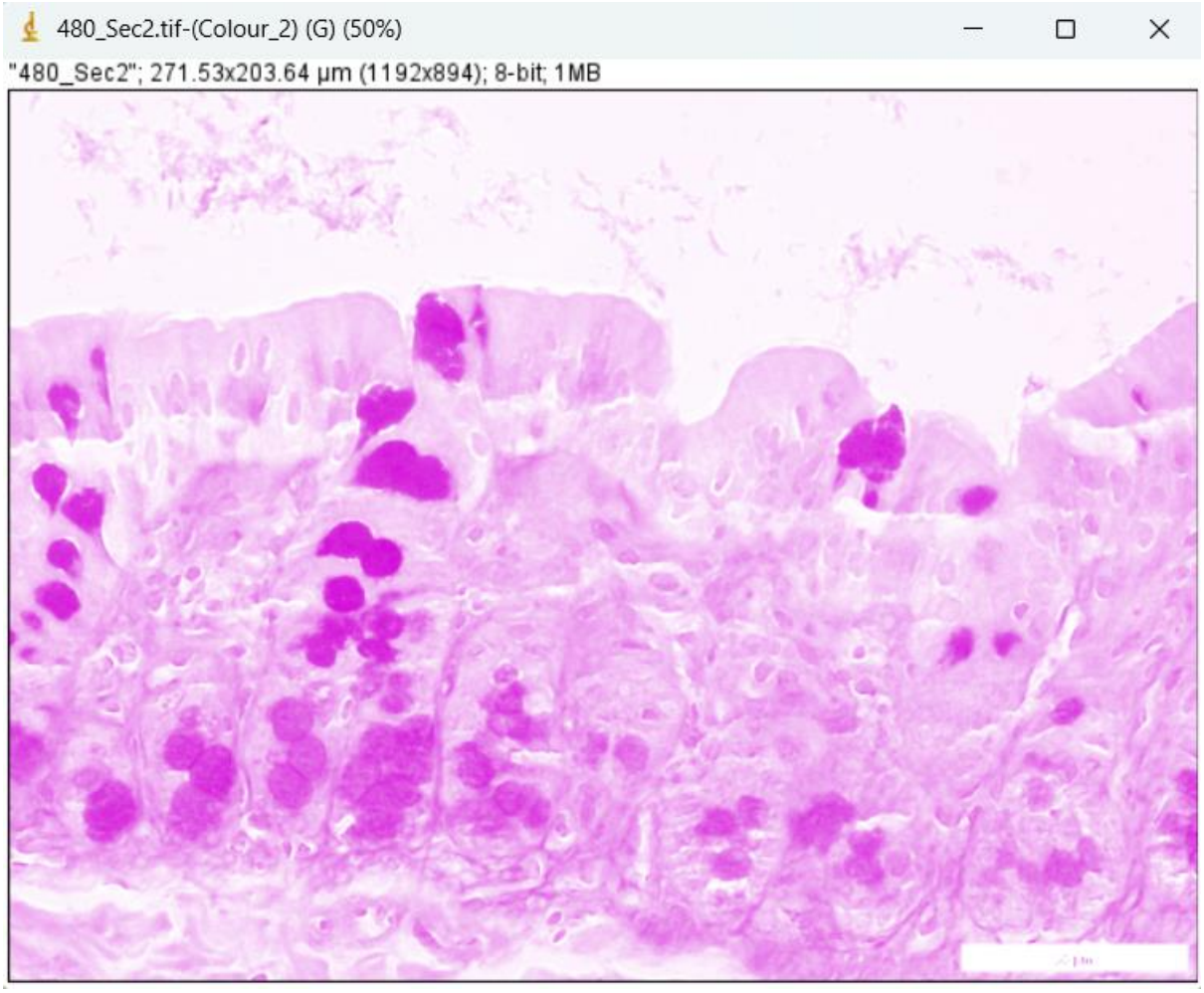


Figure 5.13: Measuring particles considered as neutral mucin

Go to Image>Adjust>Threshold. Adjust the threshold according as per need.

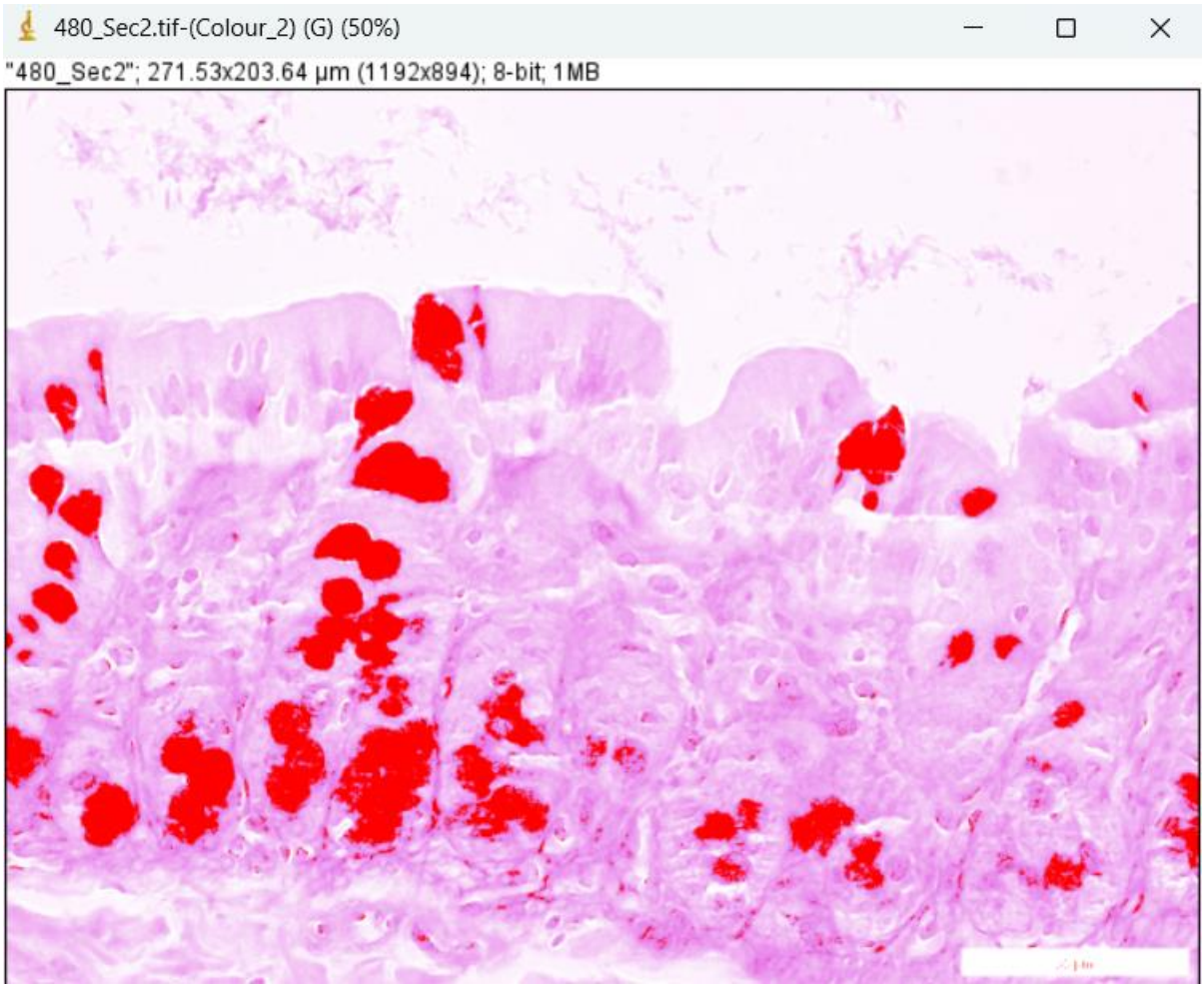


Figure 5.14: Measuring red particles considered as neutral mucin

APPENDIX II

DNA isolation

Cecal genomic DNA was extracted using a FastDNA spin kit with a bead-beating step (MP Biomedicals, Lachine, QC, Canada). In a 2 mL Lysing Matrix E tube, 500 mg feces sample, 825 µL Sodium Phosphate Buffer, and 275 µL of PLS solution were added and mixed with a shake. Then the solution was homogenized at 6.0m/s for 40 seconds. After homogenization, these were centrifuged at 14,000 x g for 5 minutes and supernatant was decanted. After decantation, 978 µL Sodium Phosphate Buffer and 122 µL MT Buffer were added to the tube and homogenized at 6.0m/s for 40 seconds. This step is repeated after resting the samples on ice for 5 minutes. Then the samples were centrifuged at 14,000 x g for 15 minutes and supernatant was transferred to clean tube. Then to the tube, 250 µL of PPS solution were added, shaken vigorously to mix, incubated on a bucket of wet ice for 10 minutes and centrifuged it at 14,000 x g for 2 minutes. Then supernatant was transferred to the binding matrix solution in a new tube, shaken gently by hand to mix, then place on a shaker/rocker for 30 minutes. After this, centrifuged samples at 14,000 x g for 2 minutes and decanted the supernatant and washed the binding mixture pellet by gently resuspending with 1 mL Wash Buffer #1. Then transferred approximately 600 µL of the binding mixture to a SPIN Filter tube and centrifuge at 14,000 x g for 1 minute and emptied the catch tube. After that, added the remaining binding mixture to the SPIN Filter tube, centrifuged as before and emptied the catch tube again. After this, added 100mL of 100% ethanol to the wash buffer 2 and added 500 µL of prepared Wash Buffer #2 to the SPIN Filter tube and gently resuspend using the force of the liquid from the pipette tip to resuspend the pellet. Centrifuged the mixture at 14,000 x g for 2 minutes and discarded the flow-through. Then centrifuged the sample again for 2 minutes to extract residual ethanol from the binding matrix and dried the sample. After drying, transferred

the SPIN Filter bucket to a clean 1.9 mL Catch Tube and added 100 µL TES. At the last step, centrifuged samples at 14,000 x g for 2 minutes to elute purified DNA into the clean catch tube and discarded the SPIN filter. Stored at -80°C for extended periods.

Quantification of DNA

DNA quantification was performed on Thermo Nanodrop Spectrophotometer. For those samples, whose DNA content was low and salt content was higher, de-salting was performed. To perform de-salting, added 10 µl of NaCl (5M in water RNAs free water) to the DNA sample and inverted 3-5 times to mix. Then added 250 µl of 100% cold EtOH to the DNA sample, inverted 3-5 times to mix and incubated the samples overnight at -20˚ C. Then centrifuged the samples at 10,000 g for 15 minutes at 4°C and decanted the liquid. After decantation, added 250 µl of 75% cold EtOH to DNA pellet, centrifuged at 10,000 g for 15 minutes at 4°C and dried the samples for 2-3 hours. Once its dried, dissolved DNA pellet in 50 µl TE Buffer and measured it with Thermo Nanodrop Spectrophotometer to ensure the DNA quantity. Then diluted the sample to get around 10 ng/uL of DNA and stored at -80˚ C before sending it to Integrated Microbiome Resource, Dalhousie University, NS.

Bioinformatics

All microbial analysis has been performed on R studio version 2023.06.1. After setting directory and importing file into R studio, data was prepared using command fnFs <- sort(list.files(path, pattern = "_R1_paired.fastq", full.names=TRUE)) for forward data and fnRs <-sort(list.files(path, pattern="_R2_paired.fastq", full.names=TRUE)) for reverse data. Then we extracted names using sample.names <- sapply(strsplit(basename(fnFs), "_"), '[',1) command.

In step 2, we inspected the forward and reverse sequence using plotQualityProfile(fnFs[1:52]) and plotQualityProfile(fnRs[1:52]) respectively. Then aggregated the data and generated quality plot

for forward and reverse data using plotQualityProfile(fnFs, aggregate = TRUE) and plotQualityProfile(fnRs, aggregate = TRUE) command, respectively.

In step 3, we filtered and trimmed the data using filtFs <-file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz")), filtRs <- file.path(path, "filtered", paste0(sample.names, "_R_filt.fastq.gz")) and out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs,trimLeft = c(17,21), truncLen=c(260, 230), out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(260, 230), maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE,, compress=TRUE, multithread=FALSE) . After this step we saved the data to prevent the risk of losing data. To save the data, we have used saveRDS(out, file="out.vitamin"), out<-readRDS("out.vitamin") command. After this step, we checked the percent output using head(out), out.d <- data.frame(out), out.per<-out.d%>%, mutate(out.percent=(100*reads.out/reads.in)), head(out.per), View(out.d) commands.

In step 4, we checked the error rate and plot error rate using errF <- learnErrors(filtFs, multithread = TRUE), errR <- learnErrors(filtRs, multithread = TRUE), saveRDS(errF, file="errF"), saveRDS(errR, file="errR") and plotErrors(errF, nominalQ=TRUE), plotErrors(errR, nominalQ=TRUE), dadaFs <-dada(filtFs, err=errF, multithread = TRUE), dadaRs <-dada(filtRs, err=errR, multithread = TRUE), saveRDS(dadaFs, file="dadaFs"), saveRDS(dadaRs, file="dadaRs").

In step 5, we merged the pairs using mergers <- mergePairs(dadaFs, filtFs, dadaRs, filtRs, verbose = TRUE), head(mergers[[1]]), saveRDS(mergers, file="mergers") and constructed sequence table seqtab <-makeSequenceTable (mergers), dim(seqtab), table(nchar(getSequences(seqtab))) commands. Then we removed sequence beyond 438:466 using seqtab <- seqtab[, nchar(colnames(seqtab))%in% 438:466] command and removed

chimeras and saved the file using seqtab.nochim <-removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE, verbose = TRUE), dim(seqtab.nochim), sum(seqtab.nochim)/sum(seqtab), saveRDS(seqtab.nochim, file = "seqtab.nochim"), write.csv(seqtab.nochim, file="seqtab.nochim"), read.csv2("seqtab.nochim").

In step 6, we summarized the ASV output using getN <- function(x) sum(getUniques(x)), track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN), sapply(mergers, getN), rowSums(seqtab.nochim)), colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR", "merged","nochim"), rownames(track)<-sample.names, head(track), track.d <- as.data.frame(track), merge.per <- track.d%>%, mutate(merge.percent=(100*merged/denoisedF)), nochim.per <- track.d%>%, mutate(nochimPer=(100*nochim/merged)), outputSum<- cbind(track,out.per$out.percent,merge.per$merge.percent,nochim.per$nochimPer), colnames(outputSum) <-c("input", "filtered", "denoisedF", "denoisedR","Merged","Nochim", "out.Percent", "Merge.Percent","Nochim.Percent"), outputSum.

Last step in this process is assigning taxonomy. We have used silva taxonomy in this study. To assign taxonomy, we have used SILVA <- "C:/Users/kshru/Desktop/HossainTest/silva_nr_v138_train_set.fa.gz", SILVA.species <- "C:/Users/kshru/Desktop/HossainTest/Silva_species_assignment_v138.fa.gz", SILVA, SILVA.species, taxa.SILVA <- assignTaxonomy(seqtab.nochim, SILVA, multithread = T), head(taxa.SILVA), taxa.SILVA.species<- addSpecies(taxa.SILVA,SILVA.species), saveRDS(taxa.SILVA.species, file ="taxa.SILVA.species"), taxa.SILVA.print <- taxa.SILVA.species, rownames(taxa.SILVA.print) <-NULL, dim(taxa.SILVA.print), head(taxa.SILVA.print), write.csv2(taxa.SILVA.print, file="SILVA.taxa"),

read.csv2("SILVA.taxa"), metadata<-

read.csv("C:/Users/kshru/Desktop/HossainTest/Hossain16SMetadataTest.csv", row.names = 1, header = TRUE), metadata, names <-rownames(seqtab.nochim), samdf <- data.frame(metadata), rownames(samdf), library(phyloseq), ps<- phyloseq(otu_table(seqtab.nochim, taxa_are_rows = FALSE), sample_data(samdf), tax_table(taxa.SILVA.species)), saveRDS(ps, file="ps.Hosaain"), ps.

APPENDIX III

Metabolite analysis

Dry, stool pellets were homogenized with a spatula and weighed out at 10-15 mg for all samples and a pooled quality control (QC) sample. Briefly, a pooled QC sample was prepared by taking 5 mg of homogenized stool sample from all 47 samples within the study population. A solution containing (70:30, MeOH:H_2O) mix with 4 μM of recovery standards (trimethylamine-d9 N-oxide, creatinine-d3, 15N2-ornithine, 15N-histidine, choline-d9, carnitine-d3, 4-fluoro-L-phenylalanine, 3-fluoro-L-tyrosine, gamma-aminobutyric acid-d6, methylhistidine-d3) was made and added to sample vials at 200 μL. Samples were then vortexed for 15 minutes at 2000 rpm and centrifuged for 10 minutes at 4000 rpm. 100 μL of supernatant was removed from vials and set aside prior to adding an additional 200 μL of the recovery standard mixture and repeating the vortexing and centrifugation process. 200 μL of supernatant was removed and combined with the first 100 μL of supernatant, yielding 300 μL of supernatant that was evaporated to dryness using nitrogen gas and subsequently reconstituted in 40 μLof a (70:30, MeOH:H_2O) mix containing internal standards (IS) 40 μM of Cl-Tyr and NMS. All reconstituted samples were frozen at -80 °C and a 20 μL aliquot was transferred into a polypropylene vial for CE-MS analysis. An Agilent 6230B time-of-flight (TOF) mass spectrometer with an electrospray ionization (ESI) source equipped to an Agilent G7100A capillary electrophoresis (CE) unit was used for all experiments (Agilent Technologies Inc., Mississauga, ON, Canada). An Agilent 1260 Infinity Isocratic pump and a 1260 Infinity degasser were applied to deliver sheath liquid. A sheath liquid composition of 0.1% formic acid in (60:40 MeOH:H_2O) and (70:30 MeOH:H_2O) at a flow rate of 10 μL/min were used for positive and negative ion mode, respectively. For real-time mass correction, reference ions purine (20μL) and hexakis(2,2,3,3-tetrafluoropropoxy)phosphazine (HP-921, 20μL) were spiked into the sheath liquid (400 μL) at 0.02% *v/v* to provide constant mass signals. The nebulizer spray was set

off during the serial sample injection before being switched on at 4 psi (27.6 kPa) following voltage application. The source temperature was set to 300 °C and drying gas was delivered at 4 L/min. The instrument was operated under a 2 GHz extended dynamic range for positive and negative modes of detection. The Vcap, fragmentor, skimmer and octupole RF voltage were set to 3500 V, 120 V, 65 V and 750 V, respectively. Separations were performed on bare fused-silica capillaries with a 50 μm internal diameter, a 360 μm outer diameter and a total length of 135 cm (Polymicro Technologies Inc.,AZ, USA). A capillary window maker (MicroSolv, Leland, NC, USA) was used to remove 7 mm of the polyimide coating on both ends of the capillary. All stool extract and QC samples were analyzed by MSI-CE-MS under two different configurations (positive and negative ionization modes). An acidic background electrolyte (BGE, 1 M formic acid with 15% vol acetonitrile, pH 1.8) was used for cationic metabolites under positive ion mode while an alkaline BGE (50 mM ammonium bicarbonate, pH 8.5 adjusted with ammonium hydroxide) was used for anionic metabolites under negative ion mode. Stool extracts were injected hydrodynamically at 100 mbar (10 kPa) alternating between 5 s for each sample plug and 75 s for each BGE spacer plug which was electrokinetically injected at 30 kV. In total, thirteen discrete samples were analyzed within one run of 45 min. The applied voltage was set to 30 kV at 25 °C for CE separations together with a gradient pressure of 2 mbar/min. A list of metabolites was selected using in-house metabolite library and un-targeted list using MZmine software. This list of m/z (mass/charge) was extracted as extracted ion electropherograms (EIEs) from TIEs using Agilent MassHunter Workstation Qualitative 13 Analysis Software (version B.10.00, Agilent Technologies.) in profile mode with a 10 ppm masswindow and a smoothing function “Savitsky-Golay quadratic/cubic function (7 pts)”. Corrective integration of EIE peaks yielded migration times (MT) and peak areas (PA) for each m/z that were transferred to Microsoft Excel (Microsoft

Office) and normalized to the MT and PA of internal standard to determine relative peak areas (RPAs) and relative migration times (RMTs), ultimately correcting for injection volume variability. Cl-Tyr and NMS were the primary internal standards used for normalization for positive and negative mode data, respectively. However, the metabolites with corresponding deuterated standards were normalized using their deuterated standards. Subsequently, all RPAs were normalized to their respective stool sample weight and duplicate RPAs from the same sample were averaged. The averaged, dry weight-normalized RPA data was used for all statistical analysis.

APPENDIX IV

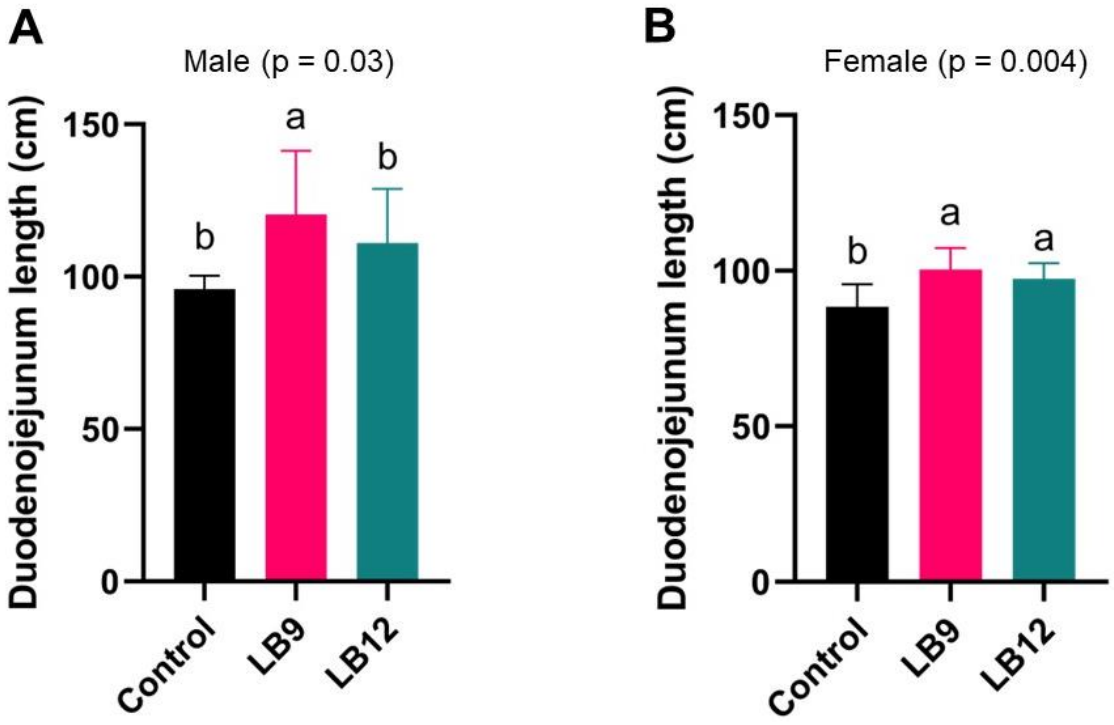


Figure 5.15: Duodeno-jejunum length of sprague-dawley rats after six weeks of exposure to vitamin-deficient diets before adjusting with body weights. Duodeno-jejunal lengths were significantly greater (p=0.03) in LB9 male rats (120.37±20.93 mm) compared to the control (95.92±4.42 mm) but no significant changes have been observed in males LB12 group. Similar findings were observed in both LB9 (100.43±6.90 mm), and LB12 (97.37±5.08 mm) females compared to the control (88.43±7.19 mm) (p=0.004). [ab] Groups with different letters are significantly different by one way ANOVA-test. Values are expressed as mean ± SD, n=8 in LB9 and LB12 and n=7 in Control in males and n= 8 / group in females.

Table 5.1: Body weights, organ weights* after six weeks of treatment with vitamin B9 and B12 deficient diet

Treatment	Body weight*	Organ weights (%)							
		Male				Female			
		Heart	Brain	Liver	Kidney	Heart	Brain	Liver	Kidney
Control	359.67±117.69	0.31±0.03	0.49±0.17	3.04±0.35	0.64±0.02	0.29±0.13	0.75±0.29	3.05±0.4	0.65±0.1
LB9	347.83±111.42	0.29±0.03	0.4±0.04	3.3±0.51	0.68±0.13	0.29±0.11	0.64±0.07	2.87±0.53	0.61±0.05
LB12	362.5±103.23	0.26±0.1	0.34±0.14	3.36±0.57	0.7±0.15	0.41±0.37	0.77±0.19	2.91±0.24	0.73±0.24
p-value	0.7	0.39	0.1	0.41	0.61	0.5	0.4	0.34	0.345

Values are expressed as mean ± SD, n=8 in LB9 and LB12 and n=7 in Control in males and n= 8 / group in females. [#] Based on treatment. * Adjusted weight and length with body weight using the formula below:

$$\%\, weight = \frac{Organ\ weight}{Body\ weight} \times 100$$

Table 5.2: Intestinal length * after six weeks of treatment with vitamin B9 and B12 deficient diet

	Organ length (%)			
	Duodeno-jejunal length			
Treatment	Male	Female	Ileum	Colon
Control	20.52±1.63	34.07±4.35	0.62±0.34	3.32±0.52
LB9	24.65±4.4	36.99±5.21	0.64±0.33	3.46±0.98
LB12	24.32±5.17	36.87±4.22	0.49±0.19	3.5±1.15
p-value	0.15	0.375	0.67	0.96

Values are expressed as mean ± SD, n=8 in LB9 and LB12 and n=7 in Control in males and n= 8 / group in females. * Adjusted length with body weight using the formula below:

$$\%\, weight = \frac{Organ\ length}{Body\ weight} \times 100$$