Doctoral Thesis

Nutritional study on the influence of dietary vitamin B6 on colon luminal environment and heart

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Abbreviations

AD	Alzheimer's disease
ALP	Alkaline phosphatase
AMD	Age-related macular degeneration
ANOVA	Analysis of Variance
AOX	Aldehyde Oxidase
ASD	Autism Spectrum Disorder
AT	Aminotransferase
B_{12}	Cobalamine
B_2	Riboflavin
B_6	Vitamin B_6
C-RP	C-Reactive Protein
CAD	Coronary Artery Disease
CD	Crohn's disease
CE-TOFMS	Capillary Electrophoresis-time of Flight Mass Spectrometry
CHD	Coronary Heart Disease
CNAs	Circulating Nucleic Acids
CRC	Colorectal Cancer
CSF	Cerebro Spinal Fluid
CVD	Cardiovascular disease
CVD	Cerebrovascular diseases
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERβ	Estrogen receptor-β
ESI	Electrospray ionization
F	Forward
FAD	Flavin Adenine Dinucleotida
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
HCY	Homocysteine
HPLC	High-performance liquid chromatography
IBD	Inflammation bowel disease
IBS	Irritable bowel syndrome
IgA	Immunoglobulin A
IL	Interlukin
KAT	Kynurenine aminotransferase
KEGG	Kyoto Encyclopedia of Genes and Genomes
MS	Mass Spectrometry
MS	Methionine synthetase
MUCs	mucins
MWCO	Molecular Weight Cut-Off
NAFLD	Non-alcoholic fatty liver diseases
NCBI	National Center for Biotechnology Information
Nrf2	Nuclear Factor (Erythroid-derived 2)
ODC	Ornithine decarboxylase
OPA	*o*-phthaldialdehyde
Orn	Ornithine
PCA	Perchloric acid
PD	Parkinson disease

PDXK	Pyridoxal kinase
PDXP	Pyridoxal phosphatase
PL	Pyridoxal
PLP	Pyridoxal 5′-phosphate
PM	Pyridoxamine
PMP	Pyridoxamine 5′-phosphate
PMS	Premenstrual syndrome
PMSF	Phenylmethylsulfonyl fluoride
PN	Pyridoxine
PNP	Pyridoxine 5′-phosphate
PNPO	Pyridoxal phosphatase oxidase
qPCR	quantitative Polymerare Chain Reaction
R	Reverse
RA	Rheumatoid Arthritis
RDA	Recommended Daily Allowances
RDI	Recommended Daily Intake
SAH	S-Adenosylhomocysteine
SAM	S-Adenosylmethionine
SE	Standard error
SLE	Systemic lupus erythematous
STZ	Streptozotin
Tau	Taurine
TCA	Tricarboxylic acid
TLRs	Toll-like receptors
TNF-α	Tumor Necrosis Factor - α
UC	Ulcerative colitis

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Chapter I
Introduction

I. Background

Vitamin B_6 (B_6) is an essential water-soluble vitamin required for normal growth and development in mammals. B_6 is the collective term for metabolically and functionally compounds including pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated forms pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP), and pyridoxamine 5′-phosphate (PMP). PLP is an active biologic form of B_6, acts as a co-factor in over 140 distinct enzyme reactions that are involved in the metabolisms of proteins, lipids, and carbohydrates, neurotransmitters, nucleic acids, one carbon units, and immune modulatory metabolites and others.

Beyond its the role as the co-factor, B_6 has the preventive roles in certain diseases including colon diseases [irritable bowel syndrome (IBS), intestinal bowel disease (IBD), colitis, colon cancer (CRC)], heart diseases [coronary bowel disease (CVD), atherosclerosis, stroke], brain diseases [Parkinson's disease (PD), schizophrenia, Alzheimer's disease (AD)], autoimmune disease [rheumatic arthritis (RA)].

Many diseases are differently expressed in men and women including colon diseases [IBS, Crohn's disease (CD), Ulcerative disease (UC), CRC], brain diseases [AD, PD, autism spectrum disorder (ASD)], autoimmune diseases [RA, systemic lupus erythematosus (SLE)].

In this study, I focused on the effects of B_6 on colon and heart diseases. Accumulating studies have suggested the defensive role of dietary B_6 on colon diseases. B6 has the potential mechanisms to prevent colon diseases include reducing cell proliferation, oxidative stress, inflammation, angiogenesis, and increased intestinal mucins. Furthermore, there is growing evidence that the incidence of colon diseases is affected by gender difference. The suggested mechanisms responsible for the effects of gender on the diseases include biological difference such as hormones and chromosomes as well as environmental factors such as nutrition and microbiota. Inflammation and metabolisms are also responsible for the influence of gender on diseases. However, the underlying mechanisms of the effects of B_6 and gender differences on colon diseases are still unclear.

Several epidemiological studies have shown the positive benefits of B_6 to heart diseases. The suggested mechanisms responsible for the effects of B6 on the heart diseases include homocysteine, purinergic receptors, inflammation marker C-Reactive Protein (CRP), and inflammatory pathway; kynurenine pathway. However, the exact mechanisms

of the effect of B_6 on heart diseases are still unclear.

II. The objective of study

The objective of this study is to determine the effects of dietary vitamin B_6 and gender difference on colon luminal environment of rats and to determine the effects vitamin B_6 on metabolite concentration in heart of rats.

Chapter II
Literature Review

I. Vitamin B_6 (B_6)

B_6 is an essential water-soluble vitamin required optimal health (Tryfiates, 1981; Merril & Henderson, 1987). There are three primary forms of B_6, pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorylated forms, pyridoxine 5-phosphate (PNP), pyridoxal 5-phosphate (PLP), and pyridoxamine 5-phosphate (PMP) (Umbreit, 1954; Johansson *et al*., 1974).

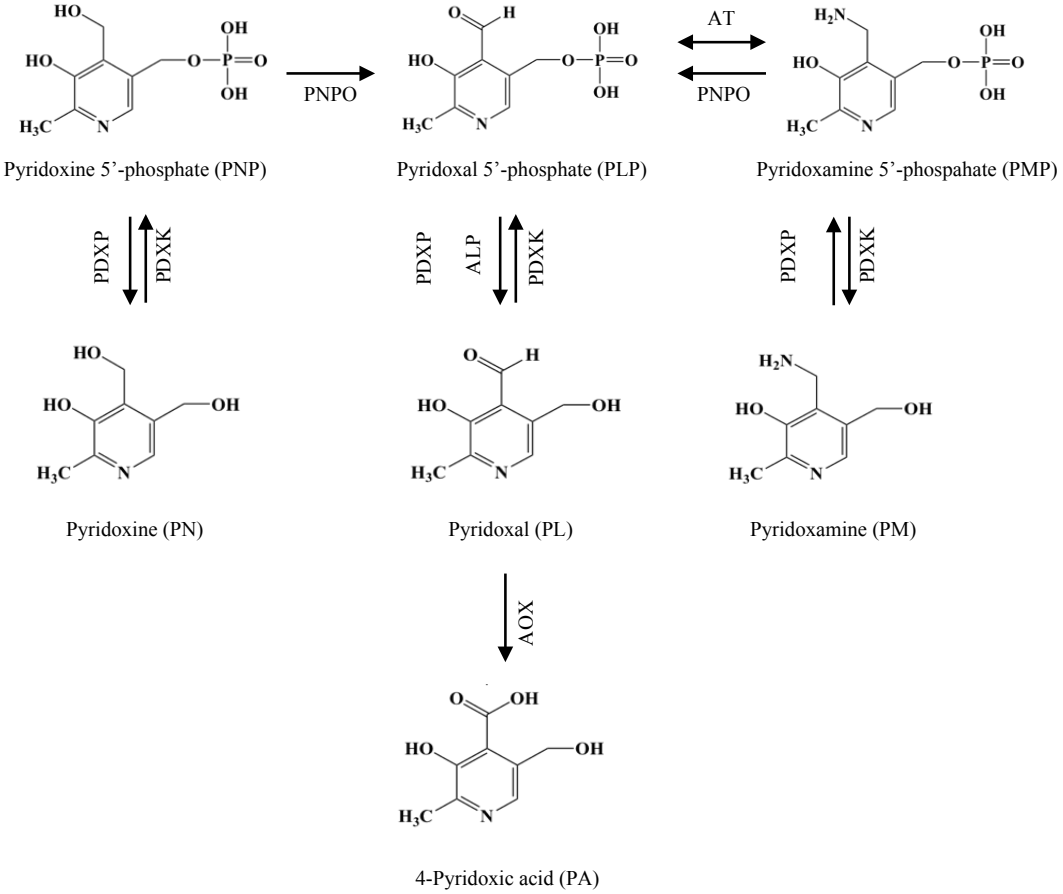

Figure 1. The basic B_6 and their phosphorylated form. Pyridoxal phosphatase oxidase (PNPO), aminotransferase (AT), pyridoxal phosphatase (PDXP), pyridoxal kinase (PDXK), alkaline phosphatase (ALP), aldehyde oxidase (AOX) (Ueland *et al*., 2015).

1. B_6 and its source

B_6 has to be obtained from the diet because a human cannot synthesize it. B_6 is present in wide variety of food including animals and plants (Vanderschuren *et al*., 2013; Ross *et al*., 2014; Combs *et al*., 2008; Domke *et al*., 2005; Mackey *et al*., 2005; Turner and Frey, 2005). For example, from animals such as meat (turkey, beef), beef liver and other organ

meats, poultry (chicken), fish (tuna, salmon), eggs, milk, milk product (cheese, yogurt). From plant such as cereals, nuts, starchy vegetable (potatoes, sweet potatoes), vegetables (spinach, cabbage, Bok choy), non-citrus fruit (banana, avocado). Since B_6 can be lost during processing and storage, it is found fortified in some products such as cereals (Ross *et al*., 2014; Gregory and Kirk, 2006). B_6 is available in multi-vitamins, in supplements containing other B complex vitamins, and as a stand-alone supplement (Simpson *et al*., 2010). The most common B_6 vitamin in supplements is PN (in the form of PN HCl, although some supplements contain PLP.

2. B_6 and its bioavailability

Bioavailability of B_6 can vary considerably from various dietary sources (Gregory and Kirk, 1981). The chemical forms of B_6 tend to vary among foods of plant and animal origin; plant tissues contain mostly PN, whereas animal tissues contain mostly PL and PM. It is estimated that in various foods about 75% of B_6 is bioavailable biologically (Tarr *et al*., 1981). The PN form of B_6 tends to be reasonably stable to storage than the PL or PM (Combs, 2008). It takes about one year for about 25% of the B_6 in various foods to be lost. The stability of B_6 in its original form can be affected by temperature, canning, moisture, and storage time. In contrast, the type of enriched PN HCl has proven to be more stable against such changes, with estimated retention of 90 to 100% (Gregory and Kirk, 2006).

3. B_6 and its requirements

B_6 requirements vary worldwide, and there are no exact amounts for these requirements. Recommended Dietary Allowance (RDA) of B_6 by Ministry of Health, Labour and Welfare (2015-2019) for adult Japanese are 1.4 mg/day and 1.2 mg/day (males and female, respectively). Meanwhile, RDA for grown-up Indonesia people is 1.3 mg/day by Regulation of the Ministry of Health of the Republic of Indonesia (2013).

4. B_6 and its metabolism

The phosphorylated B_6 in the diet is thought to be hydrolyzed to PL, PM, and PN by intestinal phosphatases (IP) before absorption (Fig. 2). PN-glucoside is hydrolyzed to PN by a glucosidase. The absorbed PN, PM and PN are rapidly cleared, principally by uptake into the liver, where they are PDXK. PNP and PMP are then converted to PLP by PNPO. PLP re-enters the circulation bound to the lysine-190 residue of albumin. Delivery of active cofactor to the tissues requires hydrolysis of circulating PLP to PL by the ectoenzyme tissue-nonspecific ALP. PL can cross the blood-brain barrier (and enter other tissues) but then needs to be re-phosphorylated by PDXK to produce active cofactor (Clayton, 2006).

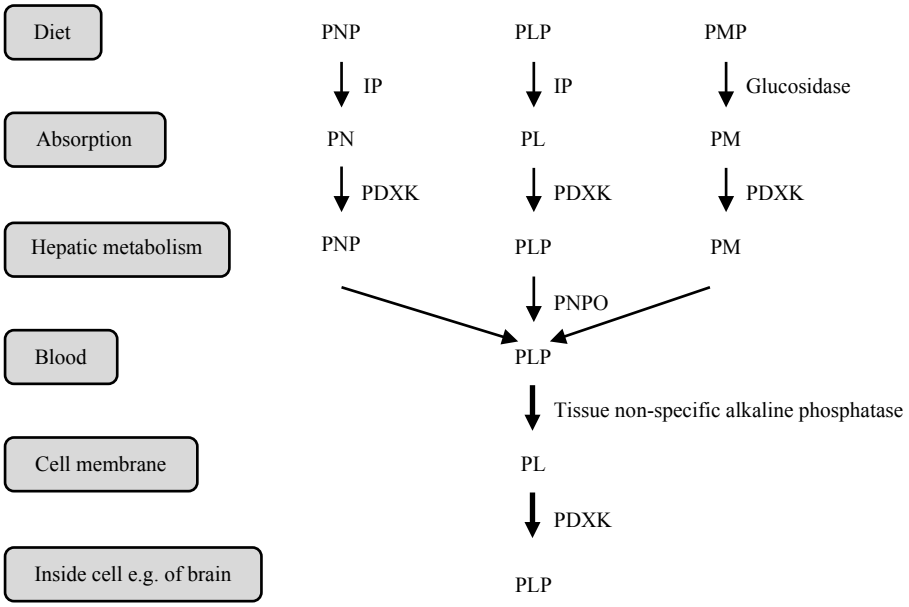

Figure 2. Conversion of dietary vitamin B_6 to intracellular pyridoxal 5'-phosphate cofactor. Intestinal phosphates (IP), pyridoxal kinase (PDXK), pyridoxal phosphatase oxidase (PNPO) (Clayton, 2006).

II. B_6 and its functions

PLP is the active biologic form of B_6 that functions as an enzyme co-factor and regulator for 140 enzyme-catalyzed reactions (Percudani and Peracchi, 2009). Many biochemical reactions are catalyzed by PLP-dependent enzymes involved in essential biological processes, such as the metabolisms of amino acids, carbohydrates, and fatty acids. In amino acid metabolisms, transamination reactions of amino acids leading to corresponding alpha-keto acids require the presence of the coenzyme PLP to catalyze the required deamination and dehydration reactions in the presence of beta-hydroxyl or sulfhydryl groups (Meister, 1965). Since PLP has a primary role in amino acid metabolisms, studies suggest that vitamin B_6 requirements are affected by protein consumption. In glucose metabolism, PLP coenzymes assist the enzymatic cleavage of glycogen during glucose synthesis. PLP is also necessary for acid catalysis in glycogenesis; glycogen phosphorylase requires PLP as a prosthetic group that serves as a general acid-base catalyst (Ross *et al*., 2014). In lipid metabolisms, PLP plays an essential role in the biosynthetic pathway of carnitine. This process is vital for transport long chain fatty acids. Also, it is important in protecting the cell from oxidative damage caused by lipid peroxidation (Ross *et al*., 2014). It could be beneficial by reducing levels of oxidative stress and preventing complications in patients with heart disease and diabetes (Jain *et al*., 2002).

The human requirement for another B vitamin, niacin, can be met in part by the conversion of tryptophan to niacin, as well as through dietary intake. PLP is a coenzyme

for a critical reaction in the synthesis of niacin from tryptophan; thus, adequate B_6 decreases the requirement for dietary niacin (Leklem, 1999).

PLP acts as a coenzyme for a critical enzyme involved in the mobilization of single-carbon functional groups (one-carbon metabolism). Such reactions are included in the synthesis of nucleic acids. The effect of B_6 deficiency on the function of the immune system may be partly related to the role of PLP in one-carbon metabolism.

1. B_6 and the kynurenine pathway

The essential amino acid, tryptophan is mainly catabolized along the so-called kynurenine pathway that produces a variety of compounds, collectively termed kynurenines. Several enzymes involved in the kynurenine pathway require PLP as co-factor (Ueland, 2016). The enzyme to convert kynurenine to other product such as tryptophan dioxygenase or indoleamine dioxygenase and the kynurenine pathway-related product such as kynurenine acids, 3-hydroxykynurenine, xanthurenic acid, anthranilic acid, 3-hydroxyanthranilic acid, and quinolinic acid related to heart disease, brain disease, and autoimmune diseases.

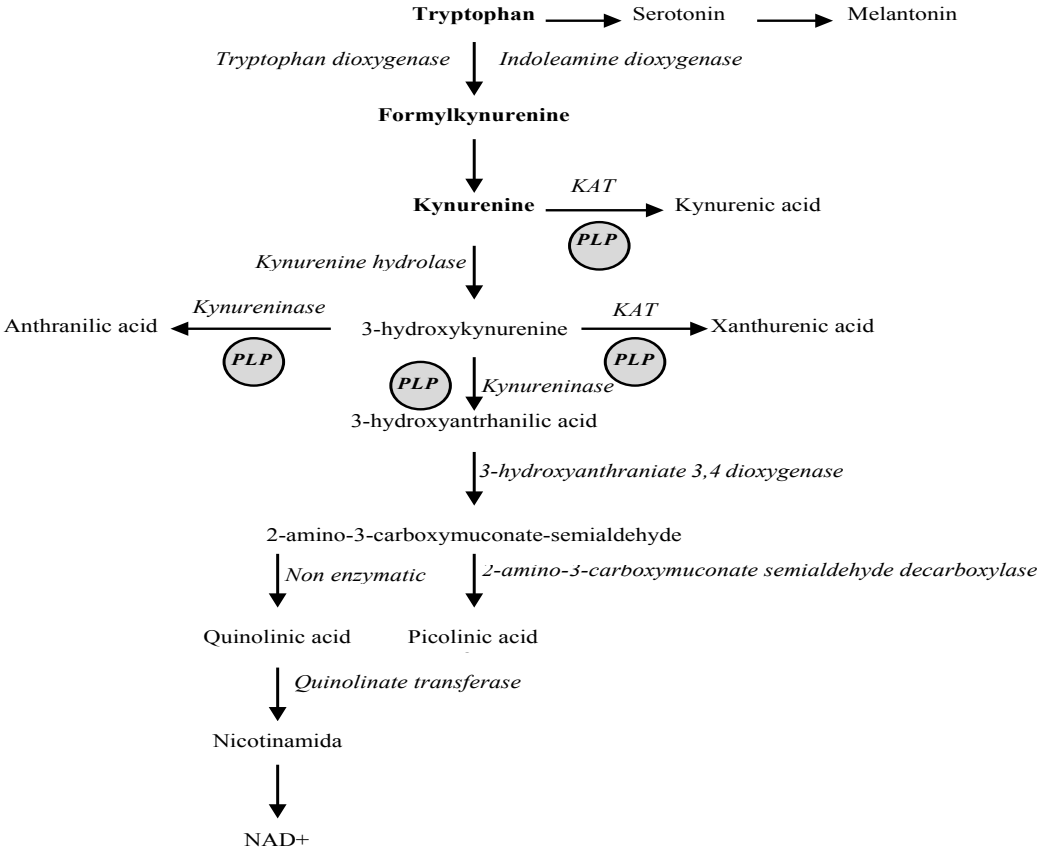

Figure 3. Tryptophan metabolism. Kynurenine aminotransferase (KAT).

2. B_6 and the homocysteine pathway

Homocysteine is a sulfur-containing non-proteinogenic amino acid. Homocysteine is not obtained from the diet. Homocysteine is an intermediate in the metabolism of methionine. Healthy individuals utilize two different pathways to metabolize homocysteine. One pathway (remethylation pathway) converts homocysteine back to methionine and is dependent on folic acid and vitamin B_{12}. The other pathway (transsulfuration pathway) converts homocysteine to the amino acid cysteine and requires two PLP-dependent enzymes (Ntaios *et al*., 2009). Homocysteine is known to be related to heart diseases, brain disease, and autoimmune disease. Many studies have suggested the correlation of elevated homocysteine with its diseases.

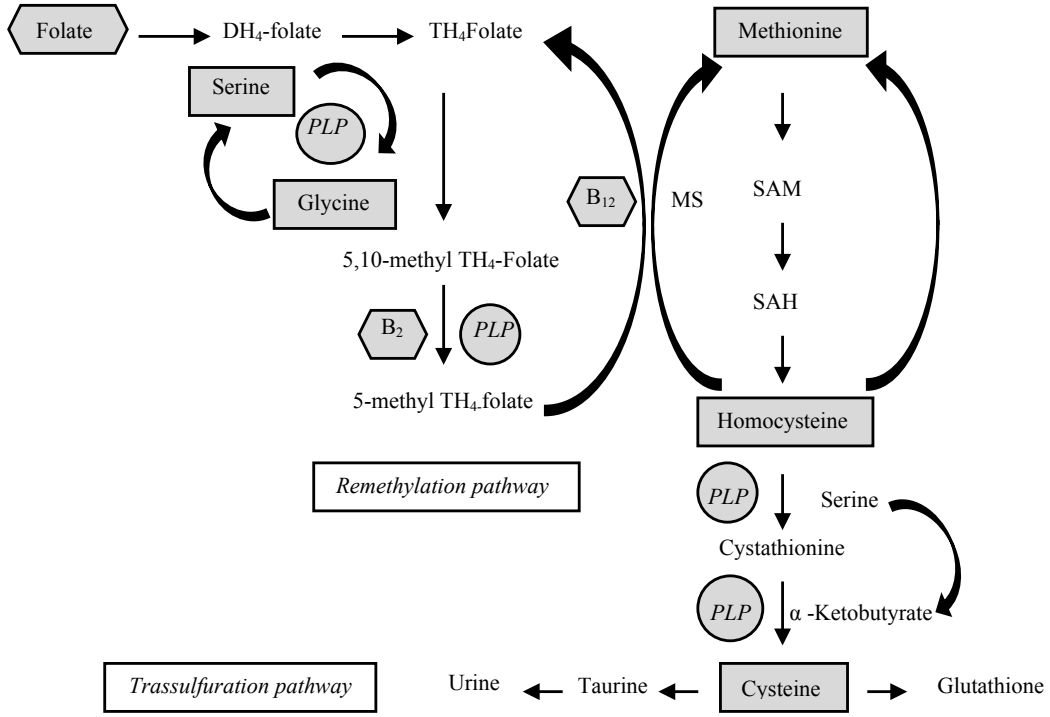

Figure 4. Methionine metabolism. Methionine synthetase (MS), S-Adenosylmethionine (SAM), S-Adenosylhomocysteine (SAH), riboflavin (B_2), cobalamine (B_{12})

III. B_6, disease and its mechanism

B6 deficiency is rare. B6 deficiency can be caused by a decrease in intake, alcohol consumption, or use medications. B6 deficiency due to food consumption is rare because of B6 is present in large quantities in various food sources. However, B6 deficiency is still common in children and the elderly. Excessive alcohol consumption can lead to low plasma PLP levels (Li, Lumeng, and Vech, 1975). In addition, use of certain drugs such as Isoniazid may also cause a B6 deficiency. Chronic diseases such as colon disease, heart disease, brain diseases and autoimmune diseases are affected by the B6 status.

1. Colon diseases

Colon diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), ulcerative colitis (UC) and colorectal cancer (CRC) are influenced by dietary B_6. Low intake of B_6 is associated with IBS symptoms (Ligarden and Farup, 2011) and modulates colonic inflammation in the interleukin (IL)-10 knockout mice of IBD (Selhub *et al*. 2013). However, dietary B_6 supplementation protects the rat colon from colitis (Mahmoud, 2011) and prevent tumor development and spreading in human peripheral blood mononuclear cells (PMBC) (Bessler and Djaldetti, 2016).

The possible mechanisms by which B_6 prevents colon diseases have been reported. The previous studies by Kato *et al*. have suggested that B_6 exhibits an anti-tumor effect by reducing cell proliferation, oxidative stress, inflammation, and angiogenesis (Komatsu *et al*., 2005; Matsubara *et al*., 2003; Komatsu *et al*., 2003; Komatsu *et al*., 2001). Okazaki *et al*. (2012) reported the dietary supplemental B_6 increase of fecal mucin that functions as the intestinal barrier for the prevention of colon diseases. Bessler and Djaldetti (2016) reported that B_6 exerts an immunomodulatory effect on human colon cells by interfering the cross-talk between immune and cancer cells. Mahmoud (2011) suggested that dietary B_6 suppresses rat colitis through a mechanism that involves a decrease in oxidative stress associated with inflammation.

2. Heart diseases

Heart diseases such as cardiovascular disease (CVD), atherosclerosis, and stroke also influence by dietary of B_6. High dietary B6 intakes were associated with reduced the risk of stroke, coronary heart disease (CHD), and heart failure among Japanese (Chui *et al*., 2010), atherosclerosis (Robinson *et al*., 1998), and coronary artery disease (CAD) (Lin *et al*., 2006). However, low plasma PLP and high the inflammation marker C-reactive protein (C-RP) levels were associated with increased CAD risk (Cheng *et al*., 2008; Friso *et al*., 2004). Low plasma PLP concentrations are associated with high values of substrate-product of the PLP-dependent enzymes in the kynurenine pathway (Ulvik *et al*., 2013; Midttun *et al*., 2010). Dietary Folate, B_6, and B_{12} are associated with low serum homocysteine, being important for preventing CVD (Desai *et al*., 2014). B_6 has an important role as a cofactor for cystathionine-synthase and cystathionine-lyase in homocysteine pathway (Midttun *et al*., 2007).

There are several possible mechanisms by which B_6 prevents heart diseases including a blockade of a purinergic receptor, reduce of inflammation marker C-RP, reduction of homocysteine levels, and reduction of inflammatory pathway; the kynurenine pathway-

related product. PLP may produce cardio-protective effects in ischemic heart disease by attenuating the occurrence of intracellular $Ca2^{+}$ overload due to the blockade of purinergic receptors (Dhalla *et al*., 2013). Higher B6 degradation utilization in the presence of an underlying inflammatory process (higher C-RP levels) may represent a possible mechanism to explain the decreased B_6 levels in CVD (Friso *et al*., 2001). The kynurenine pathway-related compound has the important role in modulating the risk of heart diseases such as CVD (Wang *et al*., 2016; Zuo *et al*., 2015; Polyzos and Ketelhuth, 2014), CHD (Eussen *et al*., 2015), CAD (Pedersen *et al*., 2013). Elevated homocysteine causes the increased risk of atherosclerotic plaques (Desai *et al*., 2014). A low level of B_6 accelerates the development of homocysteine-induced atherosclerosis in rats (Endo *et al*., 2006). A combination of folic acid, vitamin B_{12}, and PN significantly reduces homocysteine levels (Schnyder *et al*., 2001).

3. Other diseases

Brain disease such as Parkinson disease (PD), schizophrenia, Alzheimer's disease (AD) are influenced by dietary of B_6 (Shen, 2015; Sharma *et al*., 2015). The possible mechanisms by which B_6 prevents brain diseases including neurotransmitter compound (Leklem, 1999), the kynurenine pathway-related product (Nematollahi *et al*., 2016), and the homocysteine level (Selhub *et al*., 2000). Autoimmune disease such as rheumatoid arthritis (RA) (Huang *et al*., 2010; Chiang *et al*., 2005), type 1 diabetes (T1D) (Masse *et al*., 2012; Nix *et al*., 2014), SLE (Minami *et al*., 2011) are affected by B_6. The high level of homocysteine is possible mechanisms in SLE (Handono *et al*., 2014; Woolf and Manore, 2008). Other diseases are also affected by dietary B_6 such as McArdle disease; a glycogenetic myopathy (Izumi *et al*., 2010), memory impairment (Barichello *et al*., 2014), nausea and vomiting during pregnancy (morning sickness) (Jewell and Young, 2003), age-related macular degeneration (AMD); an eye disease that can cause vision loss (Christen *et al*., 2009), premenstrual syndrome (PMS), (Kashanian *et al*., 2006), carpal tunnel syndrome (Ryan-Harshman and Aldoori, 2007), tardive dyskinesia (Lerner *et al*., 2001; Lerner *et al*. 2007), breast cancer (Wu, 2013).

IV. Gender difference and nutrients metabolism

There is a metabolic difference between male and female including macronutrients (carbohydrate, protein, and fat). Varmalov *et al*. (2015) reported that sex-specific differences in lipid and glucose metabolisms. One-third of the metabolites show significant differences in their concentrations between males and females such as fatty acids, lipids,

amino acids, and proteins (Krumsiek *et al*., 2015). Micronutrients (vitamins and minerals) also have the difference between male and female. Serum 25-hydroxyvitamin D3 (25(OH)D3) level was inversely associated with the serum ferritin levels in men, but was positively associated with the serum ferritin levels in premenopausal women, and was not associated with the serum ferritin levels in postmenopausal women (Seong *et al*., 2017). Gender significantly affects vitamin D status; the lower 25(OH)D3 levels observed in females, as compared to males, play a more relevant role in conditioning the severity of CAD (Verdoia, 2015). Cellular sodium homeostasis may differ in males and females (Grikiniene *et al*., 2014). The mechanisms may be associated with gender-related differences in iron metabolism and chronic liver diseases (Harriron-Findik, 2010). Other food components such as flavonoid have metabolic differences between male and female. The inverse association of flavonoid-rich food intake with systolic blood pressure in women but not in men, showing that high consumption of polyphenol-containing plant foods may be beneficial for prevention of cardiovascular diseases in women (Mennen *et al*., 2004). Also, higher intake of various subclasses of dietary flavonoids, such as flavanols, flavonols, flavones, and flavanones, has been related to the higher bone mineral density among women but not among men (Zhang *et al*., 2014).

Factors that affect gender difference in nutrient metabolism include chromosome and sex hormones. Comitato *et al*. (2015) reported that the modulation by sexual hormones affecting glucose, amino acid and protein metabolisms and the metabolization of nutritional fats and the distribution of fat depots. Wu (2011) suggested that the reason for these sex differences in energy metabolism may relate to sex steroids, differences in insulin resistance, or metabolic effects of other hormones such as leptin. Estrogen-promoted differences in vitamin D3 metabolism, suggesting a more significant protective effect of vitamin D3-based therapeutic strategies in women (Correale *et al*., 2010).

V. Gender difference and B_6

The metabolism and status of B_6 are affected by gender difference. There is an interaction between gender difference and B_6. The binding of PLP to steroid receptors for estrogen, progesterone, testosterone, and other steroid hormones suggests that the B_6 status of an individual may have implications for diseases affected by steroid hormones, including breast cancer and prostate cancers (Leklem, 1999). Gender, B_6 supplement use, oral contraceptive use, and alcohol intake have important effects on B_6-dependent tryptophan metabolism (Deac *et al*., 2015). In Helena study, gender and age influence blood folate, B_{12}, B_6, and homocysteine levels in European adolescents (Gonzales-Gross *et al*., 2012).

VI. Gender difference, diseases and its mechanisms

1. Colon diseases

Men and women are alike in many ways. However, there are important biological and behavioral differences between the two genders. They affect manifestation, epidemiology, and pathophysiology of many widespread diseases. Chronic diseases, such as colon diseases, brain diseases, and autoimmune diseases are differently expressed in men and women.

There is growing evidence that colon diseases such as IBS, Crohn's disease (CD), ulcerative colitis (UC), and CRC are affected by gender difference. For example, compared to men, women are more likely to get IBS (Lee, 2001). Nausea was found to be reported more by female IBS patients and higher prevalence of bowel movements and looser stool in males (Anbardan *et al*., 2012). Girls appear to have an overall more severe course of the pediatric CD (Gupta *et al*., 2007), and women experience more constipation symptoms and have abnormal bowel habits more frequently than men do (McCrea *et al*., 2009). However, CD in children <10 years affects predominantly affects boys (Herzog *et al*., 2014), and the effects of UC on the incidence of colorectal cancer are more significant in male patients than those in female patients (Choi *et al*., 2016).

The mechanisms of possible gender difference in colon diseases include sex hormone and chromosome. Sex hormone in particular estrogens plays a significant in the physiology and pathology of the gastrointestinal (GI) track especially GI mucosal mast cell (Meleine and Matricon, 2014) and brain-gut-microbiota axis (Mulak *et al*., 2014). Men and women differ in susceptibility and exposure to various environmental risk factors for IBD and various endogenous sex-determined differences in immune reactions might play a role in IBD pathogenesis as well (Zelinkova and van der Woude, 2014). A possible anti-inflammatory and anti-neoplastic mechanisms of the action of estrogen receptor-β (ERβ) in the colon may lead to prevent or treat IBD (Salairo *et al*., 2012). Differences in chromosomal CNAs (circulating nucleic acids) between genders may be an important aspect in the development of CRC, and feminization may constitute a unique pathway in colorectal carcinogenesis in a subset of male patients (Ali *et al*., 2014). Indirect tumor-promoting effects of testosterone are likely to explain the disparity between the sexes in the development of colonic adenomas (Amos-Landgraf *et al*., 2014).

2. Other diseases

Brain disease, for instance, AD, PD, and Autism Spectrum Disorder (ASD) are brain diseases that are influenced by gender difference (Filon *et al*., 2016; Wooten *et al*., 2013).

The possible mechanism effect of gender difference on brain disease includes biology (sex hormones and genetic factors) and psychosocial (culture and socialization) (Zagni *et al*., 2016; Trabzuni *et al*., 2013; Martinez-Pinnila *et al*., 2016; Gallart-Palau *et al*., 2016; Picillo *et al*., 2017; Augustine *et al*., 2015; Liu *et al*., 2015). Autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) also emerge in consequence of gender difference (van Vollenhoven, 2009; Yacoub, 2004). Many diseases including metabolic diseases such as metabolic syndrome and diabetes mellitus manifest differently in male and female (Mennecozzi *et al*., 2015; Hung *et al*., 2017; Choi *et al*., 2013; Alemzadeh *et al*., 2014; Xu *et al*., 2016. Sex hormones are one of the factors causing those diseases due to gender differences (Buzzeti *et al*., 2017; Rattanavichit *et al*., 2016).

Chapter III
Study on the influence of dietary vitamin B_6 and gender difference on colonic luminal environment in rats

I. Brief introduction

Colon diseases such as intestinal bowel syndrome (IBS), Crohn's disease (CD), ulcerative colitis (UC), and colon cancer (CRC) are affected by gender difference. For example, compared to men, women are more likely to get IBS (Lee *et al*., 2001), girls appear to have an overall more severe course of pediatric CD (Gupta *et al*., 2007), and women experience more constipation symptoms and have abnormal bowel habits more frequently than men do (McCrea *et al*., 2009). However, CD in children <10 years affects boys (Herzog *et al*., 2014) predominantly, and the effects of UC on the incidence of colorectal cancer are more significant in male patients than those in female patients (Choi *et al*., 2013).

The suggested mechanisms responsible for the effects of gender on the diseases include biological difference such as hormones and chromosomes (Zelinkova *et al*., 2014; Marino *et al*. 2011) as well as environmental factors such as nutrition (Gonzales-Gross *et al*., 2012) and microbiota (Org *et al*., 2016; Markle *et al*., 2013). Inflammation (Sankaran-Walters *et al*., 2013) and metabolisms (Krumsiek *et al*., 2015; Comitato *et al*., 2015) are also responsible for the influence of gender on diseases. Higher inflammation in women is considered to be associated with higher incidence of diseases (Sankaran-Walters *et al*., 2013). Previous studies have reported the possible mechanisms of the gender effects on colon diseases. Steegenga *et al*. (2014) reported that molecular sexually dimorphic effects between males and females in the small intestine and colon of prepubescent mice determined differences in physiological functioning and disease predisposition. Sex hormones had a crucial role in the regulatory mechanisms of the brain-gut-microbiota axis involved in the pathophysiology of IBS (Mulak *et al*., 2014). Furthermore, Meleine *et al*. (2014) reported that ovarian hormones modulated IBS and estrogen receptors were found to imply colorectal carcinogenesis (Caiazza *et al*., 2015). Meanwhile, Amos-Landgraf *et al*. (2014) reported that testosterone promoted early adenomagenesis and the enhanced susceptibility of males to colonic adenomas. However, there is limited information on the role of gender difference in the colon luminal environment. An understanding of the colon luminal environment is essential because changes in mucins (Sheng *et al*., 2012), immunoglobulin A (IgA) (Mantis *et al*., 2011), and microflora (Hold *et al*., 2014) can be a sign of the onset of colon diseases. Mucins have an important role in gastrointestinal barrier

function (Sheng *et al*., 2012). IgA is one of the most important effector molecules in the gastrointestinal immune system because it is the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (Mantis *et al*., 2011).

B_6 is an essential water-soluble vitamin required for normal growth and development in mammals (Tryfiates *et al*., 1981). Pyridoxal 5'-phosphate (PLP), the biologically active form of B6, acts as a co-factor in over 140 distinct enzyme reactions that are involved in the metabolisms of proteins, lipids, and carbohydrates, neurotransmitters, nucleic acids, one carbon units, and immune modulatory metabolites and others (Mooney *et al*., 2009). Beyond its the role as a co-factor, B6 has the preventive roles in certain diseases including colon diseases, such as colitis, intestinal bowel disease (IBD), and colon cancer.

The possible mechanisms by which B_6 prevents colon diseases have been reported. Supplemental B_6 was found to protect colon DNA against damage in female rats with colitis (Mahmoud *et al*., 2011). Dietary B6 intake was shown to modulate colonic inflammation in the IL10 knockout mice of IBD (Selhub *et al*., 2013). Our previous studies have suggested that B6 exhibits an anti-tumor effect by reducing cell proliferation, oxidative stress, inflammation, and angiogenesis (Komatsu *et al*., 2005; Matsubara et al., 2003; Komatsu *et al*., 2003; Komatsu *et al*., 2001). Additionally, we reported an increase of fecal mucins in rats by supplemental dietary B_6 (Okazaki *et al*., 2012). However, there is still limited information about the effect of dietary B6 on colon luminal environment.

As mentioned above, there is growing evidence that the incidence of colon diseases is affected by gender difference. Additionally, accumulating studies have suggested the defensive role of dietary B_6 on colon diseases and the status of B6 is affected by gender difference (Deac *et al*., 2015). Thus, in this study, I hypothesized that gender difference modulates the colon luminal environment, which is dependent upon B_6 status. To examine this hypothesis, this study investigated the effects of gender difference on the colon luminal environment in male and female rats fed diets containing different levels of B_6.

II. Material and methods

1. Animals and diets

A total of 24 male and 24 female Sprague–Dawley rats (3 weeks old) were purchased from the Hiroshima Laboratory Animal Center (Hiroshima, Japan) and were maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. This study was approved by the Ethics Committee of Hiroshima University (approval No. C15-12). The rats were housed individually in an air-conditioned

room at 23°C–24°C under a 12-hour light/dark cycle (lights on from 08:00–20:00). Following acclimatization with a non-purified commercial rodent diet (MF, Oriental Yeast Co., Ltd., Tokyo) for 7 days, the male rats (mean body weight, 113 g) or female rats (mean body weight, 109 g) were randomly assigned to one of three groups (n = 8 rats per group). The rats were provided free access to experimental diets and drinking water for six weeks. The basal diet was comprised of the following components (g/kg diet) (36): α-corn starch, 302; casein, 200; sucrose, 200; corn oil, 200; cellulose powder 50; AIN-93G mineral mixture (Reeves *et al*., 1993), 35; AIN-93 vitamin mixture (PN free) (Reeves et al., 1993), 10; and L-cystine, 3. Pyridoxine (PN) HCl (Nacalai Tesque, Inc., Kyoto, Japan) was supplemented to the basal diet at 1, 7, or 35 mg PN HCl per kg of diet. The level of PN HCl diet recommended in the AIN-93 diet is 7 mg/kg PN HCl (Reeves *et al*., 1993). Meanwhile, 1 mg/kg PN HCl diet is reported to be the minimum level required to prevent growth depression caused by a B_6 deficiency (Coburn et al., 1994). Feces were collected for the final two days. At the end of the feeding period, the rats were euthanized by decapitation following anesthesia (13:00–15:00) with inhalation exposure of isoflurane (Wako Pure Chemical Industries Ltd., Osaka, Japan) in the desiccator to reduce the suffering. Blood was collected, and serum was separated by centrifugation at 2,000 × g for 20 min and stored at −80°C. The cecum was immediately excised, and its contents were removed entirely, weighed, and stored at −80°C until subsequent analysis of microflora. Colon tissue was collected, washed by 0.9% saline, and stored at −80°C until subsequent analysis of free amino acids and gene expression in colon tissue

2. Analysis

a. Serum PLP analysis

The analysis of serum PLP was conducted using high-performance liquid chromatography (HPLC) (Tsuge, 1997). Vitamin B6 from serum and heart were extracted using 3 M perchloric acid (PCA) and measured by HPLC with a fluorometric detector. PLP was converted to pyridoxic acid 5′-phosphate and measured. A 4.6mm (ID) × 250 mm (L) column packed with 10μm pore size TSK-gel ODS-120A (Tosoh) was used. The optimum mobile phases consist of the appropriate concentration of mixtures of 1%, v/v acetonitrile (CH_3CN), 0.1 M sodium perchlorate ($NaClO_4$), and 0.1 M monopotassium phosphate (KH_2PO_4). A fluorescence measurement was set at an emission wavelength of 420 nm with an excitation wavelength of 320 nm. The flow rate for the analysis was set to 0.5 ml/min. Prior to injection into an HPLC line, all of the solutions were passed through a 0.45μm pore size membrane filter, and 200μl was analyzed by HPLC.

b. Colon free amino acids analysis

The analysis of colon free amino acids in colon tissue was done by an amino acid analyzer (JLC-500; JEOL, Tokyo, Japan). To measure the concentrations of free amino acids, we homogenized the colon tissue on ice with 3% sulfosalicylic acids. After centrifugation at 2,000 × *g* at 4°C for 20 min, the supernatant was collected, filtered through a 0.22-μm-pore-size membrane filter, and immediately stored at −80°C until analysis. Quantification of free amino acids was measured by an amino acid analyzer (JLC-500; JEOL, Tokyo, Japan). Amino acid mixture standard solutions (type AN-2 and type B) were used as standard solutions (Wako, Osaka, Japan).

c. Cecal and fecal microflora analysis

The analysis of bacterial genomic DNA was done by real-time quantitative polymerase chain reaction (qPCR) using StepOneTM Real-Time PCR System (Applied Biosystems, CA, USA). Bacterial genomic DNA was isolated from cecum and feces using UltraClean Fecal DNA extraction kit (MO BIO Laboratories, CA, USA) according to the manufacturer's instructions. Bacterial groups (Table 2) were quantified by real-time qPCR using StepOne Real-Time PCR System (Applied Biosystem, CA, USA). Real-time qPCR was performed in a reaction volume of 20 μL containing 10 μL SYBR qPCR mix (Toyobo, Osaka, Japan), 200 nmol/L each of the forward and reverse primers (Parnell et al., 2012; Delroisse *et al*., 2008; Ahmed *et al*., 2007; Guo *et al*., 2008), and 2 μL cecal or fecal DNA samples. The reaction conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 55°C for 15 s, and 72°C for 30 s. The fluorescent products were detected at the last step of each cycle. Melting curve analysis was performed after amplification to distinguish the targeted PCR product from the non-targeted PCR product. Data were analyzed by the second derivative maximum method of the StepOne Real-Time PCR Software.

The plasmid copy number per microliter was determined for standard plasmid solution [ng of cut standard plasmid mixture/μL × (molecules. bp/1.0 × 10^9 ng) × 1/660 DNA length bp per plasmid = plasmid copies/μL]. Real-time qPCR was run on serial dilutions of each standard mixture to relate threshold cycle number to copy numbers of the target sequence and to generate standard curves for quantification in unknown samples. Typically, standard curves were linear across 5 orders of magnitude ($R2 > 0.98$).

d. Fecal immunoglobulin A (IgA) analysis

The analysis of fecal IgA was conducted by enzyme-linked immunosorbent assay (ELISA). For IgA extraction, 100 mg of the freeze-dried fecal matter was homogenized on ice with four mL phosphate buffered saline (pH 7.2) containing 0.1 mg/mL soybean trypsin

inhibitor (Wako, Osaka, Japan), 50 mM ethylenediaminetetraacetic acid (EDTA), and one mM phenylmethylsulfonyl fluoride (PMSF). Homogenized solution was incubated at 4°C overnight and then centrifuged at 9,000 × g for 10 min. The supernatant was further used for assaying fecal IgA concentration, and stored at –70°C until analyzed. Quantification of fecal IgA was measured using Rat IgA ELISA Quantification Kit (Bethyl Laboratories, Montgomery, Texas, USA) according to the manufacturer's protocol.

e. Fecal mucin analysis

The analysis of fecal mucins was conducted by fluorometric assay (Bovee-Oudenhoven *et al*., 1997; Crowther *et al*., 1987). Briefly, each freeze-dried fecal sample of 100 mg was used for extraction and partial purification of mucin. O-Glycosidically linked oligosaccharide chains were β-eliminated by diluted alkali, and the reducing ends of sugar chains were formed. Reducing carbohydrates were fluorescence-labeled at high temperature to produce intensity fluorescent condensate. For determination of mucins, we used a solution of 100 μL to measure the fluorescence at an excitation of 336 nm and a wavelength of 383 nm. N-acetylgalactosamine was used as a standard solution to calculate the number of oligosaccharide chains liberated from mucins during the procedure. For extraction, purification, and determination of mucins, we used the fecal mucin assay kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer's protocol.

f. Colonic gene expression analysis

The analysis of gene expression in colon tissue was done using real-time quantitative PCR (qPCR) using StepOneTM Real-Time PCR System (Applied Biosystems, CA, USA). The Qiagen Midi kit (Qiagen AB, Hilden, Germany) was used to isolate total RNA (500 and 1,200 ng/μL) from rat colon tissue according to the manufacturer's protocol; silica-gel membrane and spin-column technology were used to efficiently remove the genomic DNA for the purification of total RNA. The reverse transcriptase reaction was carried out with one μg total RNA as a template to synthesize cDNA using ReverTra Ace (Toyobo, Osaka, Japan) and random hexamers (Takara Bio, Otsu, Japan) in a final reaction volume of 20 μL, according to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed with a StepOneTM Real-Time PCR System (Applied Biosystems, CA, USA) using the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). Briefly, for qPCR analysis, SYBR-Green (containing DNA polymerase) of 10 μL, forward and reverse primers (0.8 μM each), and 1,000 ng cDNA were contained in a final reaction volume of 20 μL. The cycling parameters were as follows: an initial step at 90ºC for 10 s, followed by 40 cycles at 90ºC for 5 s, 60ºC for 10 s, and 72ºC for 10 s. The mRNA level was

normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all reactions were performed at least in duplicate. Analysis of relative gene expression levels was performed using the following formula: 2-ΔΔCT with ΔΔCT = Cq (target gene) – Cq (control).

g. Statistical analysis

Data are expressed as the average with ± standard error (SE). Two-way analysis of variance (ANOVA) and Tukey–Kramer HSD test was used with $p < 0.05$ being considered as significant. Some data underwent regression analysis, and the correlation coefficient was calculated, and $p < 0.05$ was considered statistically significant.

III. Results

1. Body weights, food intake, and PLP

Final body weights, gains in body weight, and food intake were significantly higher in the male rats than in the female rats (Table 3, $p < 0.05$, ANOVA). Additionally, serum PLP levels in the male rats were significantly higher than in the female rats (Table 3, $p < 0.05$, ANOVA)**.** Dietary Supplemental B_6 to the low B_6 diet (1 mg PN HCl/kg diet) caused higher serum PLP levels in both male and female groups ($p < 0.05$, ANOVA). There was no interaction of the effects of gender difference and dietary B_6 on serum PLP, final body weight, gains in body weight, and food intake ($p > 0.05$, ANOVA).

2. Fecal IgA and mucins

Fecal dry weights of the female rats were significantly lower than those of the male rats (Table 4, $p < 0.05$, ANOVA). Fecal levels of IgA were unaffected by B_6 and gender difference. When expressed in both per g dry feces and per 2 days, fecal levels of mucins were significantly higher in the female rats than in the male rats ($p < 0.05$, ANOVA). ANOVA analysis showed supplemental dietary B_6 significantly increased fecal mucins ($p < 0.05$), although the significant effect of B_6 was observed only in the female rats by Tukey-Kramer HSD test ($p < 0.05$). There was an interaction of the effects of gender difference and dietary B6 on fecal mucins ($p < 0.05$, ANOVA). The increase in fecal mucins by B6 supplementation appeared to be prominent in the female groups. There was no correlation of serum PLP with fecal mucins in per 2 days ($r = 0.26$, $p > 0.05$).

3. Colonic free amino acids

Colon tissue levels of free threonine were significantly higher in the female rats than in the male rats (Table 5, $p < 0.05$, ANOVA), but were unaffected by dietary level of B_6.

Meanwhile, among the female groups, the threonine levels were significantly higher in the 35 mg PN HCl/kg diet than in the 1 and 7 mg PN HCl/kg diets ($p < 0.05$, Tukey-Kramer HSD test). Colon tissue levels of free serine were significantly affected by dietary level of B_6 ($p < 0.05$, ANOVA). Although Tukey–Kramer HSD test indicated no significant effect of B_6 on the serine levels among the groups, the serine levels appeared to be higher in the 7 and 35 mg PN HCL/kg groups compared to the 1 mg PN HCL/kg group. Colon tissue levels of free tyrosine were significantly higher in the male rats than in the female rats ($p < 0.05$, ANOVA), but were unaffected by dietary B_6 ($p > 0.05$, ANOVA). Colon tissue levels of free ornithine were slightly, but significantly, higher in the male rats than in the female rats ($p < 0.05$, ANOVA), but were unaffected by dietary B_6. The levels of other free amino acids were unaffected. There was a significant correlation of fecal mucins (per 2 days) with colon tissue free threonine and serine ($r = 0.60$, $p < 0.01$, Figure 1A and $r = 0.48$, $p < 0.01$, Figure 1B, respectively). The ratios of asparagine/aspartate (Asn/Asp) and of glutamine/glutamate (Gln/Glu) were significantly higher in the male rats than in the female rats ($p < 0.05$, ANOVA), but were unaffected by dietary B_6. Meanwhile, the ratios of phenylalanine/tyrosine (Phe/Tyr) was significantly higher in the female rats than in the male rats ($p < 0.05$, ANOVA), but were unaffected by dietary B_6.

4. Cecal microflora

As shown in Table 6, the relative abundance (%) of cecal *Bifidobacterium* spp. was markedly higher in the male rats than in the female rats ($p < 0.05$, ANOVA), but was unaffected by dietary B_6, whereas the numbers of cecal *Bifidobacterium* spp. were unaffected by both gender difference and dietary B_6 ($p > 0.05$, ANOVA). The numbers and the relative abundance (%) of cecal *Lactobacillus* spp. were both markedly higher in the male rats than in the female rats (Table 6, $p < 0.05$, ANOVA), but were unaffected by dietary B_6. The numbers of other microflora examined were unaffected. There was no interaction of the effects of gender difference and dietary B_6 on the numbers of microflora examined ($p > 0.05$, ANOVA). The relative abundance (%) of cecal *Bacteriodetes* was markedly higher in the female rats than in the male rats ($p < 0.05$, ANOVA). The numbers and relative abundance of cecal *Akkermansia muciniphila* were unaffected.

5. Fecal microflora

As shown in Table 7, the relative abundance (%) of fecal *Bifidobacterium* spp. was markedly higher in the male rats than in the female rats ($p < 0.05$, ANOVA), but was unaffected by dietary B_6, whereas the numbers of fecal *Bifidobacterium* spp. were unaffected by both gender difference and dietary B6 ($p > 0.05$, ANOVA). The numbers and

the relative abundance (%) of fecal *Lactobacillus* spp. were both markedly higher in the male rats than in the female rats (Table 7, $p < 0.05$, ANOVA), but were unaffected by dietary B_6. The numbers of other microflora examined were unaffected. There was no interaction of the effects of gender difference and dietary B_6 on the numbers of microflora examined ($p > 0.05$, ANOVA). The relative abundance (%) of fecal C. cocoides and Firmicutes was significantly affected by dietary B_6 but was unaffected by gender difference. The relative abundance of fecal C. cocoides in the 7 mg PN HCl/kg male and female rats appeared to be higher than that in the 1 and 35 mg PN HCl/kg male and female rats ($p < 0.05$, Tukey-Kramer HSD test). Meanwhile, the relative abundance of fecal Firmicutes in the 1 mg PN HCl/kg female rats was significantly lower than in the 35 mg PN HCl/kg female rats ($p < 0.05$, Tukey-Kramer HSD test). The numbers and relative abundance of fecal Akkermansia muciniphila were unaffected.

6. Colonic gene expression of mucins (MUCs)

As shown in Table 8, the gene expression of MUC16 was significantly higher in the female rats than in the male rats ($p < 0.01$, ANOVA), but was unaffected by dietary B_6. Fecal levels of mucins (per 2 days) were significantly associated with the gene expression of MUC16 ($r = 0.47$, $p < 0.01$, Figure 1C), but not with those of other MUCs ($p > 0.05$).

7. Colonic gene expression of toll-like receptors (TLRs)

The gene expression of TLR8 was significantly higher in the female rats than in the male rats (Table 9, $p < 0.05$, ANOVA). In particular, the effect of gender difference on the expression of TLR8 was prominent in the 1 mg PN HCl/kg groups ($p < 0.05$, Tukey-Kramer HSD test). The gene expressions of the other TLRs were unaffected.

IV. Discussion

Our previous study indicated that supplementation of B_6 to a 30% beef tallow diet resulted in higher fecal mucins in the male rats (Yang *et al*., 2017), whereas the present study indicated no significant increase in the fecal mucins in the male rats that were given a 20% corn oil diet. Nevertheless, ANOVA analysis showed the overall increase in the fecal mucins by supplemental B_6 in the female rats. This study further indicated a significant interaction of the effects of gender difference and dietary B_6 on the fecal mucin. In other words, the increase of the fecal mucin levels in the female rats by dietary B_6 was more profound than in the male rats. At the same time, this study found no effect of gender and B_6 on fecal IgA. Mucins have the unique function of protecting and lubricating epithelial surfaces, but they implicate in additional diverse roles such as growth, fetal

development, epithelial renewal and differentiation, epithelial integrity, carcinogenesis, and metastases (Wang *et al*., 2003). Thus, in the female rats, dietary B_6 may modulate colon luminal environment through mucin production, but not IgA production. However, the physiological implication of the effect of B_6 and gender difference on fecal mucins remains to be investigated.

Supplemental threonine has been suggested to enhance the intestinal mucin production in rats (Wang *et al*., 2010; Law *et al*., 2007). Consumption of an amino acid mixture containing L-threonine, L-serine, L-proline, and L-cysteine has also been suggested to promote mucin synthesis (Faure *et al*., 2006). Therefore, in this study, colonic free amino acids were determined to know the effect of threonine on mucin production. As a result, this study indicated that the modulation of colonic free threonine and serine by gender and B_6 was associated with the alteration in the fecal mucin levels. Thus, the effect of gender and dietary B_6 on fecal mucin levels may be related to the alterations in colonic free threonine and serine. Intriguingly, the female rats had higher levels of free threonine in the colon compared with the male rats. To our knowledge, this is the first evidence of the effect of gender difference on colonic threonine in an animal study. Remond *et al*. (2009) reported that inflammation stimulated intestinal mucin production and threonine uptake in minipigs. Thus, it is of interest to test whether higher inflammation in female animals relates to higher fecal mucins and colonic threonine. Liu *et al*. (2017) reported that threonine has a vital role in intestinal mucosal protein synthesis, particularly mucin, and in intestinal integrity, immune barrier function, and oxidative stress. The present study further stated the increase of free serine in the colon by supplemental dietary B_6. A recent metabolomics study by Ramos *et al*. (2017) showed lower serine concentration in B_6-deprived Neuro-2a cells, implying that B_6 was essential for serine de novo biosynthesis in the brain. Zhou *et al*. (2017) reported that serine relieves oxidative stress through supporting glutathione synthesis and methionine cycle in mice. Serine prevents LPS—induced damage in the intestine by improving glutathione synthesis and AMPK activity in a p53-dependent manner (Zhou *et al*., 2017). Thus, further study is necessary to examine whether higher free serine in the colon by dietary B_6 supplementation is ascribed to higher biosynthesis.

This study indicated that colonic free ornithine was significantly lower in the female rats than in the male rats, although it was unaffected by dietary B6. Previous studies have suggested that the high ornithine decarboxylase (ODC) activity is associated with colon diseases such as UC and CD (Pillai *et al*., 1999) and with the malignant state of rat and human colon (Rozhim *et al*., 1984). Recently, Hardbower *et al*. (2017) found that ODC activates M1 macrophage and causes inflammation. Thus, it is of interest to test whether

the lower ornithine in the female colon related to higher ODC activity and higher inflammation.

This study further indicated that Asn/Asp ratio and Gln/Glu ratio were both significantly lower in the female rats than in the male rats. Asparagine and glutamine have been reported to stimulate ODC activity and cell proliferation (Chen *et al*., 2016; Kandil *et al*., 1995). Asparagine and glutamine are primary nutrients for the intestine by providing nitrogen, which is necessary for the DNA synthesis or cell proliferation. Thus, it is required to investigate whether lower Asn/Asp ratio and lower Gln/Glu ratio in female rats is associated with higher utilization or catabolism of asparagine and glutamine, which in turn leads to higher ODC activity and lower ornithine levels. On the contrary, asparagine has been reported to preserve intestinal barrier function and improve intestinal integrity in inflammatory in weanling piglets (Chen *et al*., 2016; Zhu *et al*., 2017), whereas glutamine decreased the LPS-induced inflammatory response in infant rat intestine (Li *et al*., 2004). Thus, further study is necessary to test whether lower Asn/Asp ratio and Gln/Glu ratio in the female rats cause lower intestinal integrity and higher inflammation. Also, our study indicated higher Phe/Tyr ratio in the female rats than in the male rats. It has been reported that inflammatory state during infection is associated with serum Phe/Tyr ratio (Wannemacher *et al*., 1976). Thus, this result raises the question whether the implication of higher Phe/Tyr ratio in the colon of female rats relates to inflammation.

Yurkovetsyskiy *et al*. (2013) reported that the male mice have a higher relative abundance of cecal Lactobacillaceae compared with the female mice or the castrated male mice. However, Fransen *et al*. (2017) indicated that the conventional female mice had a higher relative abundance of fecal *Lactobacillus* spp. than the conventional male mice. Our recent study indicated that the alteration in fecal *Lactobacillus* spp. by some dietary manipulation was not necessarily consistent with that of cecal *Lactobacillus* spp. (Yang *et al*., 2017). Therefore, we examined both cecal and fecal microflora in rats. The results showed higher relative abundance and numbers of cecal and fecal *Lactobacillus* spp. in the male rats than in the female rats, which are in agreement with those by Yurkovetsyskiy *et al*. (2013). It is unknown why our results were not consistent with the findings of Fransen *et al*. (2017). Zhang (2014) has reported that the relative abundance of fecal *Bifidobacterium* spp. is higher in male rats than in female rats; consistent with Zhang's study, our work also found that the relative abundance of cecal and fecal *Bifidobacterium* spp. is significantly higher in the male rats than in the female rats. Our results implied that the levels of beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. were both affected by gender difference. Such beneficial bacteria have been reported to have an

essential role in gastrointestinal inflammation. Liu *et al*. (2010) have reported that human-derived probiotic *Lactobacillus reuteri* strains reduce intestinal inflammation. Oral treatment with *Bifidobacterium longum* 51A reduces inflammation in a murine experimental model of gout (Vieira *et al*., 2015). Therefore, it is worthwhile to test the possibility that lower abundance of these beneficial bacteria in female rats may be associated with higher inflammation.

About the degradation of mucins, intestinal *Akkermansia muciniphila* (mucin degrader) has an important role. The present study indicated no influence of gender and dietary B_6 on the abundance of cecal and fecal *Akkermansia muciniphila*. Thus, the results did not support the possibility of involvement of *Akkermansia muciniphila* in the mechanism of the combined effect of gender and B6 on fecal mucins. However, it remains to determine the activity of mucin degrading enzyme (mucinase) to test the possibility of the involvement of mucin degradation. Recent studies have suggested that other intestinal bacteria such as some strains of *Bifidobacterium*, *Bacteroides*, and *Ruminococcus* other than *Akkermansia muciniphila* are also able to degrade mucins (Sicard *et al*., 2017; Ouwerkerk *et al*., 2013). Therefore, the possibility of the involvement of such bacteria in the alteration of mucins cannot be ruled out.

This study indicated that the gene expression of MUC16 was significantly higher in the female rats than in the male rats, although this expression was unaffected by dietary B_6. Importantly, we found that the gene expression of MUC16 had a significant positive correlation with the fecal mucins. Thus, the effect of gender difference on the fecal mucins might be mediated by the change in the gene expression of MUC16. The expressions of MUC3 were also affected by gender difference. However, the implication of such alterations is unknown.

Since MUC expression is closely associated with TLR expression (Tarang *et al*., 2012), this study further examined the gene expression of TLRs. As a result, this study indicated that TLR8 expression was significantly higher in the female rats than in the male rats, regardless of dietary B_6. The function of TLR8 in epithelial cells is to induce pro-inflammatory signals (Abreu *et al*., 2010). TLR8 expression has been associated with colon diseases such as CD (Ortiz-Fernandez *et al*., 2015), UC (Sanchez-Munoz *et al*., 2011), CRC (Grimm *et al*., 2010), IBD (Steenhold *et al*., 2009), CD, and UC (Saruta *et al*., 2009). Therefore, it might be possible that TLR8 expression is related to the incidence of gender-dependent colon diseases. However, further study is necessary to test this possibility.

V. Conclusion

We found a combined effect of gender and dietary B_6 on fecal mucin levels. Such an effect was suggested to be mediated by the alterations in colonic free threonine and serine and MUC16 expression. Furthermore, gender difference significantly affected several colonic parameters such as free amino acids, including threonine, ornithine, Asn/Asp ratio, and Gln/Glu ratio, an abundance of *Lactobacillus* spp., and expressions of MUC16 and TLR8, as being essential to colon health and diseases. This study suggests that gender difference and dietary B_6 have a significant impact on the colon luminal environment by modulating these parameters. Further investigation is necessary to investigate if such alterations in the parameters related to sex hormones.

Table and Figure

Table 1. Composition of the experimental diets

Composition (%, w/w)	mg pyridoxine (PN) HCl/kg diet		
	1	7	35
Corn oil	20.00	20.00	20.00
Casein[1]	24.00	24.00	24.00
L-cystine	0.20	0.20	0.20
Pyridoxine (PN) HCl	0.10	0.70	3.50
Cellulosa	5.00	5.00	5.00
Sucrose	19.90	19.30	16.50
Corn starch	26.3	26.3	26.3
Vitamin mixture[2] (PN free)	1.0	1.0	1.0
Mineral mixture[2]	3.5	3.5	3.5

[1]Casein: net protein content, 87% (w/w).
[2]American Institute for Nutrition (AIN-93).

Table 2. Target bacteria group, primers sequence and product size for quantitative PCR

Target bacteria group	Direction	Sequence (5' to 3')
Total bacteria	F	ACTCCTACGGGAGGCAG
	R	GTATTACCGCGGCTGCTG
Bifidobacterium spp.	F	CGCGTCYGGTGTGAAAG
	R	CCCCACATCCAGCATCCA
Lactobacillus spp.	F	GAGGCAGCAGTAGGGAATCTTC
	R	GGCCAGTTACTACCTCTATCCTTCTTC
Clostridium coccoides group	F	AAATGACGGTACCTGACTAA
	R	CTTTGAGTTTCATTCTTGCGAA
Clostridium leptum subgroup	F	GCACAAGCAGTGGAGT
	R	CTTCCTCCGTTTTGTCAA
Akkermansia muciniphila	F	CAGCACGTTGAAGGTGGGGAC
	R	CCTTGCGGTTGGCTTCAGAT
Bacteriodetes	F	GTCAGTTGTGAAAGTTTGC
	R	CAATCGGAGTTCTTCGTG
Firmicutes	F	GGAGYATGTGGTTTAATTCGA
	R	AGCAAGCTGACGACAACCATGCAC
GAPDH	F	TGACAACTCCCTCAAGATTGTC
	R	GGCATGGACTGTGGTCATGA
MUC1	F	CGGTCTTCCCTACCTTCTCC
	R	CTTGGTAGTAGCGGGAGCTG
MUC2	F	ACTGGGAATGTGACTGCTAC
	R	ACCCTGGTAACTGTAGTAAA
MUC3	F	CCAGGATTCGACAACACCTT
	R	GCCTCTTTCTTGCACACCTC
MUC4	F	GTTGGAAAGTCAGCCTCCAG
	R	CCCACAGACCTGTGTTGATG
MUC5A	F	CTACCCTGAGAGAGCGATGG
	R	CAAAGCAGTCGCACCTCATA
MUC6	F	TACCATGACCACGCATCAGT
	R	GTGATGGCAGTGATGTGACC
MUC13	F	CAAGATCTGTGATGCGTGCT
	R	GGAATAGCTTTGCACCTTGC
MUC15	F	AAAGACCACACCCGTGACTC
	R	CTGATGCATTGGGGAAAAGT
MUC16	F	AGCCTTCAGGTCTGTCTCCA
	R	GCAGCTGGGTACCATTTTGT
TLR1	F	TACCCTGAACAACGTGGACA
	R	ATCGACAAAGCCCTCAGAGA
TLR2	F	CTGCAAGCTCTTTGGCTCTT
	R	GAGTCCCGAGGGAATAGAGG
TLR3	F	CTTGCGGTACTGCTCATTCA
	R	CTGCTCACCCTGTGCATCTA
TLR4	F	CAGGGAATTAGGCTCCATGA
	R	TCCATGACAGAACGGTCAAA
TLR5	F	TCTTCGAACTTCGGCTGTTT
	R	TGTGAATCTCGTTGGCAGAG
TLR6	F	GTCTCCCCACTTCATCCAGA
	R	AGAGCCCTCAGCTTGTGGTA

TLR7	F	AGCTCTGTCTCCCTCCACCA
	R	CATGGGTGTTTGTGCTATCG
TLR8	F	TGGACTTGGGGAGAAATGAG
	R	GAAGCCAGCAGGTAGGTGAG

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MUC, mucin; PCR, polymerase chain reaction; R, reverse; TLR, toll-like receptor.

Table 3. Effects of gender difference and dietary B_6 on body weight, food intake and serum PLP in rats

Parameters	B_6 (mg/kg diet)						Two-way ANOVA (p value)		
	Male			Female			Gender Effect	B_6 Effect	Interaction
	1	7	35	1	7	35			
Final body wt (g)	434 ± 23^{a}	453 ± 12^{a}	461 ± 12^{a}	253 ± 5^{b}	276 ± 9^{b}	288 ± 8^{b}	< 0.01	< 0.05	0.96
Gains in body wt (g/6 wk)	321 ± 21^{a}	339 ± 12^{a}	348 ± 12^{a}	145 ± 4^{b}	166 ± 8^{b}	179 ± 7^{b}	< 0.01	< 0.05	0.96
Food intake (g/6 wk)	722 ± 43^{a}	801 ± 34^{a}	709 ± 35^{a}	583 ± 13^{b}	630 ± 18^{ab}	637 ± 16^{ab}	< 0.01	< 0.05	0.11
Cecal contents wt (g)	2.43 ± 0.15^{a}	2.16 ± 0.22^{a}	2.13 ± 0.21^{a}	1.30 ± 0.08^{b}	1.60 ± 0.26^{ab}	1.47 ± 0.11^{ab}	< 0.01	0.90	0.25
Cecal contents wt/100 g body wt	0.56 ± 0.03	0.48 ± 0.05	0.46 ± 0.04	0.51 ± 0.03	0.58 ± 0.09	0.51 ± 0.03	0.36	0.52	0.30
Serum PLP (nmol/L)	0.17 ± 0.07^{a}	0.55 ± 0.02^{b}	1.14 ± 0.03^{c}	0.05 ± 0.00^{a}	0.45 ± 0.02^{ab}	0.73 ± 0.05^{b}	< 0.01	< 0.01	0.06

Mean ± SE (n = 8). Values with different superscript are significantly different (p < 0.05) by Tukey–Kramer HSD test.

Table 4. Effects of gender difference and dietary B_6 on fecal weight, IgA and mucins in rats

Parameters	B_6(mg/kg diet)						Two-way ANOVA (p value)		
	Male			Female			Gender Effect	B_6 Effect	Interaction
	1	7	35	1	7	35			
Fecal dry wt (g/2 days/100g body wt)	4.01 ± 0.20^{a}	4.24 ± 0.31^{a}	4.35 ± 0.18^{a}	2.68 ± 0.14^{b}	3.02 ± 0.11^{b}	3.23 ± 0.12^{ab}	< 0.01	< 0.05	0.79
Fecal IgA (mg/g dry feces)	0.17 ± 0.07	0.22 ± 0.04	0.28 ± 0.05	0.26 ± 0.04	0.22 ± 0.03	0.20 ± 0.02	0.89	0.76	0.76
Fecal IgA (mg/2 days/100g body wt)	0.73 ± 0.28	0.96 ± 0.23	1.23 ± 0.25	0.70 ± 0.12	0.67 ± 0.11	0.66 ± 0.07	0.23	0.19	0.11
Fecal mucin (mg/g dry feces)	0.11 ± 0.03^{a}	0.19 ± 0.03^{a}	0.20 ± 0.02^{a}	0.41 ± 0.06^{b}	0.78 ± 0.07^{b}	1.01 ± 0.13^{b}	< 0.01	< 0.01	< 0.01
Fecal mucin (mg/2 days/100g body wt)	0.47 ± 0.15^{a}	0.76 ± 0.15^{a}	0.87 ± 0.09^{a}	1.10 ± 0.15^{a}	2.38 ± 0.24^{b}	3.18 ± 0.34^{b}	< 0.01	< 0.01	< 0.01

Mean ± SE (n = 8). Values with different superscript are significantly different (p < 0.05) by Tukey–Kramer HSD test.

Table 5. Effects of gender difference and dietary B_6 on colonic free amino acids in rats

Amino acids	B_6 (mg/kg diet)						Two-way ANOVA (p value)		
	Male			Female			Gender Effect	B_6 Effect	Interaction
	1	7	35	1	7	35			
Val (nmol/g tissue)	43.5 ± 3.2	41.3 ± 3.9	45.7 ± 3.8	41.6 ± 3.4	40.6 ± 3.4	41.5 ± 5.5	0.53	0.23	0.10
Leu (nmol/g tissue)	47.3 ± 3.1	51.5 ± 3.0	51.4 ± 3.4	48.3 ± 3.5	43.3 ± 4.4	48.8 ± 4.7	0.36	0.72	0.44
Ile (nmol/g tissue)	24.0 ± 1.8	26.1 ± 1.3	26.7 ± 2.4	24.5 ± 1.5	20.4 ± 3.5	18.0 ± 4.4	0.11	0.74	0.20
Met (nmol/g tissue)	16.8 ± 3.6	20.2 ± 4.6	20.5 ± 3.5	16.9 ± 1.5	17.2 ± 3.1	19.3 ± 4.0	0.76	0.40	0.78
Arg (nmol/g tissue)	40.7 ± 4.0	44.5 ± 3.5	41.6 ± 3.0	41.1 ± 3.4	41.0 ± 3.1	38.3 ± 6.9	0.54	0.80	0.87
Lys (nmol/g tissue)	109 ± 6	115 ± 9	117 ± 10	129 ± 10	135 ± 10	129 ± 12	0.06	0.78	0.88
Thr (nmol/g tissue)	215 ± 9^{a}	273 ± 20^{a}	264 ± 18^{a}	295 ± 25^{a}	284 ± 23^{a}	336 ± 23^{b}	< 0.05	0.07	0.16
Ala (nmol/g tissue)	652 ± 33	721 ± 44	679 ± 16	663 ± 35	677 ± 35	704 ± 34	0.92	0.44	0.57
Gly (nmol/g tissue)	518 ± 44	564 ± 38	532 ± 84	466 ± 45	515 ± 42	527 ± 64	0.63	0.28	0.70
Ser (nmol/g tissue)	176 ± 7	213 ± 8	210 ± 14	194 ± 14	205 ± 14	226 ± 14	0.41	< 0.01	0.25
Asn (nmol/g tissue)	103 ± 20	122 ± 16	134 ± 14	83 ± 15	94 ± 15	100 ± 16	0.13	0.25	0.90
Gln (nmol/g tissue)	673 ± 27	745 ± 53	733 ± 54	632 ± 41	635 ± 33	642 ± 70	0.20	0.40	0.51
Asp (nmol/g tissue)	488 ± 21	572 ± 38	551 ± 18	630 ± 32	610 ± 43	612 ± 53	0.07	0.53	0.18
Glu (nmol/g tissue)	805 ± 29	907 ± 69	876 ± 30	902 ± 27	934 ± 42	919 ± 33	0.21	0.18	0.59
Asn/Asp ratio	0.21 ± 0.03	0.21 ± 0.02	0.24 ± 0.03	0.13 ± 0.02	0.15 ± 0.02	0.16 ± 0.02	< 0.05	0.28	0.81
Gln/Glu ratio	0.84 ± 0.05	0.83 ± 0.03	0.84 ± 0.05	0.70 ± 0.04	0.69 ± 0.05	0.69 ± 0.05	< 0.05	0.84	0.99
Phe (nmol/g tissue)	20.8 ± 2.2	24.2 ± 2.8	23.2 ± 2.5	19.6 ± 1.3	16.8 ± 1.7	20.8 ± 2.9	0.08	0.71	0.38
Tyr (nmol/g tissue)	28.4 ± 2.2^{ab}	33.4 ± 2.9^{b}	33.6 ± 2.8^{b}	23.8 ± 2.3^{ab}	21.7 ± 1.3^{a}	23.9 ± 1.9^{ab}	< 0.01	0.31	0.11
Phe/Tyr ratio	0.73±0.05	0.72±0.05	0.69±0.04	0.87±0.10	0.77±0.05	0.87±0.09	< 0.05	0.72	0.63
His (nmol/g tissue)	15.6 ± 0.7	16.9 ± 1.1	14.9 ± 1.9	15.1± 1.8	14.3 ± 1.8	16.1 ± 1.2	0.67	0.98	0.38
Tau (μmol/g tissue)	2.44 ± 0.18	2.57± 0.11	2.62 ± 0.14	2.61 ± 0.12	2.59 ± 0.16	2.80 ± 0.12	0.47	0.14	0.61
Urea (μmol/g tissue)	6.21 ± 0.52	6.23 ± 0.63	5.95 ± 0.35	6.26 ± 0.60	6.81 ± 0.54	7.53 ± 0.72	0.28	0.49	0.20
GABA (nmol/g tissue)	5.39 ± 0.53	5.83 ± 0.61	5.38 ± 0.82	6.49 ± 0.93	6.27 ± 0.93	6.53 ± 1.01	0.33	0.98	0.84
NH_3 (μmol/g tissue)	0.95 ± 0.11	0.97 ± 0.12	1.02 ± 0.06	1.03 ± 0.05	0.92 ± 0.04	1.05 ± 0.07	0.83	0.42	0.61
Orn (nmol/g tissue)	4.8 ± 0.4	4.8 ± 0.4	4.6 ± 0.4	3.8 ± 0.3	3.6 ± 0.5	4.0 ± 0.2	< 0.05	0.87	0.62

Mean ± SE (n = 6). Values with different superscript are significantly different (p < 0.05) by Tukey–Kramer HSD test.

GABA,γ-aminobutyric acid; Orn, ornithine;Tau, taurine.

Table 6. Effects of gender difference and dietary B_6 on the abundance of microflora in cecal contents of rats

Microflora	B_6(mg/kg diet)						Two-way ANOVA (*p* value)		
	Male			Female			Gender Effect	B_6 Effect	Interaction
	1	7	35	1	7	35			
	numbers/g wet cecal contents								
Total bacteria ($x10^{14}$)	2.33 ± 0.63	3.80 ± 0.99	4.31 ± 1.74	2.70 ± 0.99	4.10 ± 1.91**	2.86 ± 1.77*	0.53	0.72	0.70
Bifidobacterium spp. ($x10^{10}$)	1.66 ± 0.86*	5.60 ± 3.51	2.67 ± 0.99	0.17 ± 0.10*	0.10 ± 0.05**	0.08 ± 0.06*	< 0.05	0.37	0.35
Lactobacillus spp. ($x10^{13}$)	2.15 ± 1.45	2.45± 0.50	6.85 ± 3.86	0.97 ± 0.52	0.35 ± 0.26**	0.21 ± 0.08*	< 0.05	0.41	0.27
Clostridium coccoides ($x10^{13}$)	5.16 ± 1.34	9.21 ± 3.21	4.58 ± 2.22	6.16 ± 2.35	9.39 ± 5.95**	6.69 ± 3.53*	0.99	0.60	0.83
Clostridium leptum ($x10^{13}$)	4.08 ± 0.92	6.95 ± 1.84	4.04 ± 1.57	6.00 ± 2.67	4.12 ± 1.15**	4.41 ± 2.20**	0.60	0.61	0.20
Akkermansia muciniphila ($x10^{12}$)	0.77 ± 0.31	2.22 ± 0.74	1.25 ± 0.54	0.41 ± 0.19	2.03 ± 0.89**	0.72 ± 0.55*	0.21	0.09	0.96
Bacteriodetes ($x10^{13}$)	0.39 ± 0.30	0.42 ± 0.19	0.33± 0.25	1.34± 0.73	0.75 ± 0.32**	0.83± 0.44*	0.18	0.60	0.56
Firmicutes ($x10^{14}$)	0.57 ± 0.20	1.27 ± 0.58	0.38 ± 0.11	1.04 ± 0.65	0.15 ± 0.08**	0.21 ± 0.14*	0.36	0.34	0.10
	numbers/total cecal contents/100g body wt								
Total bacteria ($x10^{14}$)	0.14 ± 0.04	0.17 ± 0.06	0.22 ± 0.08	0.15 ± 0.06	0.30 ± 0.15**	0.16 ± 0.11*	0.90	0.84	0.85
Bifidobacterium spp. ($x10^{10}$)	0.87 ± 0.41*	2.60 ± 1.65	1.30 ± 0.48	0.09 ± 0.05*	0.06 ± 0.03**	0.04 ± 0.03*	< 0.05	0.54	0.40
Lactobacillus spp. ($x10^{13}$)	0.11 ± 0.07	0.11 ± 0.02	0.40 ± 0.24	0.05 ± 0.02	0.03 ± 0.02**	0.01 ± 0.00*	< 0.05	0.38	0.26
Clostridium coccoides ($x10^{14}$)	0.28 ± 0.07	0.46 ± 0.17	0.19 ± 0.08	0.32 ± 0.12	0.63 ± 0.43**	0.38 ± 0.20*	0.16	0.59	0.98
Clostridium leptum ($x10^{14}$)	0.23 ± 0.05	0.34 ± 0.10	0.17 ± 0.06	0.30 ± 0.13	0.26 ± 0.08**	0.23 ± 0.10**	0.87	0.56	0.92
Akkermansia muciniphila ($x10^{12}$)	0.42 ± 0.16	1.09 ± 0.38	0.52 ± 0.21	0.24 ± 0.13	1.25 ± 0.63**	0.39 ± 0.33*	0.44	0.09	0.99
Bacteriodetes ($x10^{13}$)	0.22 ± 0.17	0.20 ± 0.10	0.13 ± 0.09	0.69 ± 0.38	0.50 ± 0.23**	0.46 ± 0.24*	0.12	0.62	0.77
Firmicutes ($x10^{14}$)	0.30 ± 0.10	0.62 ± 0.31	0.20 ± 0.07	0.52 ± 0.32	0.10 ± 0.06**	0.11 ± 0.08*	0.37	0.38	0.16
	% of total bacteria								
Bifidobacterium spp.	0.25 ± 0.17^{ab}	0.12 ± 0.05^{ab}	0.48 ± 0.17^{b}	0.10 ± 0.03^{ab}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	< 0.05	0.15	0.15
Lactobacillus spp.	7.33 ± 2.73^{ab}	6.30 ± 1.25^{ab}	11.29 ± 3.30^{b}	0.49 ± 0.18^{a}	0.09 ± 0.08^{a}	0.88 ± 0.26^{a}	< 0.01	0.22	0.41
Clostridium coccoides	23.7 ± 5.7	29.3 ± 8.1	27.3 ± 6.9	34.8 ± 10.3	46.1 ± 8.6	30.2 ± 9.9	0.17	0.48	0.70
Clostridium leptum	1.30 ± 0.25	2.60 ± 0.71	1.70 ± 0.54	2.42 ± 0.46	3.27 ± 1.01	3.26 ± 1.64	0.16	0.48	0.88
Akkermansia muciniphila	1.98 ± 0.55	4.20 ± 1.03	2.29 ± 0.62	2.43 ± 0.86	4.30 ± 0.88	4.04± 1.57	0.60	0.07	0.60
Bacteroidetes	1.71 ± 0.45^{a}	1.42 ± 0.40^{a}	1.43 ± 0.32^{a}	6.30 ± 1.39^{b}	2.65 ± 1.18^{b}	2.28 ± 0.74^{b}	< 0.01	0.10	0.07
Firmicutes	2.98 ± 0.86	1.45 ± 0.70	2.53 ± 0.91	2.27 ± 1.02*	1.83 ± 0.02**	2.51 ± 0.76*	0.54	0.46	0.81

Mean ± SE. $n = 6$~8; * and ** indicate studies using 7 and 6 animals, respectively and others are the data of 8 animals.

Values with different superscript are significantly different ($p < 0.05$) by Tukey–Kramer HSD test.

Table 7. Effects of gender difference and dietary B_6 on the abundance of microflora in feces of rats

Microflora	B_6 (mg/kg diet)						Two-way ANOVA (*p* value)		
	Male			Female			Gender Effect	B_6 Effect	Interaction
	1	7	35	1	7	35			
	numbers/g fecal dry wt								
Total bacteria ($x10^{14}$)	2.63 ± 0.85	3.92 ± 0.44	4.34 ± 1.82*	2.15 ± 0.87	2.40 ± 0.82	2.57 ± 0.90	0.22	0.51	0.77
Bifidobacterium spp. ($x10^{11}$)	0.36 ± 0.16	0.49 ± 0.25	1.31 ± 0.97	0.85 ± 0.62	0.01 ± 0.00	0.02 ± 0.02	0.28	0.67	0.22
Lactobacillus spp. ($x10^{13}$)	4.56 ± 1.85^{a}	3.82 ± 0.62^{a}	6.06 ± 2.79^{a}	2.38 ± 1.61^{b}*	0.28 ± 0.10^{b}	1.14 ± 0.50^{b}	< 0.05	0.49	0.63
Clostridium coccoides ($x10^{13}$)	4.86 ± 2.11	5.56 ± 1.81	7.28 ± 1.7	5.96 ± 2.80	1.04 ± 0.38	2.65 ± 0.93	0.12	0.43	0.17
Clostridium leptum ($x10^{14}$)	0.81 ± 0.38	1.64 ± 0.32	1.54 ± 0.92	0.29 ± 0.11	2.54 ± 1.00	1.07 ± 0.59	0.95	0.06	0.44
Akkermansia muciniphila ($x10^{12}$)	1.23 ± 0.47	2.05 ± 0.28	2.53 ± 0.74	1.07 ± 0.66	2.84 ± 0.90	1.21 ± 0. 52	0.58	0.12	0.22
Bacteriodetes ($x10^{13}$)	1.98 ± 0.65	4.61 ± 0.89	2.96 ± 0.75	2.19 ± 0.98	4.36 ± 2.20	1.83 ± 1.03	0.69	0.11	0.85
Firmicutes ($x10^{14}$)	0.75 ± 0.23	1.34 ± 0.15	1.33 ± 0.48	0.71 ± 0.30	1.68 ± 0.81	0.85 ± 0.29	0.87	0.21	0.64
	numbers/total feces for 2 days/100g body wt								
Total bacteria ($x10^{14}$)	0.60 ± 0.20	0.86 ± 0.09	1.09 ± 0.43*	0.83 ± 0.33	0.89 ± 0.33	0.91 ± 0.33	0.80	0.71	0.87
Bifidobacterium spp. ($x10^{10}$)	0.85 ± 0.39	1.06 ± 0.53	2.96 ± 2.25	3.14 ± 2.23	0.04 ± 0.02	0.08 ± 0.06	0.62	0.56	0.17
Lactobacillus spp. ($x10^{13}$)	1.04 ± 0.43	0.84 ± 0.13	1.35 ± 0.64	0.52 ± 0.31*	0.11 ± 0.04	0.40 ± 0.18	< 0.05	0.43	0.85
Clostridium coccoides ($x10^{13}$)	1.10 ± 0.48	1.20 ± 0.39	1.61 ± 0.38	2.37 ± 1.13	0.37 ± 0.13	0.92 ± 0.31	0.86	0.24	0.12
Clostridium leptum ($x10^{13}$)	1.79 ± 0.86	3.65 ± 0.74	3.45 ± 2.12	1.12 ± 0.40	6.27 ± 2.62	3.57 ± 1.82	0.65	0.09	0.54
Akkermansia muciniphila ($x10^{12}$)	0.26 ± 0.10	0.46 ± 0.07	0.56 ± 0.18	0.43 ± 0.29	1.05 ± 0.35	0.42 ± 0.20	0.41	0.89	0.15
Bacteriodetes ($x10^{13}$)	0.45 ± 0.15	1.01 ± 0.18	0.66 ± 0.17	0.87 ± 0.39	1.55 ± 0.72	0.64 ± 0.36	0.33	0.22	0.76
Firmicutes ($x10^{13}$)	1.71 ± 0.52	2.95 ± 0.30	2.95 ± 1.10	2.76 ± 1.13	6.05 ± 2.81	2.99 ± 1.06	0.25	0.27	0.54
	% of total bacteria								
Bifidobacterium spp.	0.155 ± 0.074	0.106 ± 0.057	0.305 ± 0.135	0.135 ± 0.086	0.004 ± 0.001	0.005± 0.001	< 0.01	0.42	0.24
Lactobacillus spp.	2.69 ± 0.62^{a}	2.19 ± 0.40^{a}	2.46 ± 0.38^{a}	0.30 ± 0.13^{b}*	0.14 ± 0.07^{b}	0.48 ± 0.17^{b}	< 0.01	0.64	0.82
Clostridium coccoides	8.90 ± 1.80	14.8 ± 1.80	9.30 ± 1.70	7.80 ± 1.50	15.7 ± 4.00	11.1 ± 2.40	0.76	< 0.05	0.84
Clostridium leptum	3.50 ± 1.20	5.30 ± 1.00	2.40 ± 0.80	1.80 ± 0.60	6.90 ± 2.60	5.30 ± 3.10	0.45	0.21	0.48
Akkermansia muciniphila	0.21 ± 0.11	0.39 ± 0.07	0.57 ± 0.30	0.35 ± 0.13	0.52 ± 0.12	0.11± 0.05	0.62	0.51	0.10
Bacteriodetes	0.63 ± 0.10	0.83 ± 0.08	0.70 ± 0.09	1.30 ± 0.48	0.86± 0.23	0.64 ± 0.14	0.18	0.50	0.31
Firmicutes	14.9 ± 1.3^{a}	17.4 ± 0.5^{ab}	16.1 ± 0.7^{ab}	13.8 ± 0.8^{a}	17.1 ± 1.2^{ab}	19.0 ± 1.0^{b}	0.50	< 0.01	0.12

Mean ± SE. $n = 7$~8; *indicates studies using 7 animals, respectively and others are the data of 8 animals.

Values with different superscript are significantly different ($p < 0.05$) by Tukey–Kramer HSD test.

Table 8. Effects of gender difference and dietary B_6 on relative expression of colonic genes of mucins in rats

Gene	B_6 (mg/kg) diet						Two-way ANOVA (*p* value)		
	Male			Female			Gender	B_6	Interaction
	1	7	35	1	7	35	Effect	Effect	
MUC1	0.89 ± 0.09*	0.88 ± 0.14*	0.76 ± 0.14*	0.54 ± 0.05	0.51 ± 0.06	0.58 ± 0.09	0.09	0.95	0.70
MUC2	0.46 ± 0.15*	0.52 ± 0.19*	0.50 ± 0.19*	0.14 ± 0.05	0.18 ± 0.06	0.14 ± 0.05	0.06	0.92	0.98
MUC3	0.79 ± 0.26*	0.60 ± 0.22*	0.74± 0.22*	0.22 ± 0.08	0.20 ± 0.07	0.17 ± 0.08	< 0.05	0.84	0.84
MUC4	1.34 ± 0.47*	0.93 ± 0.18*	0.95 ± 0.24*	1.42 ± 0.08	0.94 ± 0.07	0.67 ± 0.07	0.82	0.26	0.83
MUC5AC	1.49 ± 0.19*	1.80 ± 0.36*	1.30 ± 0.24*	1.38 ± 0.14	1.27 ± 0.19	1.25 ± 0.19	0.85	0.71	0.71
MUC6	0.89 ± 0.04**^{ab}	0.91 ± 0.20**^{ab}	1.08 ± 0.15*^{ab}	1.27 ± 0.20^{a}	0.74 ± 0.05^{b}	0.67 ± 0.07^{b}	0.37	0.29	< 0.03
MUC13	0.70 ± 0.10*	1.15 ± 0.25*	0.57 ± 0.12*	0.48 ± 0.07	0.63 ± 0.19	0.85 ± 0.27	0.79	0.26	0.09
MUC15	1.04 ± 0.13*^{ab}	1.67± 0.23*^{a}	0.96 ± 0.15*^{b}	1.26 ± 0.08^{ab}	1.61 ± 0.15^{ab}	1.27 ± 0.17^{ab}	< 0.05	< 0.05	0.74
MUC16	0.84 ± 0.05*	0.93 ± 0.08*	0.74 ± 0.14*	0.96 ± 0.08	1.09 ± 0.09	1.09 ± 0.11	< 0.01	0.71	0.68

Mean ± SE. $n = 6$~8; * and ** indicate studies using 7 and 6 animals, respectively and others are the data of 8 animals.

Values with different superscript are significantly different ($p < 0.05$) by Tukey–Kramer HSD test.

Table 9. Effects of gender difference and dietary B_6 on relative expression of colonic genes of TLRs in rats

Genes	B_6 (mg/kg) diet						Two-way ANOVA (*p* value)		
	Male			Female			Gender Effect	B_6 effect	Interaction
	1	7	35	1	7	35			
TLR1	1.28 ± 0.30*	1.55 ± 0.35*	1.07 ± 0.36*	1.22 ± 0.22	0.75 ± 0.11	0.68 ± 0.16	0.30	0.36	0.39
TLR2	1.00 ± 0.30*	1.32 ± 0.30*	0.27 ± 0.47*	1.08 ± 0.17	0.69 ± 0.15	0.82 ± 0.16	0.48	0.98	0.39
TLR3	0.60 ± 0.19*	0.64 ± 0.22*	0.54 ± 0.16*	0.39 ± 0.08	0.41 ± 0.11	062 ± 0.36	0.83	0.87	0.64
TLR4	1.03 ± 0.27*	1.32 ± 0.44*	1.07 ± 0.19*	0.67 ± 0.08	0.75 ± 0.10	0.86 ± 0.34	0.35	0.79	0.79
TLR5	0.78 ± 0.23*	1.03 ± 0.43*	0.95 ± 0.31*	0.64 ± 0.17	0.64 ± 0.19	0.88 ± 0.54	0.78	0.80	0.86
TLR6	1.13 ± 0.07*	2.24 ± 0.62*	1.35 ± 0.27*	1.44 ± 0. 19	1.17 ± 0.16	1.26 ± 0.10	0.78	0.39	0.10
TLR7	2.04 ± 1.31**	2.78 ± 0.93*	2.10 ± 1.29*	2.26 ± 1.05	1.19 ± 0.52	1.02 ± 0.38	0.60	0.81	0.42
TLR8	1.49 ± 0.30^{a}*	2.70 ± 0.98^{ab} *	2.07± 0.50^{ab} *	4.03 ± 0.44^{b}	3.46 ± 0.48^{ab}	2.75 ± 0.21^{ab}	< 0.01	0.46	0.16
TLR9	1.56 ± 0.72*	2.67 ± 0.99*	1.91 ± 0.90*	1.94 ± 0.60	0.93 ± 0.35	0.96 ± 0.19	0.44	0.78	0.20

Mean ± SE. n = 6~8; * and ** indicate studies using 7 and 6 animals, respectively and others are the data of 8 animals.
Values with different superscript are significantly different ($p < 0.05$) by Tukey–Kramer HSD tes

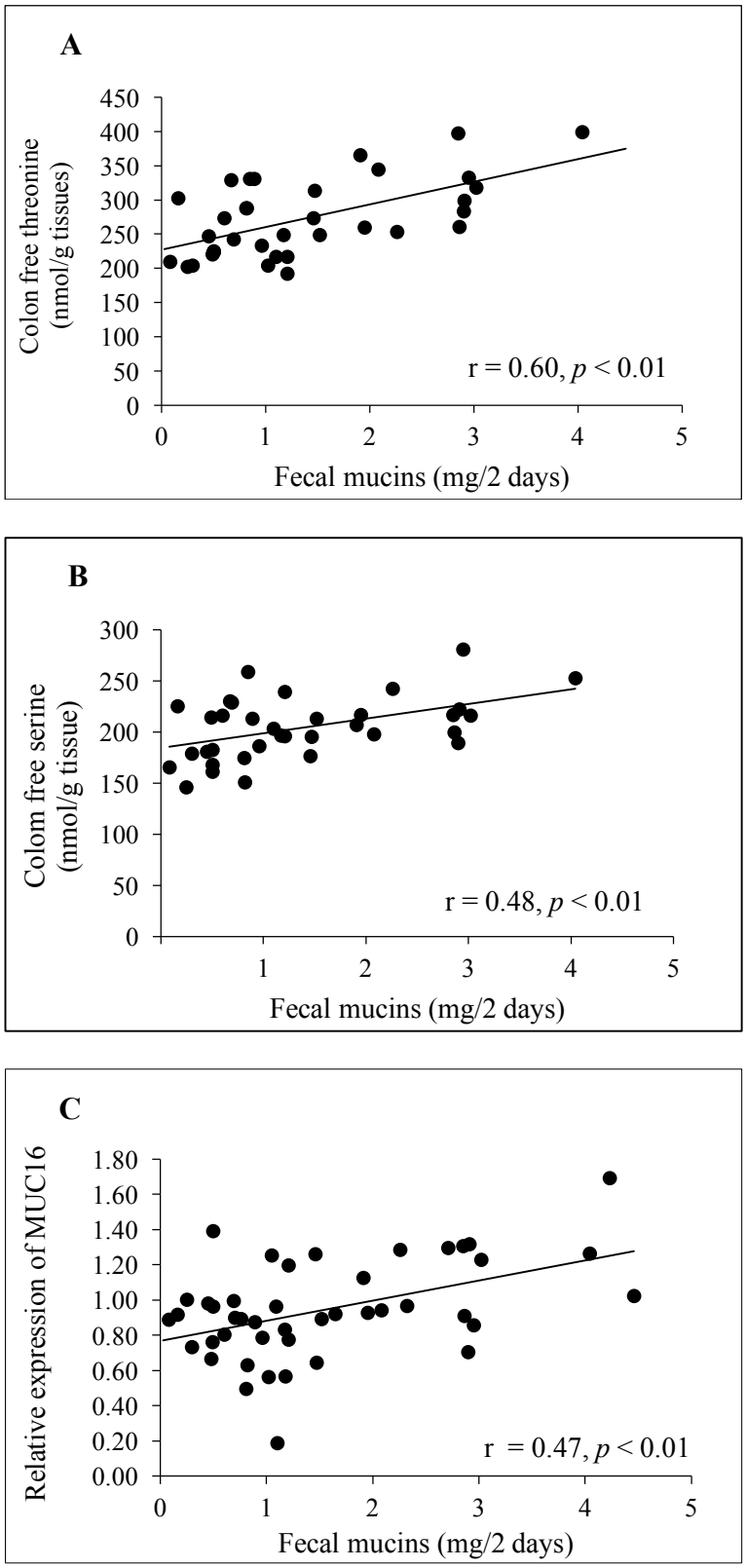

Figure 5. Correlation of fecal mucins with colon free threonine (A), serine (B) and with gene expression of MUC1 (C)

Chapter IV
Effect of dietary supplemental vitamin B_6 on the levels of anti-disease metabolites in heart

I. Brief introduction

It has been reported that a low dietary intake of B_6 is associated with a high risk of heart disease. High nutritional folate and B_6 were associated with a reduced risk of mortality from stroke, coronary heart disease (CHD), and heart failure among Japanese (Chui *et al*., 2010). Low plasma PLP concentrations are related to significant high markers of inflammation and associated with increased coronary artery disease (CAD) risk (Friso et al., 2004). Lower levels of folate and B_6 confer an increased risk of atherosclerosis (Robinson *et al*., 1998).

The suggested mechanisms responsible for the effects of B_6 on the heart diseases include elevated homocysteine causes endothelial dysfunction, promotes an innate autoimmune response, and causes accumulation of inflammatory monocytes in atherosclerotic plaques (Desai *et al*., 2014). PLP may produce heart-protective effects in ischemic heart diseases by attenuating the occurrence of intracellular $Ca2^+$ overload due to the blockade of purinergic receptors (Dhalla *et al.,* 2013). Low plasma PLP is associated with higher C-Reactive Protein (CRP) levels, an underlying inflammatory process and a possible mechanism to explain the decreased B_6 levels in CVD (Friso *et al*., 2001). The Kynurenine pathway-related compound has the important role in modulating the risk of CVD (Wang *et al*., 2016; Zuo *et al*., 2015; Polyzos and Ketelhuth, 2014), CHD (Eussen *et al*., 2015), CAD (Pedersen *et al*., 2013).

As mentioned above, there are several epidemiological studies have shown the positive benefits of B_6 to heart diseases. There is growing evidence that the low circulating PLP are related to the high risk of heart diseases. However, the exact mechanisms of the effects of B_6 are still unclear. Our group hypothesized that dietary B_6 affects heart dysfunction by modulating amino acid metabolisms. Sofya *et al*. in our laboratory first provided evidence for the increase in carnosine and anserine in skeletal muscles and heart of rats (Sofya *et al*., 2016; Sofya *et al*., 2015) by B_6 supplemental. In this study, I applied metabolomics analysis to investigated concentration of metabolites of the heart in rats fed a diet containing 1 mg (low) or 35 mg (high) pyridoxine (PN) HCl/kg for six weeks.

II. Materials and methods

1. Animals and diets

Male Sprague–Dawley rats (3 weeks old, Charles River Japan, Hino, Japan) were maintained by the Guide for the Care and Use of Laboratory Animals established by Hiroshima University established by Hiroshima University and approved by the Ethics Committee of the university (Ethical Approval No. C15-12). Rats were housed in metal cages in a temperature-controlled room (24 ± 1°C) under a 12-h light/dark cycle (lights on, 08:00–20:00 hours). Rats had free access to food and drinking water. After acclimatization with a non-purified commercial rodent diet (MF, Oriental Yeast, Tokyo) for 7 days, 26 rats (average body weight X g) were randomly divided into three groups receiving a basal diet (Masisi *et al*. 2012) mixed with 1 (deficient), 7 (recommended), or 35 (supplement) mg pyridoxine (PN) HCl/kg diet (n = 10 for the B_6 deficient and supplement groups and n = 6 for the recommended group) for 6 weeks. The level of 7 mg PN HCl/kg diet is the recommended level of dietary B_6 (Reeves *et al*., 1993), while 1 mg PN HCl/kg diet is reported to be the minimum level required for preventing growth depression caused by a B6 deficiency (Coburn, 1994). At the end of the feeding period, all rats were sacrificed by decapitation under isoflurane anesthesia (between 01:00 p.m. and 03:00 p.m.) after removal of food (08:00 a.m.). Trunk blood from each rat was collected in a glass tube immediately after decapitation. Serum was obtained by centrifugation at 2000g for 20 min and stored at −80°C. Heart tissue was removed immediately, weighed, and stored at −80°C until analyzed. Heart samples were quickly dissected, frozen in liquid nitrogen, and immediately stored at −80°C.

2. Analysis of metabolites in heart of rats by metabolomics analysis

Samples from two groups that were the B_6 recommended and B_6 supplement groups (n = 5/group) were applied to metabolomics analysis performed by Human Metabolome Technologies Inc (Tsuruoka, Yamagata, Japan). Briefly, according to the manufacturer's instruction and the details can be found elsewhere (Soga and Heiger, 2000; Soga *et al*., 2002; Soga et al., 2003) each heart tissue was homogenized under cooling condition in 750 μL of 50% acetonitrile aqueous solution (v/v) containing an internal standard (20 μmol/L). Then the homogenate was volumed up to 1.5 mL, followed by centrifugation (2,300g, 4°C, 5 min). The supernatant was ultrafiltrated using a five kDa MWCO centrifugal filter unit (Millipore) at 9,100g, 4°C, 120 min, followed by evaporation to dryness. Capillary electrophoresis-time of flight mass spectrometry (CE-TOFMS) system (Agilent Technologies) was used. Separations were carried out on a fused silica capillary (50 μm i.d.

× 80 cm total length). For cationic metabolites, MS conditions were sample injection: pressure injection 50 m-bar with 10 sec; CE voltage: positive, 27 kV; ionization: electrospray ionization (ESI) in positive mode; capillary voltage: 4,000 V; and scan range: m/z 50-1,000. For anionic metabolites, MS conditions were sample injection: pressure injection 50 m-bar with 10 sec; CE voltage: positive, 30 kV; ionization: ESI in negative mode; capillary voltage: 3,500 V; and scan range: m/z 50-1,000. Data analysis was performed with Welch t-test. A value of $P<0.05$ was considered statistically significant. Functional pathway analysis of metabolites was characterized based on databases from the KEGG database and the human metabolome database.

Statistical analysis

Data are expressed as means ± SEM. The two-group comparison was performed with Student's t-test, Welch t-test or nonparametric Wilcoxon test, as appropriate. Statistical significance of the difference among means was estimated at $P<0.05$. Data analysis was performed using Excel Statistics 2016 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan).

3. Analysis of carnosine, anserine, β-Alanine, histamine, and GABA in heart of rats by HPLC analysis

Carnosine (Wako Pure Chemical, Osaka, Japan), Anserine (Wako Pure Chemical, Osaka, Japan), β-Alanine (Nacalai Tesque, Inc., Kyoto, Japan), histamine (Nacalai Tesque, Inc., Kyoto, Japan), γ-Aminobutyric acid (GABA) (Nacalai Tesque, Inc., Kyoto, Japan), and homocarnosine (Wako Pure Chemical, Osaka, Japan were measured by an *o*-phthaldialdehyde (OPA)-HPLC method (Kamisaki *et al*., 1990) as described before with some modification. Briefly, heart tissue and serum were extracted using 0.05 N perchloric acid and acetonitrile, respectively. A Cosmosil $5C_{18}$-MS-II column (4.6 × 250 mm; Nacalai Tesque) with an isocratic elution of 0.1 mol/L sodium citrate (pH 3.5)-acetonitrile-methanol (60:30:10 (v/v) for β-alanine and GABA; 75:15:10 (v/v) for anserine, carnosine, and homocarnosine) and a fluorometric detector with Ex of 350 nm and Em of 440 nm were used to monitor the compounds. For the B_6 deficient and supplement groups, supernatants of each two rats from the same group were combined to obtain pooled samples for the analysis (n = 5), while each supernatant from each rat in the recommended group was used as it was (n = 5-6).

Statistical analysis

Data are expressed as means ± SE. Tukey's multiple-range test was used to compare

means after one-way ANOVA. Statistical significance of the difference among means was estimated at $P<0.05$. Data analysis was performed using Excel Statistics 2010 for Windows (Social Survey Research Information Co., Ltd, Tokyo, Japan).

III. Results

1. Body weight and food intake

Final body weights, gains in body weight, and food intake did not affect by dietary B_6 (Table 10, $P>0.05$).

2. Concentration of several metabolites in heart of rats by metabolomics analysis

The results in Table 11 showed all the concentration of metabolites in heart of rats. The concentrations of carnosine, anserine, homocarnosine, histamine, and 1H-imidazole propionic acid were increased by high B_6 diet (35 mg PN HCl/kg) compared to low B_6 diet (1 mg PN HCl/kg) (+154%, +109%, +75%, +131%, and +33%, respectively, $P<0.05$). The concentration of alanine, serine, isoleucine, leucine, valine, and methionine were increased by high B_6 diet (35 mg PN HCl/kg) compared to low B_6 diet (1 mg PN HCl/kg) (+36%, +46%, +21%, +11%, +13%, and +23%, respectively, $P<0.05$). The concentrations of malic acid, fumaric acid, and argininosuccinate were increased by high B_6 diet (35 mg PN HCl/kg) compared to low B_6 diet (1 mg PN HCl/kg) (+33%, +30%, and 33%, respectively, $P<0.05$). The concentrations of ornithine were decreased by high B_6 diet (35 mg PN HCl/kg) compared to low B_6 diet (1 mg PN HCl/kg) (-23%, $P<0.05$). The concentrations of b-alanine, GABA, γ-butyrobetaine, carnitine, adenine, and flavin adenine dinucleotide (FAD) were increased by high B_6 diet (35 mg PN HCl/kg) compared to low B_6 diet (1 mg PN HCl/kg) (+100%, +100%, +34%, +29%, 25%, and 17%, respectively, $P<0.05$).

There was a strong correlation of carnosine with anserine, β-Alanine, GABA, histamine (r = 0.97, 0.93, 0.77, 0.86, $P<0.01$), anserine with β-alanine, GABA, histamine (r = 0.97, 0.82, 0.87, $P<0.01$), GABA with β-alanine, histamine (r = 0.90, 0.78, $P<0.01$), β-alanine with histamine (0.84, $P<0.01$).

3. Concentration of carnosine, anserine, β-Alanine, GABA, and histamine in heart of rats by HPLC analysis

The results in Table 12 demonstrated that concentration of carnosine, anserine, histamine β-alanine, and GABA in the heart of rats. The level of carnosine was significantly higher in the 7 and 35 mg PN HCl/kg diet (+89% and +116%, respectively, $P<0.005$) than

in the 1 mg PN HCl/kg diet. The concentration of anserine was significantly higher in the 7 and 35 mg PN HCl/kg diet (+180% and +335%, respectively, $P<0.005$) than in the 1 mg PN HCl/kg diet. The concentration of histamine in 7 and 35 mg was significantly high, and the level of histamine in the 1 mg PN HCl/kg could not be detected. The concentration of β-alanine was significantly higher in the 7 and 35 mg PN HCl/kg diet (+108% and +93%, respectively, $P<0.005$) than in the 1 mg PN HCl/kg diet. The concentration of GABA was significantly higher in the 7 and 35 mg PN HCl/kg diet (+137% and +164%, respectively, $P<0.005$) than 1 mg PN HCl/kg diet.

There was a significant correlation of carnosine with anserine, β-Alanine, GABA (r = 0.65, 0.75, 0.73, respectively, $P<0.01$), anserine with β-Alanine and GABA (r = 0.58, 0.60, respectively, $P<0.01$), GABA with histamine (r = 0.62, $P<0.01$).

IV. Discussion

There were over 500 metabolites detected, and 21 metabolites were affected by dietary B6. The first group is the metabolites related to imidazole peptides were carnosine, anserine, homocarnosine, histamine, and IH-imidazole-4 propionic acid. The results of carnosine and anserine were consistent with our previous study (Sofya *et al*., 2015). The second group is the amino acids such as alanine, serine, isoleusine, leucine, valine, methionine. The third group is the metabolite in the TCA cycle and urea cycle such as malic acid, fumaric acid, arginosuccinic acid, and ornithine. The fourth group is the other metabolites such as β-alanine, GABA, γ-butyrobetaine, carnitine, adenine, and FAD. These metabolites except for ornithine were significantly increased by the high B_6 diet, whereas ornithine was enhanced by the low B_6 diet. Among those metabolites, carnosine, anserine, β-alanine, GABA, histamine, fumaric acid, malic acid, carnitine, and adenine are known to have a heart-protective effect.

Human study showed that oral administration of carnosine increased exercise tolerance and quality of life in patients with chronic heart failure (Lombardi and Metra, 2015). Many animal studies demonstrated that carnosine has a heart-protective effect including anti-inflammatory and anti-ischemic effect on the heart (Fleisher-Berkovich *et al*., 2009; Stvolinsky and Dobrota, 2000). Carnosine and anserine act as antioxidant, pH-buffering, anti-glycation, and ergogenic factors (Boldyrev *et al*., 2013; Caruso *et al*., 2012). The protective effect of histamine for cardiovascular disease (CVD) is mediated through activation of histamine 3 receptor (H3 receptor). H3 receptor is a therapeutic strategy in CVD characterized by hyperadrenergic activity, such as myocardial ischemia and congestive heart failure (Morrey *et al*., 2008). Endogenous histamine plays protective role

in the evolution from myocardial infarction (MI) to heart failure (Chen, 2017). In human study, low GABA concentrations in blood and CSF might cause early neurological deterioration in patients with acute systemic stroke (Davalos *et al*., 2000). In animal study, by using the spontaneously hypertensive rats, GABA showed an antihypertensive effect. GABA rice reduced hypertension and could prevent of cardiovascular and cerebrovascular diseases associated with high blood pressure (Nishimura *et al*., 2015). GABA could enhance the Baroreceptor sensitivity (BRS) function, adjusting heart rate to reduce the blood pressure fluctuation (Ma *et al*., 2015). GABA inhibited noradrenaline release from sympathetic nerves in the mesenteric arterial bed via presynaptic GABA receptors (Hayakawa *et al*., 2002). Pre-supplementation of β-alanine in rats on left main coronary occlusion was associated with a 57% reduction in infarct size to risk area ratio (McCarthy and DiNicolantonio, 2014). Fumarate has been reported to exert cardioprotective effect via activation of the Nrf2 antioxidant pathway (Ashrafian *et al*., 2012). Malate was demonstrated to reduce myocardial infarct size, serum levels of TNF-α, and platelet aggregation (Tang *et al*., 2013). Carnitine reduced blood pressure and attenuated the inflammatory process associated with arterial hypertension (Miguel-Carrasco *et al*., 2008). Adenine reversed myocardial infarction-induced reduction of artery pressure and left ventricular systolic pressure and elevation of left ventricular end-diastolic pressure (Sun *et al*., 2010).

Carnosine is synthesized from histidine and β-alanine by carnosine synthase, while anserine is produced from carnosine by carnosine-N-methyltransferase (Andersen *et al*., 2016). Homeostasis of carnosine and anserine in mammalian heart is controlled by circulating β-alanine (Blancquaert *et al*., 2016). In this study, high B_6 diet markedly elevated the concentrations of carnosine, anserine, and their precursor; β-alanine. This increase in β-alanine by high B_6 diet was also consistent with our previous study (Sofya *et al*., 2015). There was a substantial correlation of β-alanine with carnosine and anserine. Thus, increased carnosine and anserine by dietary B_6 might be caused by increased β-alanine. Homocarnosine is a dipeptide of histidine and GABA. The previous study reported that the availability of GABA affects homocarnosine synthesis (Petroff, 2002). In this study, homocarnosine levels had a strong correlation with those of GABA. Thus, increased homocarnosine by dietary B_6 might relate to higher GABA. Histamine is derived from the decarboxylation of histidine; a reaction is catalyzed by the enzyme histidine decarboxylase, a PLP-dependent enzyme. Thus, the higher histamine levels by high B6 diet might be mediated by higher conversion from histidine. The concentrations of β-alanine and GABA was increased by dietary B_6 and inversely associated with ornithine concentration.

Ornithine is converted by ornithine decarboxylase to β-alanine in polyamines pathway. Meanwhile, GABA is synthesized by aminotransferase from ornithine through glutamic acid pathways. Thus, high B_6 diet might increase the formation of β-alanine and GABA from ornithine through the two pathways. γ-Butyrobetaine is a precursor for carnitine. Therefore, the higher concentration of γ-butyrobetaine by high B_6 diet might be ascribed to the increased level of carnitine. The underlying mechanisms of the alterations of other metabolites such as alanine, serine, isoleucine, leucine, valine, methionine, fumaric acid, malic acid, argininosuccinic acid, butyrobetaine, carnitine, adenine, and FAD by dietary B_6 are still unclear at the present.

V. Conclusion

We found the effect of supplemental dietary of vitamin B_6 on several metabolites that related to imidazole peptides, amino acids, TCA and urea cycle, and others. The high level of vitamin B_6 increased the concentration of all metabolites except ornithine that has a high concentration in a low level of vitamin B_6. Several these metabolites have a protective effect on the heart such as carnosine, anserine, β-alanine, GABA, histamine, fumaric acid, malic acid, carnitine, and adenine. The results of this study suggest that the possible mechanism of the heart-protective effect is ascribed to increased heart-protective factors by dietary B6. This study provides a novel mechanism why adequate vitamin B6 status is essential to maintain the optimal heart health.

Table 10. Effect of dietary B6 on body weight and food intake in rats

	B6 (mg/kg diet)		
	1	**7**	**35**
Final body wt (g)	412 ± 9	444 ± 9	437 ± 13
Gains in body wt (g/6 wk)	306 ± 10	339 ± 9	331 ± 12
Food intake (g/6 wk)	731 ± 10	787 ± 15	772 ± 24

Mean ± SE (1 and 35 mg/kg diet group): n = 10; 7 mg/kg diet group: n = 6).

Table 11. Relative peak areas of detected compounds from rat heart by metabolomics analysis

Compound name	Relative peak area		Comparative Analysis		
	1mg B6/kg	35mg B6/kg	35 mg B6/kg vs 1mg B6/kg		
			Ratio	*p*-value	
Metabolites related to imidazole peptides					
Carnosine (10^{-3})	2.8 ± 0.5	7.1 ± 0.7	2.51	0.002	**
Anserine (10^{-3})	3.3 ± 0.3	6.9 ± 0.5	2.07	0.0004	***
Homocarnosine (10^{-4})	0.8 ± 0.1	1.4 ± 0.1	1.90	0.005	**
Histamine (10^{-3})	1.3 ± 0.2	3.0 ± 0.4	2.24	0.017	**
1H-imidazole-4-propionic acid (10^{-4})	0.3 ± 0.0	0.4 ± 0.1	1.65	0.028	*
Metabolites related to amino acids					
Alanine (10^{-2})	88 ± 4.6	120 ± 2.4	1.31	0.002	**
Serine (10^{-2})	26 ± 6.9	38 ± 6.7	1.43	0.030	*
Isoleucine (10^{-2})	14 ± 0.5	17 ± 0.3	1.18	0.004	**
Leucine (10^{-2})	28 ± 0.9	31 ± 0.8	1.12	0.028	*
Valine (10^{-2})	24 ± 0.8	27 ± 0.7	1.14	0.015	*
Methionine (10^{-3})	5.7 ± 0.3	7 ± 0.3	1.24	0.021	*
Metabolites related to TCA and Urea cycle					
Malic acid (10^{-2})	15 ± 0.1	20 ± 0.1	1.35	0.0008	***
Fumaric acid (10^{-3})	2.6 ± 0.0	3.3 ± 0.0	1.27	0.002	**
Argininosuccinic acid (10^{-4})	0.3 ± 0.1	0.4 ± 0.1	1.43	0.001	**
Ornithine (10^{-3})	2.2 ± 0.0	1.7 ± 0.0	0.76	0.004	**
Other metabolites					
β-Alanine (10^{-4})	0.6 ± 0.0	1.2 ± 0.1	1.89	0.0002	***
GABA (10^{-4})	0.4 ± 0.0	0.8 ± 0.0	2.28	0.005	**
γ-Butyrobetaine (10^{-3})	3.8 ± 0.3	5.1 ± 0.2	1.35	0.005	**
Carnitine (10^{-2})	56 ± 2.7	72 ± 3.3	1.28	0.006	**
Adenine (10^{-4})	0.8 ± 0.1	1 ± 0.0	1.20	0.039	*
FAD (10^{-4})	0.6 ± 0.0	0.7 ± 0.0	1.18	0.009	**

P values were calculated by the Welch's *t* test with ***:$P<0.001$, **:$P<0.01$ and *:$P<0.05$ indicating significant differences between 1 mg B6/kg group and 35 mg B6/kg group.

Table 12. Effect of dietary B6 in heart of rats by HPLC

Compound (nmol/g tissue)	B6 (mg/kg diet) 1	7	35	P-value
Carnosine	344 ± 51^{a}	652 ± 83^{b}	742 ± 68^{b}	$P < 0.005^{**}$
Anserine	50 ± 6^{a}	139 ± 42^{b}	215 ± 74^{b}	$P < 0.05^{*}$
Histamine	0.0 ± 0.0^{a}	21.3 ± 1.8^{b}	25.3 ± 4.3^{b}	$P< 0.005^{**}$
β-Alanine	12.0 ± 1.0^{a}	24.9 ± 1.8^{b}	23.1 ± 1.2^{b}	$P < 0.0001^{**}$
GABA	3.04 ± 0.35^{a}	7.23 ± 0.70^{b}	8.03 ± 0.65^{b}	$P < 0.001^{**}$

Mean ± SE (n = 5). Values with different superscript are significantly different ($p < 0.05$) by Tukey's multiple range test.

Chapter V
General Conclusion

B_6 is an essential water-soluble vitamin required for normal growth and development in mammals. PLP is the biologically active form of B_6, acts as a co-factor in over 140 distinct enzyme reactions that are involved in the metabolisms of proteins, lipids, and carbohydrates. Beyond its the role as the co-factor, B_6 has the preventive roles in certain diseases including colon diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), colitis, and colorectal cancer (CRC), as well as heart diseases such as cardiovascular disease (CVD), atherosclerosis, and stroke. However, the underlying mechanisms of these preventive effects of B_6 on colon and heart diseases are still unclear.

In the first study, I mainly focused to investigated the effect of dietary supplemental B_6 and gender difference on colon luminal environment. I hypothesized that gender difference modulates the colon luminal environment, which is dependent upon B_6 status. To examine this hypothesis, male and female rats were fed a diet containing 1 mg (low), 7 mg (recommended), or 35 mg (high) pyridoxine HCl/kg diet for six weeks. As a result, dietary B_6 significantly increased fecal mucins, and the effect was particularly profound in the female rats. The fecal mucin levels were significantly correlated with colon free threonine and serine and with gene expression of colon MUC16, implying that the combined effect of gender and dietary B_6 on fecal mucins was mediated by the alteration in the levels of such amino acids and MUC16 expression. At the same time, this study found no effect of gender and B_6 on fecal IgA. Mucins are considered to play a beneficial role in intestinal barrier function and in preventing colon cancer. Thus, dietary B_6 and gender may modulate colon luminal environment through mucin production, but not IgA production. The abundances of cecal and fecal *Akkermansia muciniphila* (mucin degrader) were unaffected. Thus, *Akkermansia muciniphila* is not likely to be responsible for the alteration in mucins. This study further showed the significant effects of gender difference on colon free amino acids such as threonine, ornithine, asparagine/aspartate ratio, glutamine/glutamate ratio, cecal and fecal *Lactobacillus* spp. levels and colon gene expressions of MUC16 and TLR8, the factors relating to colon health and diseases. My findings suggest that dietary B_6 and gender difference may have an impact on colon diseases by modulating these parameters.

In the second study, I investigated the effect of dietary supplemental vitamin B_6 on the concentrations of anti-disease metabolites in the heart. I hypothesized that dietary B6 improves heart dysfunction by modulating amino acid metabolisms. To examine this

hypothesis, I applied metabolomics analysis to investigated concentrations of metabolites of the heart in rats fed a diet containing 1 mg (low) or 35 mg (high) pyridoxine (PN) HCl/kg for six weeks. As a result, the first group affected by supplemental B_6 was the metabolites related to imidazole peptides including carnosine, anserine, homocarnosine, histamine, and 1H-imidazole-4-propionic. The second group was the metabolites related to amino acids including alanine, serine, isoleucine, leucine, valine, and methionine. The third group was the metabolite related to the TCA and urea cycles including malic acid, fumaric acid, argininosuccinic acid, and ornithine. The fourth group is the other metabolites including β-alanine, GABA, γ-butyrobetaine, carnitine, adenine, and FAD. These metabolites except for ornithine were significantly increased by high B_6 diet, whereas ornithine was increased by the low B_6 diet. Among those metabolites, carnosine, anserine, homocarnosine, histamine, malic acid, fumaric acid, GABA, histamine, carnitine, and FAD are known to have heart-protective effects. The results suggest that the possible mechanism of the heart-protective effect of B_6 is ascribed to increased heart-protective factors. My study explains the reason why adequate B_6 status is essential to maintain the optimal heart health.

In conclusion, these study demonstrated supplemental B_6 increased fecal mucins in rats in a gender-dependent manner. This combined effect of B_6 and gender was suggested to be mediated by modulating colon free threonine and serine and gene expression of MUC16. During this study, I found the gender difference modulated several parameters, including colon free amino acids, mucins, microflora, and expressions of MUC16 and TLR8, important to colon diseases. The results imply that the effects of gender on colon diseases are mediated through alterations in such parameters. Further, I found the increased levels of several heart-protective factors such as carnosine, anserine, homocarnosine, histamine, GABA, and β-alanine in the heart of rats by supplemental dietary B_6. The possible mechanisms of increase in carnosine, anserine, homocarnosine, and GABA by supplemental B_6 was discussed. Collectively, the results imply the novel mechanisms of anti-heart disease effect of dietary B_6 by elevating such protective factors. Taken together, my Ph.D. studies give an insight into the understanding of the novel mechanisms of the beneficial effects of dietary B_6 on colon and heart.

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