Potential role of dietary supplementation of indole compounds in prevention of life style related disease

Ph.D. Dissertation

Potential role of dietary supplementation of indole compounds in prevention of lifestyle related disease

(食品に含まれるインドール化合物による生活習慣病予防に関する研究)

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LIST OF PAPERS

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ABBREVIATIONS

Ccl2	C-C motif chemokine 2
Cxcl1	Chemokine (C-X-C motif) ligand 1
Cxcl2	Chemokine (C-X-C motif) ligand 2
Cxcr3	Chemokine (C-X-C motif) ligand 3
DAI	Disease activity index
DSS	Dextran sodium sulfate
FRB	Fermented rice bran
Foxp3	Forkhead box P3
IBD	Inflammatory bowel disease
Il-1β	Interleukin 1- beta
Il-17	Interleukin -17
Il-6	Ineterleukin-6
MPO	Myeloperoxidase
MUC	Mucin
RB	Rice bran
Reg3γ	Regenerating islet-derived protein 3-gamma
SCFA	Short chain fatty acids
TBARS	Thiobarbituric acid reactive substances
TA	Tryptamine
TDC	Tryptophan decarboxylase
Tnf-α	Tumor necrosis factor- alpha
UC	Ulcerative colitis
W	Tryptophan
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin

GENERAL INTRODUCTION

In human nutrition, fruits and vegetables contribute a significant part, as they are most vital sources of nutrients. Epidemiologic study revealed that higher intake of fruits and vegetables is responsible for lower incidence of life style related disease (Boeing *et al*., 2012). Currently, life style related disease is a major cause of health problems, generally affects the individuals after prolong exposure to an unbalanced or unhealthy lifestyle practices, mainly related to economic transition, rapid urbanization, smoking, drinking alcohol, westernized diet and insufficient physical activity (Scarborough *et al*., 2011). The typical lifestyle related disease is including the chronic cardiovascular disease (CVD), type 2 diabetes, cancer, gastrointestinal problem and metabolic syndrome, and these diseases are the leading cause of death globally (Yach *et al*., 2004). Besides, the life style disease is a major concern of medical economic burden. For example, In UK, more than 35% of total deaths are due to CVD and in 2006‑07, 46% of total National Health Service (NHS) costs (over £3 billion) were due to diseases related to poor diet, physical inactivity, smoking, alcohol and overweight/obesity (Scarborough *et al*., 2011).

Consuming fruits and vegetables may suppress or prevent the diseases, especially they have possessed a variety of health enhancing potential nutrients including vitamins, trace minerals, and dietary fiber, and many other classes of biologically active compounds, for example indole compounds like tryptophan, tryptamine and serotonin (Sugimoto *et al*., 2016).

Serotonin has the ability to modulate the activity in normal physiological level like gut function, the immune and inflammatory responses, the differentiation process of blood

stem cells, and also plays important roles in prevention of metabolic syndrome, by regulating adipocyte differentiation and lipid conditioning in the body (Kinoshita *et al*., 2010; Watanabe *et al*., 2011).

More recently, several studies conduct the beneficial effects of tryptophan in intestinal inflammation especially in inflammatory bowel disease (IBD), such as ulcerative colitis (UC) (Shizuma *et al*., 2013; Kim *et al*., 2010). UC is a chronic disease that is characterized by diffuse inflammation of the rectal and colonic mucosa (Ahmed *et al*., 2016), which is mainly occurred due to lifestyle practices in Western and Asian countries rather than genetic factors, increasingly hygienic environments, usage of antibiotics, intake of Western-style diets (especially high fat and low dietary fiber), reshape the intestinal microbiome to initiate dysbiosis, resulting the incidence and prevalence of UC (Ng *et al*., 2013; Seril *et al*., 2003). The occurrence of UC is approximately 4.9 to 505, 5.3 to 63.6 and 37.5 to 248.6 per 100000 people in Europe, Asia and North America respectively (Ahmed *et al*., 2016; Asakura *et al*., 2009). The incidence of UC in Japan is comparatively lower than the western countries but the number of cases increases in recent years. From the latest update of the Japanese National IBD registry, incidence of UC was found 121.9 per 100,000 persons in 2013 (Ng *et al*., 2016). This rapid increase in incidence, coupled to the considerable socioeconomic changes in Asia, has given rise to an opportunity to study the etiology of UC and importance of investigating the dietary components in the prevention of UC and minimizing the medical cost. As UC has no cure, generally lifelong medical care is required. Therapeutic approaches for UC including aminosalicylates, antibiotics and glucocorticoids, have demonstrated variable efficacy, adverse side effects, and potential

long-term toxicity (Atreua and Neurath, 2008; Faubion *et al*., 2001). Therefore, it is important to develop alternative beneficial strategies for better UC treatment.

The symbiotic relationship between host and the trillions of microbial cells that stay in the gastrointestinal tracts are interconnected on a complex molecular network, in where microbial metabolites act as major mediators of this network (Zhang and Davies, 2016). Dietary tryptophan which is unabsorbed by the cells, finally metabolized by the gut microbiota present in the lower intestine, produced several important metabolites, indole-3-aldehyde, kynurenine, indole-3-acetic acid, those can act as the ligand for aryl hydrocarbon receptor, Ahr (Zelante *et al*., 2013; Cheng *et al*., 2015). Ahr is widely renowned to regulate the adaptive immunity in order to avoid exorbitant immune response due to production of proinflammatory cytokines and chemokines like Il-17 (Quintana *et al*., 2008). In murine model of experimental colitis, generally colitis is initiated by the direct epithelial-cell damage by dextran sodium sulfate (DSS) in the large intestine, which ultimately triggers abnormal intrusion of microbial flora into the cell and initiates immune responses (Zindl *et al*., 2013). Dietary tryptophan may be an effective agent for mucosal immune-modulating agent, inducing rapid recovery and prevention of DSS model of colitis, via the down-regulation of inflammatory mediators and restoration of immune homeostasis and thereby prevent the colitis (Hisamatsu *et al*., 2012). Tryptamine is another tryptophan metabolites produced by the commensal bacteria in the large intestine, can also act as ligand for Ahr (Vikström et al., 2012), but the effect of tryptamine after tryptophan supplementation during DSS induced colitis stage still has limited information. Thus this study described the physiologically relevant doses with significant potency of tryptamine in prevention of colitis.

Rice bran is a component of raw rice that is obtained when it is removed from the starchy endosperm in the milling process (Islam *et al*., 2008) and it has recognized to possess immense potential as amelioration of life style related disease (Jariwalla, 2001), although its consumption is limited due to smell. In this context, rice bran fermented by *Aspergillus kawachii* and *Lactobacillus sp*. is an excellent option for treating UC, as it is enriched with indole compound especially tryptamine and as well as short chain fatty acids (SCFAs), for example acetic acid, propionic acid, butyric acid and also lactic acid. Generally for SCFAs production, gut microbiota works as a community, which may be ensured by the symbiotic association's microbiome and host (Basson *et al*., 2016). Thus, fermented rice bran plays an important role in maintaining a healthy symbiotic host–microbe relationship to develop the mucosal immunity for intestinal homeostasis to prevent inflammation.

Indole compounds are important orchestrators of host physiology and pathophysiology through the control of a large range of metabolic and inflammatory processes, which can be applied in the future as a therapeutic option. In future, metagenomics, metabolomics, proteomics, and transcriptomics studies will offer us to elaborate our understanding of dietary components of indole compounds in the prevention of life style related disease.

AIM OF THE STUDY

Indole compounds may be important for disease prevention, are widely distributed in a vast number of fruits, vegetables, grains and legumes. Although it's very necessary to select the right choices of food which includes the most highly content of indole compounds. Tryptophan plays an important role in the prevention of ulcerative colitis, although its mechanism of action has not been fully revealed. Besides, fermented rice bran is another excellent option for treating UC. Serotonin (5-HT) can prevent metabolic syndrome although the fate of 5-HT after orally administered 5-HT is still remaining as unknown. In particular, the following objectives of this study are as follows:

1. To determine the indole compounds level in commonly available fruits and vegetables in Japan.
2. To determine the mechanism of action of supplementary tryptophan and Ahr to prevent the DSS induced the experimental colitis.
3. To determine role of rice bran fermented by *Aspergillus kawachii* and *Lactobacillus* in the prevention of DSS induced experimental colitis.
4. To determine the 5-HT distribution and excretion after oral administration of 5-HT.

CHAPTER 1

Literature review

1.1 Indole compounds

Indole compounds are widely distributed in the natural environment and they have a wide range of biological activities including antihypertensive, anti-proliferative, antitumor, anti-inflammatory, antimicrobial, antifungal activities, etc. Indole compounds have a bicyclic structure, consisting of a six membered benzene ring fused to a five membered nitrogen containing pyrrole ring (Kaushik *et al*., 2013). Chemical structures of several indole compounds are shown in **Figure 1.1**.

1.2 Tryptophan

Tryptophan is an indole derivative, which is known to play essential biological roles, most of them being linked to metabolic pathways. Tryptophan is required by all forms of life for protein synthesis but human do not possess the enzymatic machinery to synthesize it, thus human have to take it as an essential amino acid (Kim *et al*., 2010; Moffett and Namboodiri, 2003).

1.3 Tryptamine

Tryptamine is a monoamine alkaloid that is wide spread in nature and abundantly found in animals, plants or even fungi (Szabo, 2015). It brings an indole ring structure, and is structurally similar to the amino acid tryptophan and it plays an important role as a

neuromodulator or neurotransmitter (Barker *et al*., 2012). Tryptamine is a potent Ahr activator and may be both a direct Ahr ligand and a precursor of a downstream Ahr ligand(s) (Quintana and Sherr, 2013).

1.4 Serotonin

Serotonin is a monoaminergic neurotransmitter with functions of modulating central and peripheral activities. It was originally recognized as a vasoconstrictor substance in blood serum and later chemically identified as 5- hydroxytryptamine (5-HT) by Rapport *et al*. (1958). In the human system, serotonin is produced from tryptophan via two steps. Initially, tryptophan is converted to 5-hydroxytryptophan (5-HW) by the action of tryptophan hydroxylase, (EC 1.14.16.4). 5-HW is then decarboxylated by aromatic L-amino acid decarboxylase (EC 4.1.1.28) to form serotonin, a reaction in which tryptophan hydroxylase acts as the rate-limiting enzyme (Veenstra-Vander Weele *et al*., 2000). In plants, serotonin is synthesized via a different pathway, where tryptophan is first converted into tryptamine by tryptophan decarboxylase (*TDC*; EC 4.1.1.28) and then, tryptamine is converted to serotonin by the action of tryptamine 5-hydroxylase (Schröder *et al*., 1999).

1.4.1 Serotonin prevents life style related disease

Serotonin has been involved in glucose and lipid metabolism and adipocyte differentiation in vitro and regulates the fat and feeding in *Caenorhabditis elegans*, which ensured that peripheral serotonin has play important roles in metabolic

homeostasis (Watanabe *et al*., 2011; Srinivasan *et al*., 2008). For example, in mice, serotonin induces hypoglycemia and hyperinsulinemia at 30 min after injecting glucose (Sugimoto *et al*., 1990) and by insulin secretion through serotonylation of GTPase within the pancreatic β-cells (Zawalich *et al*., 2004).

1.5 Aryl hydrocarbon receptor (Ahr)

The Ahr is a ligand-activated transcription factor that is different from members of the steroid receptor superfamily, belongs to the member of the basic helix-loop-helix (bHLH) superfamily of DNA binding proteins and is activated by ligand binding and by dimerization with the Ahr nuclear translocator (Arnt) (Quintana and Sherr, 2013; Sato *et al*., 2013). Ahr is initially discovered as the receptor minimizing the harmful effects of toxic environmental chemicals such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, or TCDD) (Quintana and Sherr, 2013) and it has higher mRNA expression in liver, kidney, lung, heart, thymus, and placenta (Fernandez-Salguero *et al*., 1995). Dioxin-inducible genes regulated by the Ahr includes both the phase I and II xenobiotic-metabolizing enzymes (Nebert *et al*., 2004; Omiecinski *et al*., 2011).

1.6 Dextran sodium sulfate (DSS)

DSS is a water-soluble, negatively charged sulfated polysaccharide with a different molecular weight ranged from 5 to 1400 kDa, which can initiate colitis in murine model resembling to human UC (Chassaing *et al*., 2014), by dissolving (0.25-5%) in drinking water. The underlying mechanism by which DSS initiates intestinal inflammation is still unknown, but it generally causes damage and ulceration to the epithelial monolayer

lining the large intestine and increases intestinal permeability to begin the abnormal influx of microbes and the activation of inflammatory cells (Takamura *et al*., 2010). The DSS induced colitis model is very popular in experimental colitis research because of its rapid use, easy, reproducibility and convenient administration. Different phases of disease like acute, chronic and relapsing models of experimental intestinal inflammation can be achieved by altering the concentration and frequency of administration of DSS.

1.7 Ulcerative colitis (UC)

UC is the typical progression of inflammatory bowel disease (IBD) that mainly affects the innermost lining of the large intestine (Monteleone *et al*., 2013). The major complications of UC include: causing severe and long lasting symptoms like diarrhea, abdominal pain, rectal bleeding, weight loss and fatigue, perforated bowel—weaken intestinal wall, toxic megacolon—colon becomes enlarge and swollen (Bouguen *et al*., 2015) and also extra intestinal complications like musculoskeletal (arthritis), dermatologic, hepatopancreatobiliary, ocular, renal, and pulmonary disease (Levine and Burakoff, 2011). UC significantly hampers a patient's quality of life and responsible for higher financial burden (Chami *et al*., 2014). For example, in USA, it was estimated that yearly cost of UC treatment ranged from $4982 to $15,020 per patient (Gibson *et al*., 2008).

1.7.1 Role of dietary factors in UC

Study revealed that breastfeeding provides protection against IBD in offspring in their later life, as breastfeeding invests immunity to the child's intestinal immune system in their developing stage (Klement *et al*., 2004). Global variation in dietary habits probably explains the differences in the risk of IBD occurrence across geographical regions and the increase in disease incidence in migrant and developing populations. Diet habits may be effective to limit inflammation because prolonged consumption is associated with a very low incidence of severe adverse effects. Therefore, the occurrence and severity of IBD may be connected to dietary factors (Asakura *et al*., 2008), for example, excessive consumption of carbohydrates and red meat has been reported to increase the UC incidence (Baumgart and Carding, 2007).

1.9 Interleukin 22

Interleukin (IL)-22 is expressed by both the adaptive immune system cells (dendritic cells and natural killer cells) and innate lymphocytes such as $CD4^+$ T cell subsets, $CD8^+$ T cells (Aujla and Kolls, 2009). IL-22 promotes antimicrobial, proliferative and anti-apoptotic pathways and tissue regeneration (Li *et al*., 2014). IL-22 is also played crucial roles in IBD pathogenesis by promoting intestinal wound healing and proliferation of intestinal epithelial cells (Sugimoto *et al*., 2008). Il-22 also serves as a strong activator of the pleiotropic transcription factor Stat3 (signal transducer and activator of transcription 3), which promotes antibacterial peptide generation (Pickert *et al*., 2009).

1.10. Ahr control adaptive immunity

Ahr plays a vital role in the control of the adaptive immune response notably in the differentiation and activity of specific T-cell subpopulations (Quintana and Sherr, 2013). Besides, Ahr is a potential regulator of FoxP3 expression and Treg differentiation and suppress the interleukin Il-17 expression in Zebrafish, which finally suppress the inflammation (Quintana *et al*., 2010). $FoxP3^{+}$ in Tregs plays a central role in the control of immune reactivity to differentiate the self and non-self antigens (Sakaguchi *et al*., 2010).

1.11. Mucin genes

Mucins are large glycosylated proteins produced by epithelial tissues mainly involved in protection of all mucosal surfaces. They play an important protective role as they have ability to form gel which is responsible for a physical, chemical and immunological barrier between the environment and the organism. Mucins can be largely divided into 2 structurally different families: the secreted (MUC1, MUC3, MUC4) and the membrane (cell surface)-associated mucins (MUC2) (Ringel and Löhr, 2003).

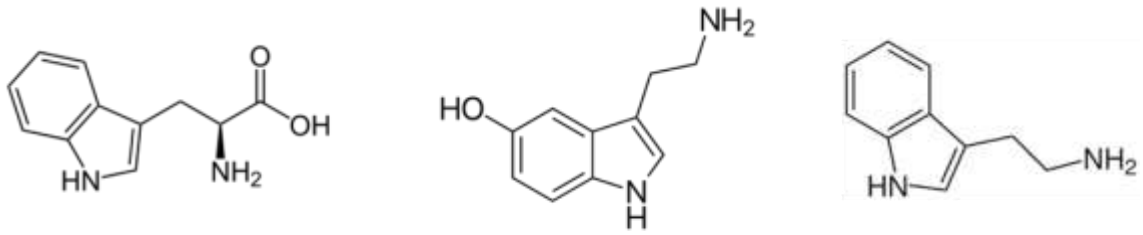
1.12 Short-chain fatty acids

Short-chain fatty acids (SCFAs), the end products of fermentation of dietary fibers by the anaerobic microbiota presented in the large intestine, have been shown to bring multiple valuable effects on mammalian energy metabolism and as well as anti-

inflammatory capacity. SCFAs play a significant role between diet, gut microbiota, and host energy metabolism (den Besten *et al*., 2013). Among the SCFAs, acetic acid, propionic acid and butyric acid are well notable as they participate in regulation of intestinal barrier (Cummings *et al*., 1987). All three SCFAs are ligands for G- protein-coupled receptors, GPR43 and GPR41 (Brown *et al*., 2003). Butyrate is also a low-affinity ligand for GPR109A (Thangaraju *et al*., 2009). Additionally, both propionate and butyrate inhibit histone deacetylase (*HDAC*) activity and thereby potential to exclude mutated epithelial cells through apoptosis induction (Zhang and Davies *et al*., 2016). Besides, lactic acid bacteria (LAB) has the superoxide anion radical scavenging capacity which also prevents murine IBD (Kuda T *et al*., 2014).

1.13 Myeloperoxidase (MPO)

Myeloperoxidase as a pro-inflammatory marker, is found in high content in neutrophils, where it is used for converting hydrogen peroxide (H_2O_2) to hypochlorous acid (HOCl). HOCl is a reactive oxygen species that chronically damages biomolecules, making it cytotoxic both to pathogenic cells such as bacteria and normal tissue cells. Neutrophils use HOCl, as a weapon against pathogens that exhibits an inflammatory response, which indicated that MPO could be a good measure of neutrophil presence in the site of inflammation and severity of the inflammation (Bradley *et al*., 1982).



Tryptophan Serotonin Tryptamine

Figure 1.1: Chemical structure of indole compounds.

CHAPTER 2

Simultaneous analysis of serotonin, tryptophan and tryptamine levels in common fresh fruits and vegetables in Japan using fluorescence HPLC

2.1 Introduction

Indole compounds are widely distributed in plants and possess morphological, physiological and molecular functions to plants (Pelagio-Flores *et al*., 2011). Tryptophan is acted as the precursor of serotonin synthesis as well as tryptamine is the intermediate product of this pathway in plants. Serotonin was first discovered as a vasoconstrictor that is released from platelets during blood coagulation and later, as an important monoamine neurotransmitter in the brain (Mück-Seler and Pivac, 2011), which affects appetite, sleep, anxiety, sexual behavior, mood, and social interactions (Tecott *et al*., 1995; Leonard, 1996). Recently, serotonin is well known for not only its neurotransmitter activity but also a hormone with various extraneuronal functions, especially in prevention of life style related disease (Watanabe *et al*., 2016).

Thus, there is enormous interest in determination of serotonin and its precursor tryptophan and tryptamine levels in the fruits and vegetables. There are several methods already established to estimate their level including thin layer chromatography, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography with ultraviolet detection (HPLC-UV), and HPLC with mass spectrometry (HPLC-MS). However, HPLC with fluorescence detection is still the method of choice because of its high accuracy and sensitivity, relatively low

cost of analysis, simple sample pretreatment requirements, and ease-of-use in comparison with HPLC-UV (Wang and Chan, 2014). Serotonin content has been estimated for a variety of plants by using RIA (Feldman and Lee, 1985) and HPLC-UV (Ly *et al*., 2008); however, to the best of our knowledge, the levels of serotonin and its precursors, tryptophan and tryptamine, in conventional foodstuffs has not been reported. Therefore, the purpose of this study was to precisely determine the levels of serotonin and its precursors, tryptophan and tryptamine, in commonly consumed foods in Japan using the fluorescence HPLC.

2.2 Materials and methods

2.2.1. Chemicals

All chemicals used were of analytical grade. Serotonin, tryptamine, L-tryptophan, and 5- hydroxy tryptophan (5-HW) were supplied by Sigma (St. Louis, MO, USA). Perchloric acid (PCA) and formic acid were supplied by Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile and water (HPLC grade) were supplied by Kanto Chemical Co. (Tokyo, Japan).

2.2.2. Sample preparation

Fresh fruits and vegetables were purchased from local markets at different times and washed carefully. For single food analysis, eight uniform fruits or vegetables were cut, pooled, and thoroughly mixed using a blender. After blending, three 1.0-g aliquots were

taken for further analysis. These aliquots were mixed with 50.0µL of an internal standard (5-HW; 1.0 mg/mL) and 4.0 mL of 0.2 M PCA, and homogenized further by using a polytron homogenizer. A 1.0-mL aliquot was then taken from each sample and centrifuged at 12,000xg for 10 min and supernatant was stored at −30°C until further analysis. Sample extraction was optimized by using 0.2 M PCA acid, which was obtained from a commercially available ELISA kit for serotonin extraction in biological samples (Immunotech, Marseille, France).

2.2.3 HPLC condition

The supernatant were injected on the HPLC column (in an Atlantis C18 column (4.6 X 50 mm, 5µm, Waters, Milford, MA, USA) at 30°C with a flow rate of 1.0 ml/min. A mixture of water, acetonitrile and 100mM of $HCOONH_4$ were used as mobile phase. Linear gradients were applied and separations were performed with 10 mM $HCOONH_4$ (pH 3.4) from 0–5 min and a linear gradient of 0–25% acetonitrile in 10 mM $HCOONH_4$ (pH 3.4) from 5–15 min; the run was continued for column washing (90% acetonitrile for 9 min), followed by re–equilibration (10 mM $HCOONH_4$ (pH 3.4) for 8 min). The total HPLC run time for each sample was 32 min, and the injection volume was 20 µL. Representative chromatogram picture is shown in **Figure 2.1**.

2.2.3.1 Preparations of standard solutions and method validation

Stock solutions of the standard analytes, at a concentration of 500 mg/mL, were prepared using Milli-Q water and appropriate volume from this solution was further

diluted to get standards of working concentrations. Calibration curves were constructed using the peak area ratio of standard analyte (tryptophan, serotonin and tryptamine) to internal standard (5- hydroxy tryptophan) using the concentration 0.25-1.0 μg/ mL (n=3). Limit of detection (LOD) was determined by injecting standard solutions, in where the response was three times as high as the threshold was selected as the LOD. The Limit of quantification (LOQ) for each standard was selected from the LOD as follows: LOQ = (10 × LOD)/3 (FDA 1994). The LOD for serotonin, tryptophan, and tryptamine were 0.00156, 0.1, and 0.0075, respectively, while the LOQ was 0.0052, 0.34, and 0.025 mg/mL, respectively. The analytical curves were constructed by calculating the regression line, and the linearity was defined by the coefficient of correlation (R^2). All analytical curves were linear within the studied range, with an R^2 value of >0.99. Four different concentration levels (0.25 - 2 μg/mL) were prepared from standard solution and then injected into the HPLC of each for 3 times. The linearity of the proposed method was estimated using calibration curve to calculate coefficient of correlation, slope and intercept values. Percent recovery (%R) of analyte was estimated successive analysis (n = 3) for three different concentrations (0. 5 μg/mL, 1 μg/mL and 10 μg/mL) of each standard, using the formula [% Recovery = (Recovered conc. /Injected conc.) x 100]. The recovery percentages of serotonin, tryptophan and tryptamine were found 92.13, 101.61 and 98.26% respectively. Besides, intra- and inter-day repeatability of responses after several replicate injections and expressed as percentages of relative standard deviation (RSD) using the formula [RSD (%) = (Standard deviation/Mean) x 100 %] and it was found to be less than 2% in all standard analytes.

2.3. Results

2.3.1 Analysis of indole compounds levels in fruits

Serotonin levels in the fruits tested ranged from 0.05–9.52 μg/g of fresh weight (**Table 2.1**). The highest serotonin levels were detected in kiwi and banana. Pineapple also contained relatively high levels of serotonin. However, the serotonin levels detected in pineapple and banana in the current study were lower than the levels reported in a previous study (Feldman and Lee, 1985). The distribution of serotonin is not uniform, and the levels are known to increase as the fruits ripen in many species, including tomato, although the inverse is true for pineapple (Ramakrishna *et al*., 2011). Among the fruits tested, watermelon had the highest tryptophan level. Grapes (green) did not contain detectable levels of serotonin, consistent with the results reported in another study (Huang and Mazza, 2011). However, green grapes contained relatively high levels of tryptophan, while avocado and pineapple had intermediate tryptophan levels in comparison with other fruits. In all the other fruits tested, the tryptophan levels were less than 10.0 μg/g of fresh weight. The highest tryptamine level was found in kiwi, whereas tryptamine was not detected in purple or green grapes. Peach, avocado, mikan (*Citrus unshiu*), and grapefruit had intermediate tryptamine levels (1–3 μg/g of fresh weight), whereas other fruits had less than 1.0 μg/g of fresh weight.

2.3.2. Analysis of indole compounds levels in leafy vegetables

Serotonin levels ranging from 0.07–0.19 μg/g and tryptamine levels of 0.26–0.71 μg/g of fresh weight were detected in spinach, lettuce, cabbage, and Chinese cabbage. Tryptophan levels were much higher, ranging from 6.46–32.00 μg/g of fresh weight

(**Table 2.2**). The highest tryptophan content was found in spinach, and its serotonin content was similar to that reported in another study (Feldman and Lee, 1985).

2.3.3. Analysis of indole compounds levels in flower and fruit vegetables

Among the flower and fruit vegetables, cherry tomato had the highest serotonin levels (**Table 2.3**), although other flower and fruit vegetable samples had less than 1.0 μg/g. In contrast, their tryptophan levels ranged from 12.63–36.12 μg/g. The highest levels of tryptophan were detected in broccoli. Cherry tomato had the highest tryptamine levels, followed by okra, tomato, bell pepper, and cauliflower. Conversely, broccoli, cucumber, and bitter melon had less than of 1.0 μg/g of tryptamine. The serotonin content in eggplant, broccoli, and cauliflower was similar to the levels detected in another study (Feldman and Lee, 1985).

2.3.4. Analysis of indole compounds levels in root and shoot vegetables

All root and shoot vegetables had significantly lower levels of serotonin (<1.0 μg/g of fresh weight) in comparison with other vegetables (**Table 2.4**). Potato had the highest level of tryptophan, followed by onion, spring onion, asparagus, ginger, and sweet potato. Spring onion had the highest tryptamine levels, and relatively high levels were detected in carrot and onion. Asparagus, sweet potato, potato, daikon radish, and ginger had lower tryptamine levels (<1.0 μg/g of fresh weight).

2.4 Discussion

Serotonin is an important phytochemical with hormonal, antioxidant, immunoactive, and neuroactive biological properties (Rayne 2010). The results obtained in this study

indicate that most of the fruits analyzed may be considered good sources of serotonin. Similarly, most of the vegetables analyzed were found to be good sources of tryptophan. The intermediate product, tryptamine, was found at relatively low levels in all foods. As such, there are two rate-limiting factors involved in serotonin synthesis in plants: tryptophan levels and the induction of *TDC*. In our study, foods with high tryptophan levels were found to contain relatively lower amounts of tryptamine. One hypothesis that might explain this result is that overexpression of *TDC* increases serotonin levels in plants. In any case, more detailed studies are needed to elucidate the role of these compounds in all plants, especially because they are not distributed equally. Previously, it was reported that tomato contained 3.2 ± 0.6 μg/g of serotonin (Feldman and Lee, 1985); however, in the present study the values obtained were higher. Both genetic and environmental factors control amine biosynthesis in tomato, even in different tomato samples grown under identical environmental conditions (Choi *et al*., 2014). Interestingly, commercially processed peeled tomato and bottled tomato juice had lower serotonin levels (1.987 ± 0.24 and 1.267 ± 0.22μg/g, respectively) than fresh tomato. Here, 5-HW was used as an internal standard, which was found undetectable in the tested fruits and vegetables. 5-HW was added to the samples at known and constant concentrations to enable accurate quantification. This indicated that all plants analyzed may synthesize serotonin from tryptophan via a single pathway, and not via 5-HW like in animals. Therefore, the results of this study may be used to further understand the nutritional value of our food, which will help to make food choices that ultimately improve our health benefits.

Table 2.1: Serotonin, tryptophan and tryptamine level in fruits

Fruit name:	Serotonin	Tryptophan	Tryptamine
Kiwi (*Actinidia deliciosa*)	9.52 ± 0.62	3.32 ± 0.13	6.38 ± 1.24
Banana (*Musa acuminata*)	9.48 ± 0.09	26.15 ± 0.37	0.959 ± 0.28
Pineapple (*Ananas comosus*)	9.11 ± 0.13	19.83 ± 4.08	1.24 ± 0.15
Avocado (*Persea americana*)	5.37 ± 0.41	16.27 ± 3.49	1.70 ± 0.52
Mikan (*Citrus unshiu*)	2.14 ± 0.08	9.18 ± 0.72	1.61 ± 0.42
Grapefruit (*Citrus paradisi*)	0.97 ± 0.42	7.07 ± 1.36	1.33 ± 1.00
Peach (*Prunus persica*)	0.22 ± 0.17	7.91 ± 4.04	3.75 ± 0.35
Grape (purple) (*Vitis vinifera*)	0.18 ± 0.03	5.04 ± 2.34	ND
Cherry (*Prunus avium*)	0.17 ± 0.06	7.85 ± 2.39	0.67 ± 0.06
Apple (*Malus pumila*)	0.15 ± 0.03	2.44 ± 1.27	0.84 ± 0.40
Kaki (*Diospyros kaki* [Thunb.])	0.11 ± 0.05	3.72 ± 0.81	0.08 ± 0.01
Pears (*Pyrus nivalis* Jacq.)	0.07 ± 0.03	2.67 ± 0.22	0.32 ± 0.08
Strawberry (*Fragaria ananassa*)	0.05 ± 0.003	19.58 ± 0.22	ND
Watermelon (*Citrullus lanatus*)	0.06 ± 0.02	30.77 ± 4.49	0.74 ± 0.16
Grape (Green) (*Vitis vinifera*)	ND	25.36 ± 0.47	ND

Values are shown as the mean ±SD μg/g of fresh weight (n=3).

Table 2.2: Serotonin, tryptophan and tryptamine level in leafy vegetables

Vegetable name:	Serotonin	Tryptophan	Tryptamine
Spinach (*Spinacia oleracea*)	0.19 ± 0.07	32.00 ± 1.02	0.26 ± 0.09
Lettuce (*Lactuca sativa*)	0.12 ± 0.03	13.18 ± 1.18	0.35 ± 0.08
Cabbage (*Brassica oleracea var. capitata*)	0.15 ± 0.03	7.38 ± 0.80	0.62 ± 0.20
Chinese cabbage (*Brassica rapa ssp. pekinensis*)	0.07 ± 0.02	6.46 ± 0.21	0.71 ± 0.21

Values are shown as the mean ±SD μg/g of fresh weight (n=3).

Table 2.3: Serotonin, tryptophan and tryptamine level in root and shoot vegetables

Vegetable name:	Serotonin	Tryptophan	Tryptamine
Asparagus (*Asparagus officinalis*)	0.55 ± 0.26	29.55 ± 1.34	0.79 ± 0.41
Carrot (*Daucus carota*)	0.34 ±0.01	7.19 ± 1.97	1.72 ± 0.63
Potato (*Solanum tuberosum*)	0.26 ± 0.14	64.47 ± 1.54	0.40 ± 0.24
Ginger (*Zingiber officinale*)	0.12 ± 0.01	20.07 ± 1.53	0.11 ± 0.04
Sweet potato (*Ipomoea batatas*)	0.11 ± 0.05	12.81 ± 1.34	0.63 ± 0.34
Spring onion (*Allium wakegi*)	0.08 ± 0.03	32.93 ± 1.15	2.77 ± 1.07
Daikon raddish (*Raphanus sativus*)	0.07 ± 0.03	4.53 ± 0.23	0.20 ± 0.05
Onion (*Allium cepa*)	0.06 ± 0.02	36.47 ± 0.47	1.33 ± 0.06

Values are shown as the mean ±SD μg/g of fresh weight (n=3).

Table 2.4: Serotonin, tryptophan and tryptamine level in flower and fruit vegetables

Vegetable name:	Serotonin	Tryptophan	Tryptamine
Cherry tomato (*Solanum lycopersicum var. cerasiforme*)	12.44 ± 0.19	16.01 ± 0.98	3.06 ± 0.09
Tomato (*Solanum lycopersicum*)	8.81 ± 0.08	12.63 ± 0.30	2.75 ± 0.068
Cauliflower (*Brassica oleracea L. var. Botrytis*)	0.23 ± 0.12	20.12 ± 0.79	1.46 ± 0.14
Okra (*Abelmoschus Esculentus*)	0.32 ± 0.14	14.33 ± 2.04	2.93 ± 0.51
Eggplant (*Solanum melongena L.*)	0.20 ± 0.07	6.73 ± 0.62	0.22 ± 0.06
Broccoli (*Brassica oleracea L. var. italica Plenck*)	0.17 ± 0.04	36.12 ± 0.31	0.94 ± 0.54
Bitter melon (*Momordica charantia*)	0.13 ± 0.05	32.58 ± 0.82	0.37 ± 0.04
Cucumber (*Cucumis sativus*)	0.06 ± 0.02	9.60 ± 0.21	0.44 ± 0.02
Bell pepper (*Capsicum annuum*)	0.02 ± 0.02	26.39 ± 0.51	2.29 ± 0.16

Values are shown as the mean ±SD μg/g of fresh weight (n=3).

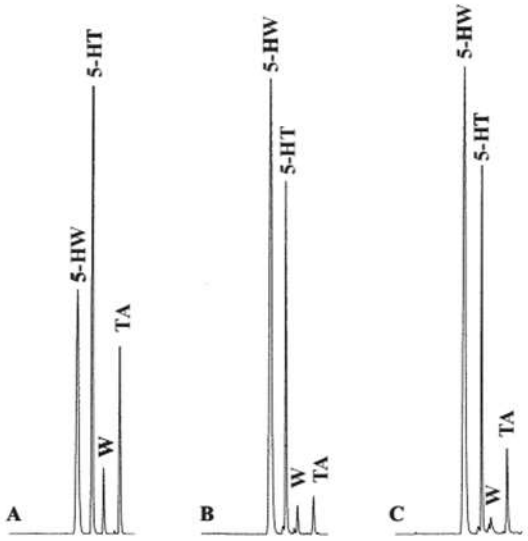


Figure 2.1: Representative chromatogram. (A) Standard profiles of 5-hydroxytryptophan (5-HW), serotonin (5-HT), tryptophan (W), and tryptamine (TA) (1 μg/ml each). (B) Tomato. (C) Kiwi.

CHAPTER 3

Dietary tryptophan alleviates dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice

3.1. Introduction

Tryptophan is an essential amino acid for mammals that is readily obtained through the diet and is regarded as a key regulator of metabolic pathways, and recent findings have demonstrated that tryptophan plays a role in protecting gut health (Hasimoto *et al*., 2012; Kim *et al*., 2010; Palego *et al*., 2016). In the body, tryptophan can enter several metabolic pathways, most notably protein-synthesis, serotonin, and kynurenine pathways or microbiota-mediated degradation in the GI tract (Keszthelyi *et al*., 2009; Zelante *et al*., 2013). Microbiota derived tryptophan metabolites can act as ligand for Ahr (Williams *et al*., 2014). Ahr mediates several toxic effects, including endocrine disruption, tumor development, cell differentiation, thymic atrophy, and immune suppression, and as compared to wild-type (WT) mice, Ahr-deficient (knockout; KO) mice are more susceptible to toxicological effects, including advancement of cardiac hypertrophy, impairment of embryonic development, skin lesions, and abnormal liver development (Furumatsu *et al*., 2011; Fernandez-Salguero *et al*., 1995). Ahr regulation by its natural ligands has been shown to lead to an abolishment carcinogenesis in the mouse intestine, where Ahr functions as a critical player in mucosal barrier defense, most notably because of its role in interleukin (Il)-22 production by innate lymphoid cells (ILCs) (Lee *et al*., 2011; Zindl *et al*., 2013).

Thus this study utilized the DSS mediated experimental colitis to investigate the further the role of dietary tryptophan in the prevention of colitis and the potential mechanisms underlying its effects.

3.2. Materials and methods

3.2.1. Materials

AIN-93M standard diet components were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Oriental Yeast Co., Ltd. (Tokyo, Japan). We purchased dextran sulfate sodium salt (DSS; MW > 40 KD) from Sigma-Aldrich (St. Louis, MO, USA), tryptophan from Wako Pure Chemicals (Tokyo, Japan). The amounts of tumor necrosis factor-α (Tnf-α) and Il-6 in mouse serum were determined using commercially available mouse enzyme-linked immunosorbent assay (ELISA) kits from Diaclone SAS (Besancon Cedex, France) and R&D Inc. (Minneapolis, MN, USA), respectively. Activity of myeloperoxidase (MPO) in tissue was measured by MPO assay kit from Biovision (Milpitas, CA, USA).

3.2.2. Ahr knockout generation

For each tail biopsy, a piece less than 3 mm in length was cut from the tail tip with sharp surgical scissors and tail was then incubated with lysis buffer (0.1M Tris- HCl (pH 8.0), 0.5M EDTA, 5M NaCl, 10% SDS) and also Proteinase K for incubate 55℃ for 12 hour and further purified by ethanol extraction and PCR was performed using specific PCR primers targeting a specific DNA sequence of gene of interest. Ahr wild

and knockout genes were confirmed by the presence of Ahr wild and neomycin (Neo) gene fragment size. The amplicon sizes for wild and Neo gene were 249 and 500 bp, respectively. Products were resolved with 1% agarose gel electrophoresis and were visualized with GelRed Nucleic Acid Stain (Biotium, Inc., Fremont, CA, USA). Primer list for genotyping is illustrated in **Table 3.1**.

3.2.3. Animals

Female C57BL/6N WT and KO mice (10–12 weeks old) were used in experiments and all mice were bred and maintained from littermates. The KO mice were kindly provided by Dr. Frank J. Gonzales (Fernandez-Salguero *et al*., 1995). Mice were housed in plastic cages (3–4 mice per cage) containing paper-chip bedding, under controlled temperature (23 ± 2°C), 50% ± 10% humidity, and a 12:12-h preset light-dark cycle. The Animal Research-Animal Care Committee of Tohoku University approved the experimental plan of this study. All experiments were conducted under the guidelines issued by this committee in accordance with Japanese governmental legislation (2005).

3.2.4. Experimental groups

Mice were provided a control diet (AIN93M standard diet for rodents) or the control diet supplemented with 0.5% tryptophan (experimental diet) (Shizuma *et al*., 2013). Composition of diets was illustrated in **Table 3.2**. To investigate the functional interaction between the tryptophan diet and Ahr, mice were allocated to 4 groups: control diet, WT and KO groups (n = 8 and 7, respectively); and 0.5% tryptophan diet, WT and KO groups (n = 8 each). The mice received these diets starting at 4 days before

the initial DSS administration; after 4 days, fresh feces were collected for determining fecal tryptamine levels (Takayama *et al*., 2011). On the day of DSS administration (Day 0), initial body weight was measured, and DSS colitis was induced in all groups by including 3.5% DSS in drinking water for 8 days. Consumption of the DSS solution, food intake, and body weight of the animals were monitored and recorded daily. The disease activity index (DAI) was obtained based on a combination of stool consistency and fecal bleeding, as described by Nishiyama *et al*. 2012 as shown in **Table 3.3**. After 8 days, mice were sacrificed, and their serum, colon, and spleen were collected. The colon was cut into several pieces and preserved for MPO assays, examination of thiobarbituric acid-reactive substances (TBARS), and histopathological and RNA analyses. For RNA analysis, the middle colon tissue was stored in RNAlater solution (Life Technologies Japan Ltd., Tokyo, Japan).

3.2.5. Histopathological analysis and scoring

The distal colon tissue was fixed in 10% formalin solution for 24 h and then preserved in 70% ethanol solution at 4°C for use in histological analysis. Fixed tissue samples were embedded in paraffin and sectioned, and the 4-µm -thick sections were stained with hematoxylin and eosin (H&E). To evaluate the degree of colitis, we modified a previously validated histopathological-score grading system as shown in **Table 3.4**.

3.2.6. Colonic MPO activity, lipid peroxidation, and measurement of serum proinflammatory cytokines

The activity of MPO, an enzymatic marker generally used to quantify the presence of inflammatory cell infiltration in colon tissue, was measured as per the instructions of the

manufacturer. Briefly, the colonic samples (after removing any visible feces or fat by using bent forceps) were thawed and homogenized on ice in 20 volumes of PBS containing 0.1% NP40, and the remainder of the assay was performed according to the manufacturer's instructions. MPO concentrations in the colon are expressed here as mU/mg of tissue. Lipid peroxidation was measured based on the formation of TBARS (Ohkawa *et al*., 1979). Briefly, samples were homogenized in 1.15% KCl solution and centrifuged, and the obtained supernatants were mixed with 0.1 ml of 8.1% SDS solution and 0.75 mL of 20% acetic acid solution and shaken vigorously for 1 min; mixed with 0.75 mL of 0.8% thiobarbituric acid solution; shaken vigorously for 1 min; and finally boiled for 1 h. Samples were mixed with 0.5 mL of H_2O and 2.5 mL of 1-butanol and pyridine (15:1), shaken vigorously for 1 min, and centrifuged. Subsequently, the absorbance of the supernatant was measured at 532 nm, and the amount of TBARS was expressed as the number of TBARS nmol/mg of tissue. Lastly, the proinflammatory cytokines Tnf-α and Il-6 were measured using the ELISA technique.

3.2.7. HPLC analysis for tryptamine content in feces, serum, and colon

HPLC analysis and the extraction procedure were performed as previously described method and materials in chapter 2.

3.2.8. Quantitative RT-PCR

RNA was isolated from colon tissue by using the commercially available reagent Isogen (Nippon Gene, Co., Ltd, Tokyo, Japan), and then RNA cleanup was performed using the RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) with optional DNaseI

treatment, according to the manufacturer's instructions. After RNA isolation, the ratio of absorbance at 260 and 280 nm was determined, and 4 μg of RNA was used to synthesize cDNA as previously described by Sato *et al*., 2013. Aliquots of the obtained cDNA were then used as templates for the subsequent quantitative PCR performed using a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified using gene-specific primers and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). The relative gene-expression levels were normalized to the amount of eukaryotic elongation factor-1α1 (EEF1A1) mRNA (Sato *et al*., 2013). The primers used in the study are listed in Table 3.4.

3.2.9. Statistical analyses

Data are presented as means ± standard error of the mean (SEM). SigmaPlot version 12.5 (San Jose, CA, USA) was used for statistical analysis. Two-way ANOVA was used to compare the effects of the diet difference (control and 0.5% tryptophan) on the two genotypes (WT and KO), and when significant interactions were observed, individual means of columns were compared using Tukey's multiple comparison. Repeated-measure-based parameters (such as body weight change over time) were analyzed using two-way ANOVA for repeated measures followed by Tukey's test. $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Dietary tryptophan attenuates clinical activity of colitis

Average food and tryptophan intake and DSS-containing water consumption did not differ in a statistically significant manner among the groups (**Figure 3.2**). The body weight change measured for the different groups is shown in **Figure 3.3A**, and these findings agree with the results of the DAI measurement (**Figure 3.3B**). Watery stool was detected and rectal bleeding was severe in mice that received the control diet and in the KO mice that received the tryptophan diet. At the end of the observation period, all mice were sacrificed and all colons were collected for measuring their length and also spleen length and weight in order to determine tryptophan association with colonic inflammation. In all groups except the WT group that received the tryptophan diet, the colon was significantly shortened and spleen length and weight were significantly increased (**Figure. 3.3 C–E**).

3.3.2. Dietary tryptophan reduces histopathological score, MPO activity and TBARS level

Representative H&E-stained sections are shown in **Figure 3.4 A–D**. Epithelial outer-layer disruption, crypt-cell damage, and inflammatory cell infiltration into the mucosa and submucosa were observed in the groups that received the control diet and in the KO group that received tryptophan diet (**Figure 3.4 A–B, D**), whereas a stable epithelial outer layer was detected in the WT group that received the tryptophan diet (**Figure. 3.4 C**). The histological score was higher in the groups that received the control diet and in the KO group that received the tryptophan diet than in the WT group that received the tryptophan diet (**Figure 3.4 E**). Dietary tryptophan significantly suppressed the colonic

MPO activity and TBARS level only in the WT group but not all others groups as shown in (**Figure 3.4 F-G**).

3.3.3. Dietary tryptophan suppresses proinflammatory cytokines and chemokines in WT mice

All animals treated with DSS showed significantly increased levels of the proinflammatory cytokines Tnf-α and Il-6 in the serum as compared to the levels in the WT mice that received the tryptophan diet (F**igure 3.5 A–B**). To investigate the role of tryptophan supplementation on local immune responses, we used quantitative RT-PCR to measure the mRNA expression of proinflammatory cytokines and inflammatory mediators involved in the pathogenesis of colitis. The mRNA levels of *Il-6, Tnf-α, Il-1β, Ccl2*, *Cxcl1*, and *Cxcr3* were elevated in the groups that received the control diet and in the KO group that received the tryptophan diet, but was decreased in the WT group that received the tryptophan diet (**Figure 3.5 C–H**).

3.3.4. Dietary tryptophan increases Ahr ligand levels and *Il-22* and *Stat3* mRNA expression

Tryptamine levels in feces before DSS administration were increased in both the WT and KO groups that received the tryptophan diet but not the control diet (**Figure. 3.6 A**). Serum and colonic tryptamine levels were measured at the end of the DSS administration (**Figure 3.6 B-C**), and the tryptamine level in the colon was found to be higher in the tryptophan-diet groups than in the control-diet groups (**Figure 3.6 C**). Serum tryptophan level was found higher only in tryptophan receiving groups (**Figure 3.6 D**). We further investigated the role of Ahr and the tryptophan diet during colitis, and our results showed that the tryptophan diet significantly enhanced the mRNA level

of Ahr in WT mice (**Figure 3.6 E**), and that the induction of Ahr also led to increased *Il-22* and *Stat3* expression (**Figure 3.6 F-G**). Furthermore, *Il-22/Stat3* induction resulted in increased mRNA levels of the antibacterial peptide *Reg3γ* and mucus-associated mucins in the WT group that received the tryptophan diet, but not in other groups (**Figure. 3.7 A–E**). Besides, colonic mRNA expression of *Foxp3* and *Il-17* to examine the adaptive role of Ahr induction by tryptophan; the tryptophan diet upregulated *Foxp3* expression but downregulated *Il-17* mRNA expression only in the WT group (**Figure 3.7 F–G**).

3.4. Discussion

Our findings indicated that dietary tryptophan protects against DSS-induced colitis in mice. For example, tryptophan supplementation reduced body weight loss, disease activity indices, and local inflammatory-cytokine and chemokines expression and improved epithelial structural integrity in WT but not Ahr KO mice. This is because tryptophan metabolites, not tryptophan itself, act as ligands for Ahr (Opitz *et al*., 2011). Generally, only a small fraction of L-tryptophan is converted into tryptamine by intestinal flora, but this amount is substantially increased after L-tryptophan administration (Vikström *et al*., 2012). Fecal excretion of tryptamine was found to be similar in both tryptophan-diet groups before DSS treatment, but colonic tryptamine levels were relatively higher in WT than KO groups, although both groups received tryptophan, which might be due to dysbiosis in the KO group after DSS treatment.

Besides, colonic MPO activity and TBARS level were significantly increased in the control-diet groups and the tryptophan-diet KO group, but not in the WT group that

received the tryptophan diet. In histological analysis, during colitis colonic inflammation is characterized by crypt-cell damage, mucosal ulceration, erosion, and neutrophil infiltration into the mucosal tissue (Kitajima *et al*., 2000), which found higher obtained here in the control-diet groups and the tryptophan-diet KO group, but not the WT group that took the tryptophan diet.

Tryptophan diet markedly increased *Ahr* mRNA expression in WT mice but not in the Ahr KO mice, and the lack of protective effect on the KO mice also revealed that the inability of the endogenous Ahr ligands to act as bioregulatory molecules in the KO groups resulted severe colitis. In normal physiological healthy gut, Ahr is important for the organogenesis of postnatal lymphoid tissues to expand the intraepithelial lymphocytes cells (IELs), and Il-22-producing innate lymphoid cells (ILCs) (Monteleone *et al*., 2013). Thus, Ahr activation upregulates *Il-22* production, which is crucial for epithelial-layer integrity. In a model of experimental *Citrobacter rodentium* infection, Il-22-deficient mice showed severe intestinal epithelial-layer damage as well as systemic bacterial burden and substantially increased mortality (Zheng *et al*., 2008). Here, colonic *Il-22* mRNA expression was increased in the WT group that received the tryptophan diet. At the initial stage of epithelial-cell damage, the recruitment of granulocytes and macrophages was correlated with a notable increase in Il-22 expression by ILCs in the middle and distal colon, which is critical for limiting further epithelial-cell damage (Zindl *et al*., 2013). However, further study is required to elucidate the role of tryptophan in inducing Il-22 production by either directly regulating Il-22 expression or regulating the production and development of type 3 ILCs through Ahr. The KO mice exhibited a considerable deficit in ILCs, which resulted in

diminished Il-22 secretion and inadequate protection against intestinal bacterial infection (Lee *et al*., *2011*). Activated Il-22 also increased the colonic mRNA expression of *Stat3* (Sugimoto *et al*., 2008), which is found similar in this study. Stat3, a central modulating agent of tissue homeostasis after colitis induction, upregulates the expression of the antibacterial peptide *Reg3γ* and the formation of the mucus layer through mucin gene expression (Dorofeyev *et al*., 2013; Hoebler *et al*., 2006). Here, this elucidated that Ahr-mediated induction of Il-22/Stat3 enhanced the expression of *Reg3γ* and upregulated *Muc1*, *Muc2*, *Muc3*, and *Muc4* in the WT group that received the tryptophan diet only but not others.

Here, Ahr upregulated the expression of *Foxp3* in the WT group receiving on the tryptophan diet. Foxp3 programs the development and function of the $CD4^{+}CD25^{+}$ Tregs that are essential for the active suppression of autoimmunity (Fontenot *et al*., 2003). However, the colonic mRNA expression of *Il-17* produced by Th17 cells was higher in all groups than in the WT group that received the tryptophan diet. In this study, we found that *Foxp3* and *Il-17* mRNA expression was reciprocally regulated, which agrees with the results of a previous study (Singh *et al*., 2011). Thus, the tryptophan diet successfully established a balance between effector and regulatory T cells through Ahr and dietary tryptophan able to prevent the progression of gut inflammation in a murine colitis model.

Table 3.1: Primer list for genotyping

Gene	Forward	Reverse
Ahr	5'-gccactcagagaccactgacggat-3'	5'-gcaaacatgaagggcagcgacg-3'
Neo	5'-agaggctattcggctatgac-3'	5'-caccatgatattcggcaagc-3'

Table 3.2: Diet composition for control and tryptophan diet

Composition	**Control diet**	**Tryptophan diet**
tert-butylhydroquinone	0.008	0.008
L- Cysteine	1.8	1.8
Choline bitartrate	2.5	2.5
Vitamin mixture	10	10
Mineral mixture	35	35
Soybean oil	40	40
Cellulose	50	50
Sucrose	100	100
Casein	140	140
Cornstarch	620.70	615.0
Tryptophan	-	5
Total (g)	**1000.0**	**1000.0**

Table 3.3: Disease activity index (DAI) scoring

Score	**Diarrheal score**	**Bloody stool score**
0	Normal stool	Normal colored stool
1	Mildly soft stool	Brown stool
2	Very soft stool	Reddish stool
3	Watery stool	Bloody stool

The sum of the two parameters is defined as DAI

Table 3.4: Histological damage scoring system

Parameters		**Score**	**Histology features**
(I)	Surface epithelial loss	0	No damage
(II)	Crypt destruction	1	Localized and mild
(III)	Inflammatory cell infiltration in mucosa and submucosa	2	Localized and moderate
		3	Extensive and moderate
		4	Extensive and severe

The sum of the scores of three parameters was defined as the histological damage score

Table 3.5: Primer list for mRNA expression

Gene	Forward	Reverse
Tnfα	5'-gacgtggaactggcagaagag-3'	5'-tctggaagccccccatct-3'
IL-1β	5'-ctgtgtctttcccgtggacc-3'	5'-cagctcatatgggtccgaca-3'
Il-6	5'-agaggagacttcacagaggatacc-3'	5'-aatcagaattgccattgcacaac-3'
Il-22	5'-ggagacagtgaaaaagcttg-3'	5'-agcttcttctcgctcagacg-3'
Stat3	5'-ttgtgatgcctccttgatcgt-3'	5'-ctggcaaggagtgggtctctag-3'
Ccl2	5'- gttggctcagccagatgca-3'	5'-agcctactcattgggatcatcttg -3'
Cxcl1	5'-ttgtgcgaaaagaagtgcag-3'	5'-tacaaacacagcctcccaca-3'
Cxcl2	5'-ccaaccaccaggctacagg-3'	5'-gcgtcacactcaagctctg-3'
Cxcr3	5'-gctgctgtccagtgggtttt-3'	5'-agttgatgttgaacaaggcgc-3'
FoxP3	5'-accacacttcatgcatcagc-3'	5'-acttggagcacaggggtct-3'
Reg3γ	5'-ttcctgtcctccatgatcaaaa-3'	5'-catccacctctgttgggttca-3'
Ahr	5'-acatcacctatgccagccg-3'	5'-gacttaattccttcagcgggga-3'
Muc1	5'-tcgtctatttccttgccctg-3'	5'-attacctgccgaaacctcct-3'
Muc2	5'-gctgacgagtggttggtgaatg-3'	5'-gatgaggtggcagacaggagac-3'
Muc3	5'-cgtggtcaactgcgagaatgg-3'	5'-cggctctatctctacgctctcc-3'
Muc4	5'-cagcagccagtggggacag-3'	5'-ctcagacacagccagggaactc-3'
Il-17	5'-ctc cag aag gcc ctc agactac-3'	5'-gct ttc cct ccg cat tga cacag-3'
Eef1a1	5'-gatggccccaaattcttgaag-3'	5'-ggaccatgtcaatggcag-3'

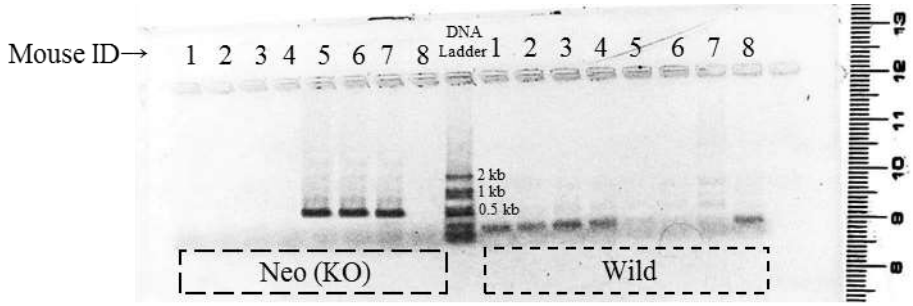


Figure 3.1: Gel electrophoresis picture of genotyping. AHR deficient mice is identified on the presence Neo gene (amplicon size 500 bp) and wild gene (amplicon size 249 bp) by gel electrophoresis.

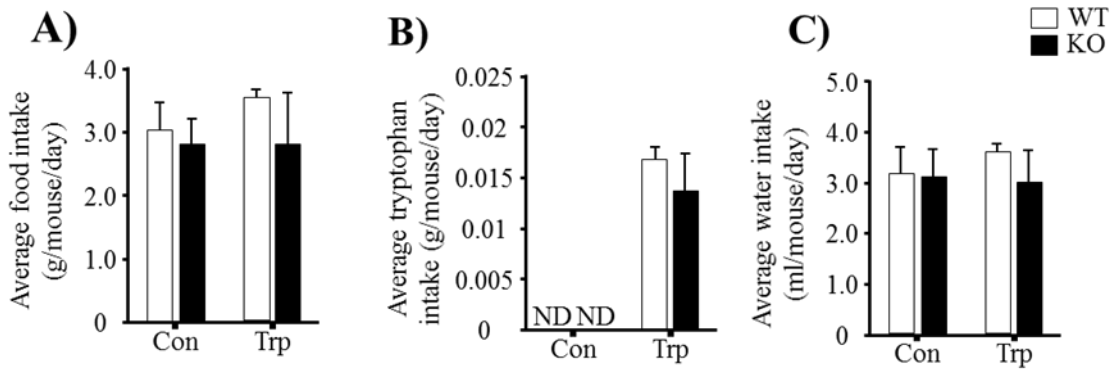


Figure 3.2: Average food and water intake. Average food (A), tryptophan (B) and water intake after 8 days of DSS treatment (P>0.5). Data are expresses as Mean ±SD (n=7-8 per group). ND= Not determined.

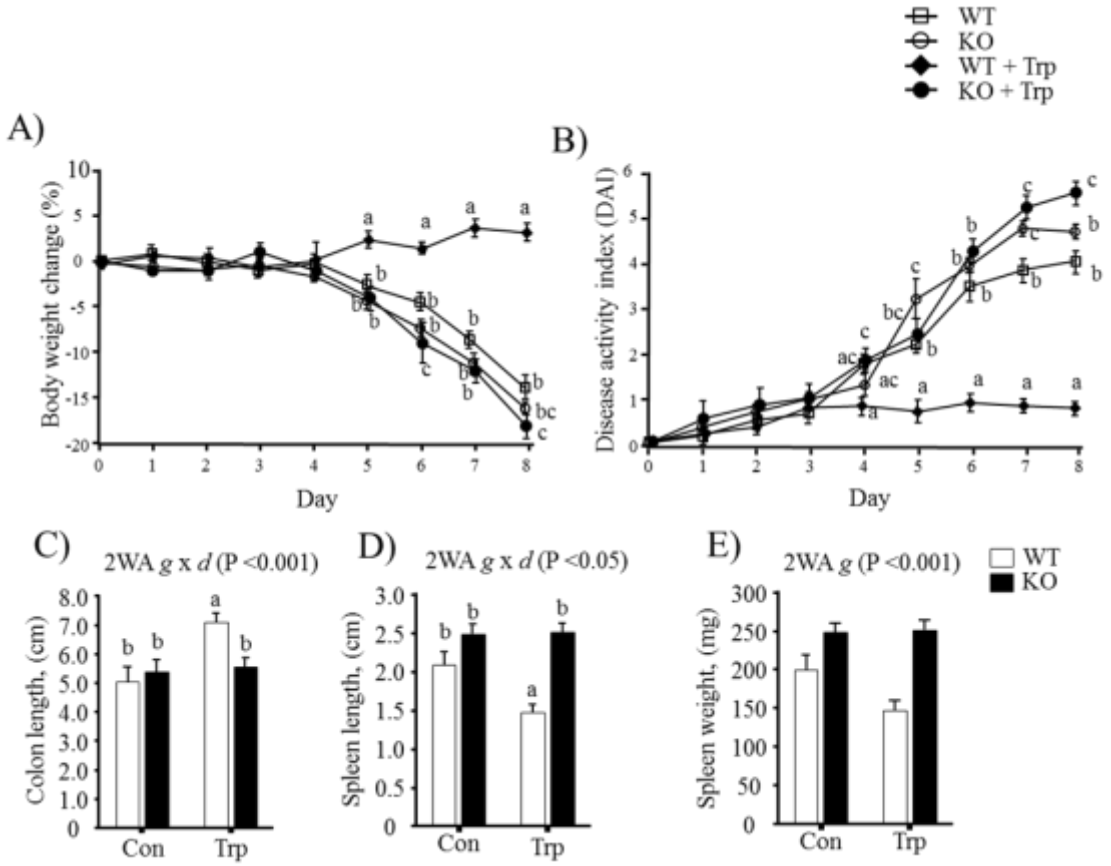


Figure 3.3: General observation and clinical index of colitis. Body weight change (%) from Day 0 to Day 8 (A). The rate of body weight gain or loss in each mouse was calculated using this formula: Body weight change (%) = [{(Weight each day)-(Weight at Day 0)}/(Weight at Day 0)] ×100 [26]. Disease activity index (B) , Colon length (C), spleen length (D), and spleen weight (E). For (A) and (B), two-way repeated ANOVA was performed, means ± SEM (n = 7–8). For (C–E), two-way ANOVA was performed followed by the Tukey multiple-comparison test; values are expressed as means ± SEM (n = 7–8). Different letters indicate significant differences. g = Genotype; d = Diet; 2WA gxd = 2 way ANOVA with a significant interaction between genotype and diet.

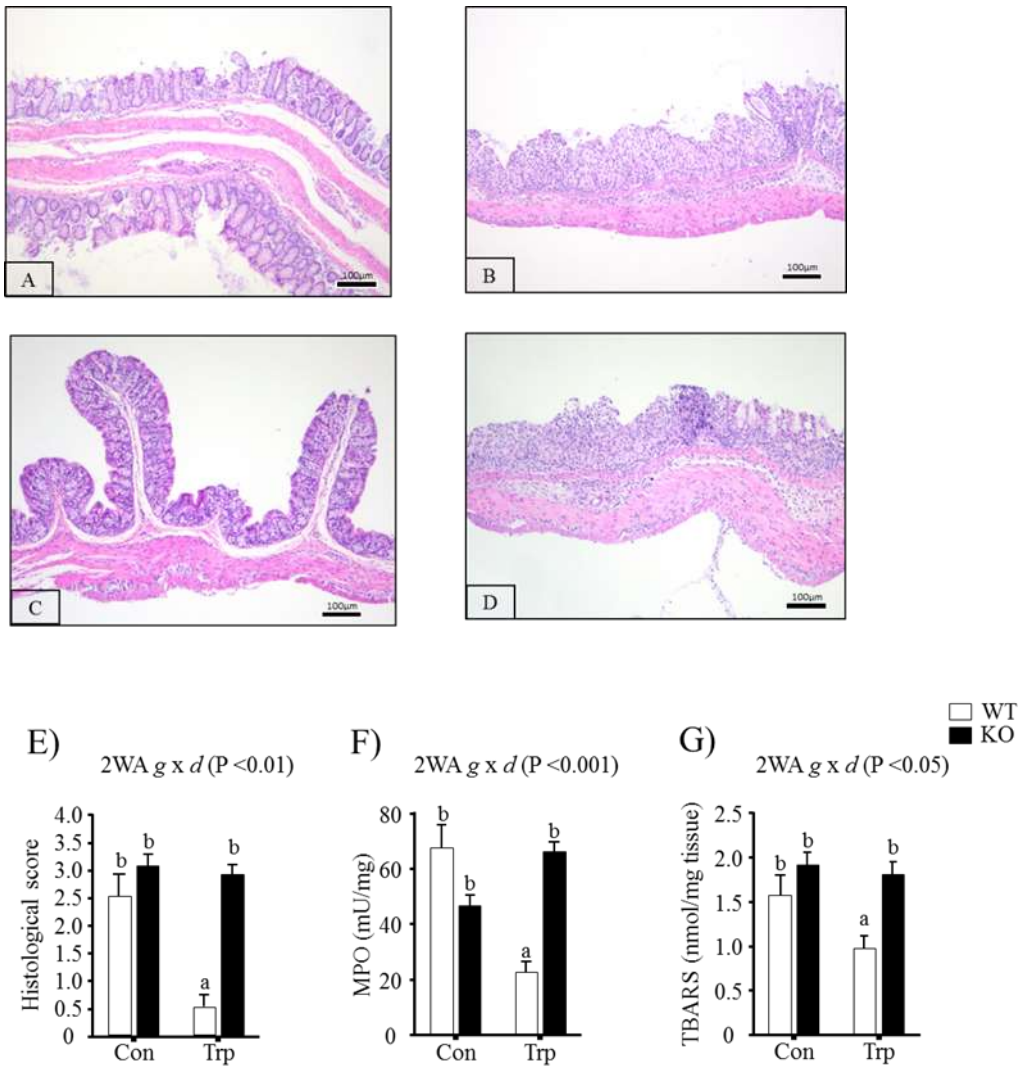


Figure 3.4: Histological and colitis-induced marker assessment. Histology (H&E staining; magnification, ×100) of colon ic-sample sections. WT (A), KO (B), WT + Trp (C), KO + Trp (D), histological score (E), colonic MPO activity (F), and TBARS level (G). Two-way ANOVA was done followed by Tukey multiple-comparison test; means ± SEM (n = 4). Different letters indicate significant differences. g = Genotype; d = Diet; 2WA gxd = 2 way ANOVA with a significant interaction between genotype and diet.

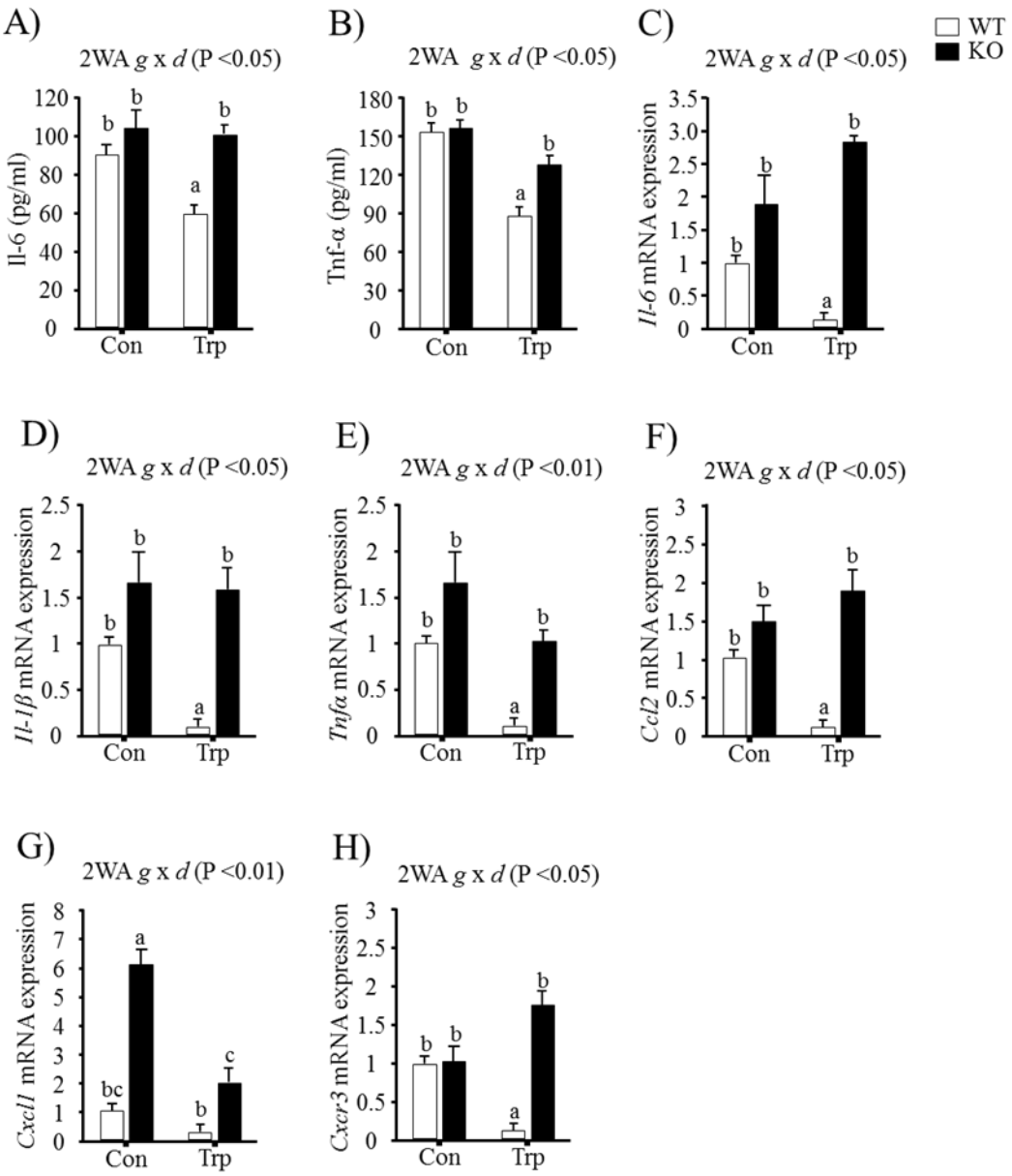


Figure 3.5: Role of tryptophan diet on proinflammatory mediators. Serum Il-6 (A) and Tnf-α (B) were measured using ELISA. Colonic mRNA expression of proinflammatory cytokines (C–E), chemokines (F-G), and a chemokine receptor (H) was measured using qRT-PCR. Two-way ANOVA was performed followed by Tukey multiple-comparison test; values are expressed as means ± SEM (n = 3–4). g = Genotype; d = Diet; 2WA gxd = 2 way ANOVA with a significant interaction between genotype and diet.

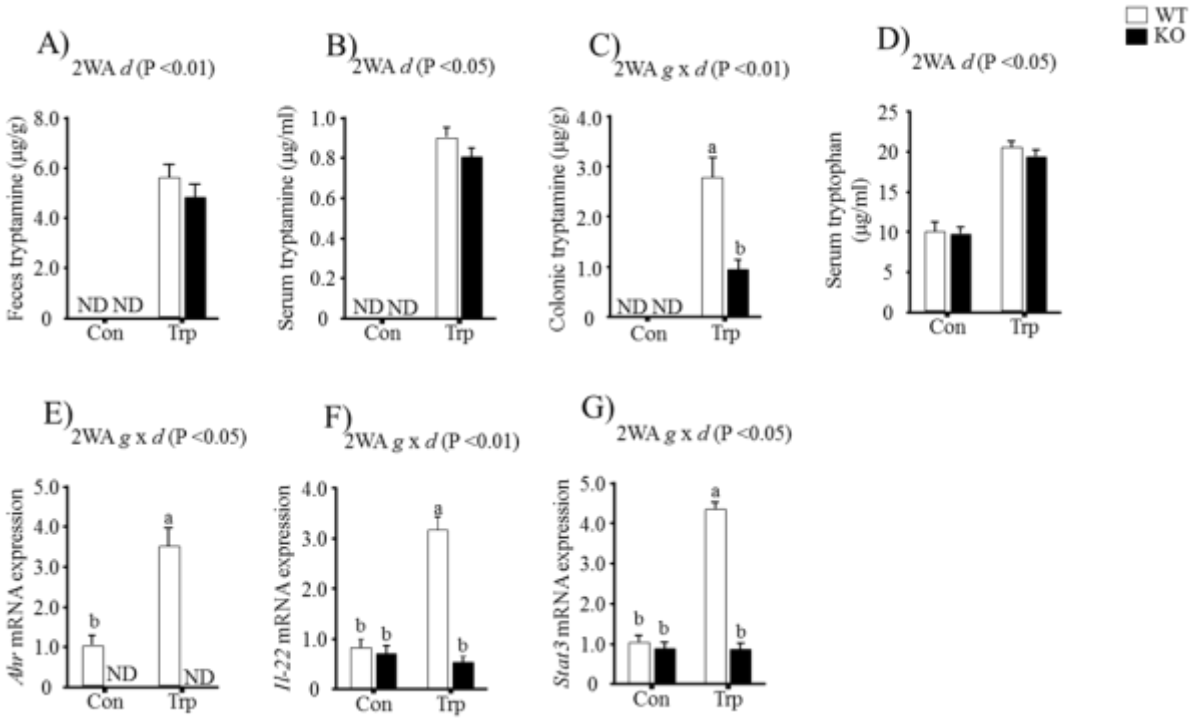


Figure 3.6: Role of tryptophan diet on tryptophan metabolites tryptamine production. Tryptamine in fresh feces before DSS treatment (A), and serum tryptamine (B), and colonic tryptamine (C) after DSS treatment. Limit of detection of tryptamine was 0.0075 µg/ml. Serum tryptophan level (D). Colonic mRNA expression of Ahr (E), Il -22 (F), and Stat3 (G) was measured using qRT-PCR. Two-way ANOVA was performed followed by Tukey multiple-comparison test; means ± SEM (n = 3). Different letters indicate significant differences. g = Genotype; d = Diet; 2WA gxd = 2 way ANOVA with a significant interaction between genotype and diet; ND = not detected.

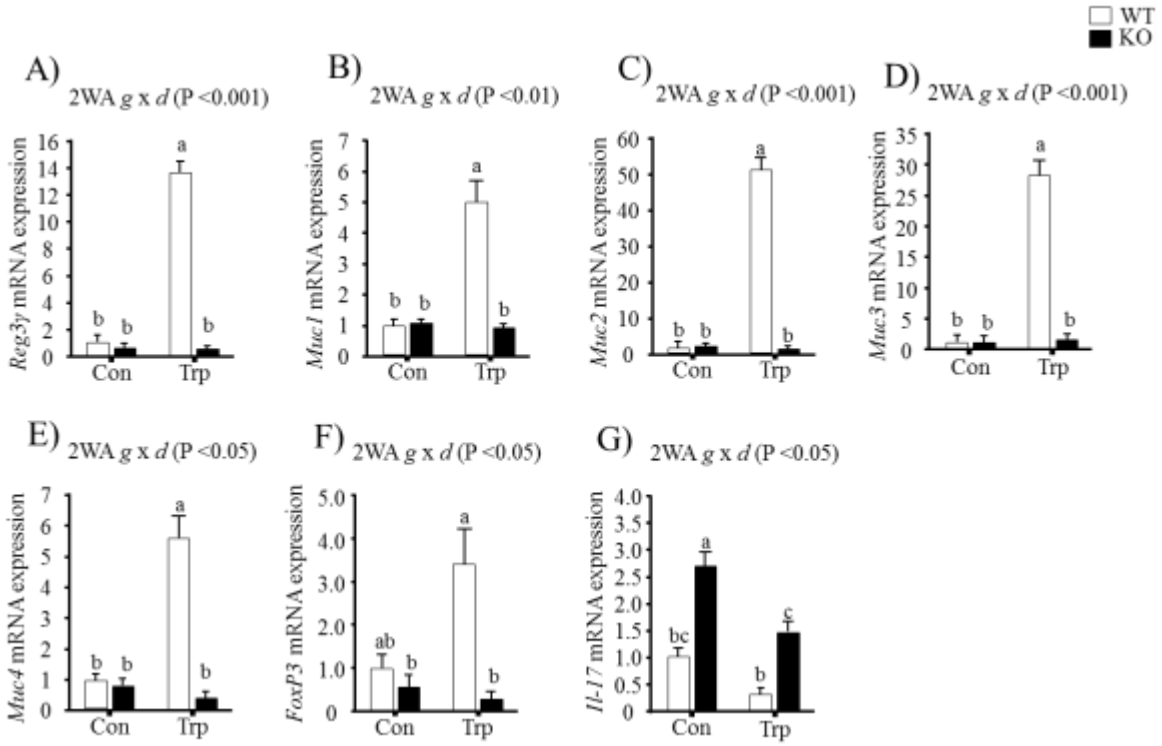


Figure 3.7: Role of tryptophan diet on antibacterial peptide production and adaptive immunity. Colonic mRNA expression of Reg3γ (A), mucins (B–E), Foxp3 (F), and Il-17 (G) was determined using qRT-PCR. Two-way ANOVA was performed followed by Tukey multiple-comparison test; means ± SEM (n = 3). Different letters indicate significant differences. g = Genotype; d = Diet; 2WA gxd = 2 way ANOVA with a significant interaction between genotype and diet.

CHAPTER 4

Protective effects of rice bran fermented by *Aspergillus kawachii* and *Lactobacillus* in dextran sodium sulphate-induced ulcerative colitis in mice

4.1 Introduction

Rice is one of the major cereal foods eaten as a staple food worldwide, especially in Asian countries (Tomita *et al*., 2008). Rice bran (RB), comprising about 10–15 % by weight of rough rice and presently available in most regions of the world as a by-product of rice polishing (Kondo *et al*., 2016). RB is a rich source of bioactive components especially dietary fiber, vitamins, and antioxidant that have potentials to promote gastrointestinal health (Choi *et al*., 2014). The usages of RB are limited and RB is discarded or used as animal feed. Methods were introduced for example, fermentation or enzymatic process or fractionalization of RB to increase its quality or making edible for human as dietary supplementation. The processed RB or the fermented RB (FRB) are becoming a food material with higher enrichment of ingredients like protein, fibers and phenolic compounds compared to the usual raw bran, and become novel prebiotics for probiotic properties.

FRB has inhibitory effects in colon, liver, and urinary bladder carcinogenesis in rodents and it has antioxidative and chemopreventive activities (Tomita *et al*., 2008). Several studies have investigated the anti-colitis effects of processing RB, in animal models, 1) RB oil (Islam *et al*., 2008), 2) brown rice fermented by *Aspergillus oryzae* (Ochiai *et al*.,

2013; Kataoka *et al*., 2008), 3) enzyme-treated rice fiber (Komiyama *et al*., 2011), and 4) RB fermented by *Saccharomyces cerevisiae* and *Lactobacillus plantarum* (Kondo *et al*., 2016). There are still differences in ingredients enrichment due to different fermentation process and methods. In this study, a unique FRB is introduced, in where *Aspergillus kawachii* and *Lactobacillus* were used during fermentation process, which enriched many ingredients as well as active compounds notably tryptophan and tryptamine than raw RB. Tryptophan is an essential amino acid, which may be considered as an effective candidate against UC (Hisamatsu *et al*., 2012; Kim *et al*., 2010). On the other hand, tryptamine is another important indole compound that can act as ligand for Ahr which finally controls the autoimmunity during colitis.

Thus this study investigated the effects of FRB supplementation in a murine model of dextran sulfate sodium (DSS) induced ulcerative colitis. DSS-induced colitis is characterized by weight loss, bloody diarrhea, and immune cell infiltration, as well as an increased production of inflammatory mediators resembles of human colitis (Ren *et al*., 2015). Besides, this study also investigated the ability of FRB to increase short-chain fatty acids (SCFAs) production, which is strongly associated with colonic health in human. Increased levels of SCFAs, notably butyric acid have been shown to induce colonic Tregs, act as energy source for enteric epithelium, limit innate immune cell-driven inflammation, and improve dysbiosis in the gut and thereby protect the mucosal health (Furusawa *et al*., 2013; van Eijk *et al*., 2009).

4.2 Materials and methods

4.2.1. Materials

HPLC grade acetic, propionic, butyric, lactic and crotonic acid were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). The other chemicals and solvents were described in method and materials in chapter 3.

4.2.2 Preparation of FRB

RB was kindly provided from Sunbran Company (Tendo, Japan). FRB was produced by dual fermentation using fungi and lactic acid bacteria. Initially, RB was steamed and cooled to 30°C and then *Aspergillus kawachii* (10^6 spores/g) was inoculated in it and kept in a fermentation chamber for 44 h. The solid-state culture obtained was named as RB koji, in where rice powder was added in a ratio of 2:1 and saccharified using a 4-volume of water at 56 °C for 12 h, heated at 85 °C for 15 min, and then cooled to about 30°C. Secondly, the saccharified culture solution was inoculated with a mixture of lactic acid bacteria (*Lactobacillus brevis, Lactobacillus rhamnosus,* and *Enterococcus faecium*) at a concentration of 0.01 % (w/w) and the solution was kept at 37 °C overnight and finally heated at 85 °C for 15 min to produce the FRB and then filtered and lyophilized. The lyophilized powder was kept at−30 °C until use (Alauddin *et al*., 2016). The major micronutrients difference between RB and FRB are described in Alauddin *et al*., 2016.

4.2.3 Animals and treatment

Male C57BL/6N aged 10-12 weeks were used in the experiment and all were maintained from littermates with free access to chow diet. The mice were housed in a pathogen-free mouse colony (temperature, 23±3°C; relative humidity, 55±10%; lighting cycle, 12 h/d) and had free access to diet and drinking water. Before starting the DSS treatment, mice were given control diet, RB and FRB diet to the respective mice groups (n=8). After 4 days, fresh feces were collected for tryptophan and SCFAs determination. Acute colitis was produced by 3% DSS mixing with drinking water for consecutive 12 days. Body weight, food intake, DSS intake, stool consistency and rectal bleeding were observed daily, and mice were sacrificed on the following day to examine the severity of colitis.

4.2.4 Disease activity index determination

Described in method and materials in chapter 3.

4.2.5 MPO and TBARS determination

Described in method and materials in chapter 3.

4.2.6 Histopathology and histological analysis

Described in method and materials in chapter 3.

4.2.7 Tryptophan and tryptamine analysis

Tryptophan and tryptamine content in RB and FRB in diet as well as feces and serum tryptophan and tryptamine level were determined by fluorescence HPLC as described in method and materials in chapter 2.

4.2.8 SCFAs analysis in feces and colon by HPLC

SCFA levels were quantified according to a previously published protocol (Hoshi *et al*., 1994) with a slight modification. Briefly, 100 mg fresh fecal samples were weighed and suspended in 2 mL of 10mM NaOH solution containing internal standard, crotonic acid (CA) homogenized with a homogenizer for about 1 min and centrifuged for 20 min at 1500×*g* at 4℃. Supernatants were collected and fat soluble compound were removed by chloroform extraction. Prior to analysis, samples were further diluted (10 x) by 20 mM NaH_2PO_4, pH 2.7. Separations were performed using the isocratic profile 20 mM NaH_2PO_4 in an Atlantis C18 column (4.6x50 mm, 5µm, Waters, Milford, MA, USA) at 30°C with a flow rate of 0.5 mL/min. The total HPLC run time for each sample was 35 min, and the injection volume was 20 µL , detected wave length at 214 nm. Fecal water content was calculated by the weight difference between before and after lyophilization (Freeze Dryer FD-550, Trap cooling temp. -45°C, Voltage: 200V, 3P for 24 h).

4.2.8.1 Method validation and working solution preparation

The analytical method for SCFAs was validated using the following parameters: specificity, linearity, sensitivity limit of detection (LOD) and limit of quantification

(LOQ), and recovery percentages determination (FDA 1994). Stock solutions (1.0 M) of lactic acid, acetic acid, propionic acid and butyric acid were prepared by dissolving appropriate amounts of each compound in Milli-Q water. The stock solutions were appropriately diluted to obtain (1 -500 mM) of working standard solutions of each. In addition to working standard solutions, internal standard was set up using the crotonic acid (Hoshi *et al*., 1994), which was freshly prepared for HPLC optimizations. A stock solution of 1.0 M crotonic acid was prepared by dissolving 0.5g of crotonic acid in 5.0 ml Mill-Q water and the resulting solution was stored at 4 °C until analysis.

The standard calibration curves for SCFAs was constructed variations of ranges 2.5- 20 mM for lactic acid, 5-40 mM for acetic acid, 312.5-2500 mM for propionic acid and 62.5-250 mM for butyric acid and fixed concentration of internal standard. The intraday and interday accuracy and repeatability were determined by analyzing three replicates of standard at different concentrations (2.0 mM for lactic acid, 4.0 mM for acetic acid, 10 mM for propionic acid and 20 mM for butyric acid) three times a day and for 3 consecutive days, respectively. Retention-time precision was determined by 3 injections of a standard samples. Samples were spiked with standards at three different concentrations of each standard and following the extraction, recovery percentages were calculated as described in chapter 2 materials and methods section and results are shown in **Table 4.2**.

4.2.9 RNA extraction and quantification of Tnf-α, Il-1β and Il-6

Described in method and materials in chapter 3.

4.2.10 Statistical analyses

Numerical data are expressed as mean ± SEM. Statistical evaluation was one way ANOVA followed by Dunnets test. The analysis was performed using SigmaPlot software version 12.5 (San Jose, CA, USA). A $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 General observation of colitis

Average food and DSS containing water intake during period of study are shown in **Figure 4.1**. During the DSS treatment, significant body weight loss and increased DAI were observed in control and RB diet receiving group but not in FRB group (**Figure 4.2 (A-B)**)**.** Both control and RB mice started to show clinical signs of illness earlier and more severely than FRB group following DSS challenge. Besides, significant shortened of colon, enlarged spleen length and weight were also found in control and RB receiving group than FRB group as shown in **Figure 4.2 (C-E)**. Shortening of the colon in mice is correlated with histological changes, and colon length is often used as a morphologic marker for degree of inflammation (Tanaka *et al*., 2007).

4.3.2 Effect of FRB on histology, MPO activity and TBARS level

Representative H&E sections and histological score were illustrated in **Figure 4.3 (A-D)**, which indicated acute inflammation occurred in the colon tissue of mice with markedly increased histological score in control and RB groups but not in FRB. After DSS challenge, MPO activity and TBARS value were found higher in control and RB group but lower in FRB group as shown **in Figure 4.3 (E-F)**.

4.3.3 FRB diet suppresses the pro-inflammatory cytokines, chemokines

The pro-inflammatory cytokines Tnf-α and Il-6 in serum and their corresponding mRNA expression in the colon were found higher levels in control and RB groups than FRB group as shown in **Figure 4.4 (A-B)**. Besides, FRB significantly reduced mRNA levels of the chemokines *Ccl2*, *Cxcl1*, *Cxcl2* and chemokine receptor *Cxcr3* than control and RB group, as shown in **Figure 4.4 (C)**.

4.3.4 Effect of FRB diet in tryptophan and SCFAs production

Tryptophan content was found 5.5 and 31.3 mg/100 g in RB and FRB groups respectively, in where tryptamine content were found 3. 78 and 32.49 mg/100 g in RB and FRB respectively. Higher tryptophan and tryptamine containing FRB diet increase the tryptophan and tryptamine levels in the feces before DSS administration as shown in **Figure 4.5**, which may increase their availability in the serum also after the DSS administration. FRB diet increased the production of fecal SCFAs before DSS administration and colonic SCFAs after DSS administration as shown in **Table 4.3**. Total water contents were found 61, 69 and 76% in control, RB and FRB group respectively.

4.4. Discussion

In this study, FRB prevents DSS induced experimental colitis by improving clinical indices of colitis. Histological analysis revealed less amount of crypt damage and inflammatory cell infiltration in FRB group compare to RB and control groups. Proinflammatory cytokines and chemokines which amplify in the inflammatory cascade of inflammatory mediators (Islam *et al*., 2008) were diminished in FRB group. Besides, MPO activity and TBARS level were significantly lower in the FRB fed group than control and RB groups, indicating that DSS induced neutrophil infiltration in the inflamed mucosa was inhibited by FRB supplementation as well as less amount of reactive metabolites were produced than control and RB groups. During fermentation, FRB enriched almost 10 times higher tryptamine, which may be acted as ligand for Ahr. Ahr has been highlighted as an immunological regulator in DSS induced inflammation by producing IL-22 production, which in turn regulates epithelial barrier function (Hisamastu *et al*., 2012). Besides, higher tryptophan content in serum in the FRB group was observed. Lower tryptophan level is responsible for severe IBD complications (Hisamatsu *et al*., 2012; Kim *et al*., 2010) and supplementation of tryptophan is a novel therapeutic strategy for IBD treatment (Shizuma *et al*., 2013).

There is limited study describing the SCFAs production by processed RB during UC (Mishiro *et al*., 2013). Here, a significant amount of SCFAs production was seen in FRB compare to RB and control diet supplementation groups both in feces and colon as shown in **Table 4.3**. The differences in the SCFAs especially butyric and lactic acid clearly reflected the amelioration potency of FRB than RB. Generally, absorption of

SCFAs is rapid and the colon absorbs more than 95% of SCFAs that are produced (Topping *et al*., 2001). SCFAs, in particular butyric acid is known to induce apoptosis of T cells through the inhibition of *HDAC*, thus eliminate the source of inflammation in the colon (Steliou *et al*., 2012; Zimmerman *et al*., 2012). Acetic, propionic and butyric acids enhance the intestinal tight junction barrier integrity (Suzuki *et al*., 2008; Hung *et al*., 2016). Thus the increased SCFAs level by FRB may contribute to characterize and construct a symbiotic intestinal environment, and prevent the development of IBD (Komiyama *et al*., 2011).

Fermentation process using *Aspergillus kawachii* increases the flavonoids (Morimoto *et al*., 2009), which are not only responsible for smell and flavor but also regulate innate immunity, inhibit the production of proinflammatory cytokines and thus reduce experimental colitis (Herath *et al*., 2003; Morimoto *et al*., 2009). In the future, more scientific evidences, such as different fractions of FRB and active components should be studied to clarify the mechanism of actions. Metabolomics analysis of FRB may also reveal multiple potential protective mechanisms including immunomodulation to prevent UC and thus FRB will be emerged as a functional food.

Table 4.1: Diet composition of control, RB and FRB

Composition	Control diet	RB	FRB
tert-butylhydroquinone	0.008	0.0072	0.0072
L- Cysteine	1.8	1.62	1.62
Choline bitartrate	2.5	2.25	2.25
Vitamin mixture	10	9	9
Mineral mixture	35	31.5	31.5
Soybean oil	40	36	36
Cellulose	50	45	45
Sucrose	100	90	90
Casein	140	126	126
Cornstarch	620.70	558.63	558.63
RB	-	100	-
FRB		-	100
Total (g)	**1000.0**	**1000.0**	**1000.0**

Table 4.2: Method validation

Compounds	Retention time	LOD, µM	Equation	R^2	Recovery %	RSD %
Lactic acid	3.04± 0.009	15.62	Y= 0.0045x+0.0059	0.99	96.26	1.45
Acetic acid	3.85± 0.004	31.25	Y=0.0036x+ 0.0012	0.99	103.14	0.61
Propionic acid	9.65± 0.009	156	Y=0.0041x- 0.0048	0.99	92.25	1.88
Butyric acid	30.53± 0.05	62.5	Y= 0.0042x+ 0.003	0.99	91.65	1.15

Table 4.3: The concentration of SCFAs (µmol/g in fresh weight)

	Feces (Before DSS treatment)			Colon (After DSS treatment)		
	Control	RB	FRB	Control	RB	FRB
Lactic acid	1.65±0.40	5.09±0.86	6.11±0.81*	ND	ND	0.77±0.05*
Acetic acid	1.49±0.39	3.71±0.54	5.43±1.38	2.24±0.30	2.23±0.16	3.64±0.22
Butyric acid	ND	0.34±0.15	0.87±0.13*	ND	ND	0.55±0.02*
Propionic acid	0.81±0.22	2.67±0.30	9.75±1.50*	3.80±0.25	4.63±0.37	5.69±0.12*

ND. Not detected. Values are expressed as mean ± SEM (n=4). *p< 0.05 compared to control.

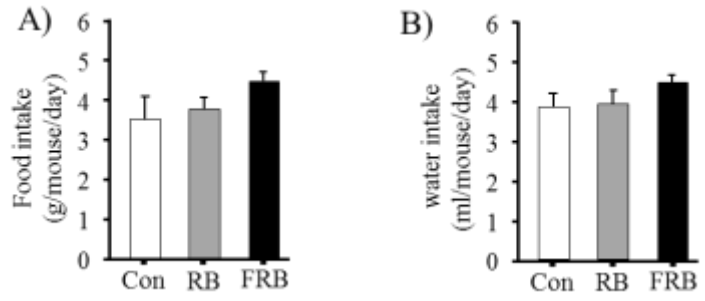


Figure 4.1: Food and DSS containing water intake. Average food; (B) DSS containing water intake (P>0.05). Data is shown as mean ±SEM (n=8).

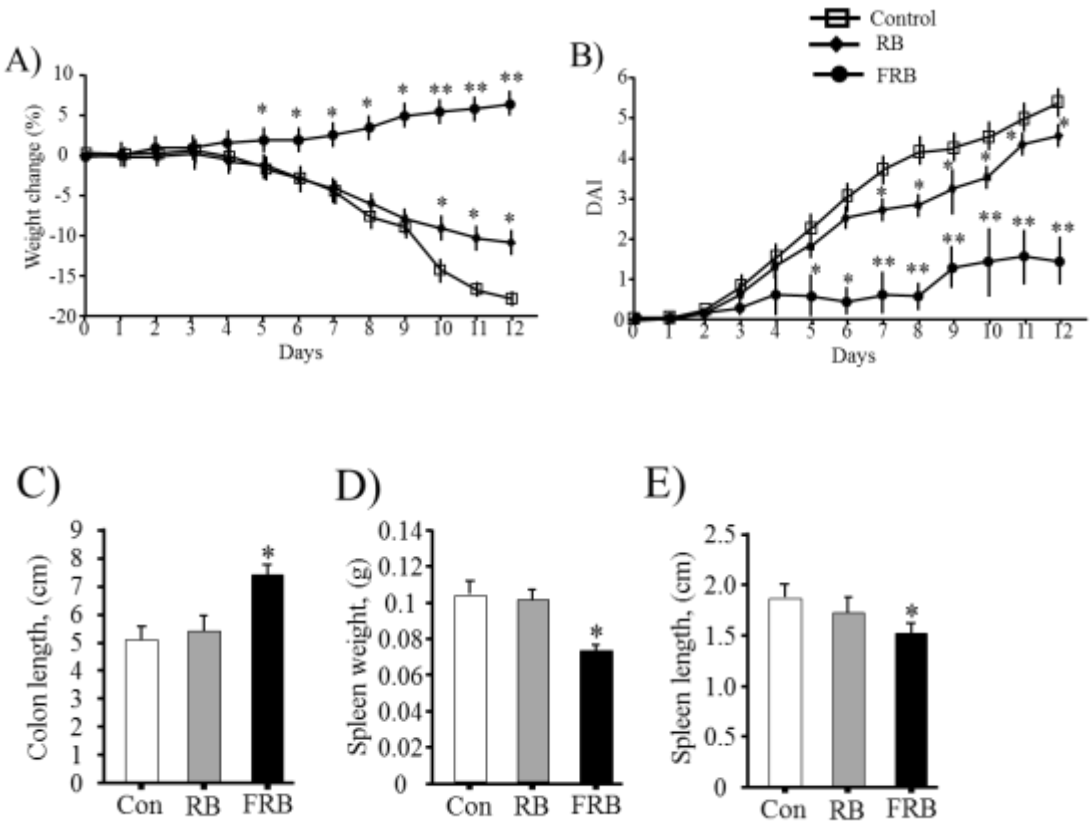


Figure 4.2: General observation and clinical indices of colitis (A) Body weight change (%) determination. (B) DAI, (C) colon length, (D) spleen weight, (E) spleen length. Values are expressed as mean ± SEM (n=8). *p< 0.05; **p< 0.01 compared to control.

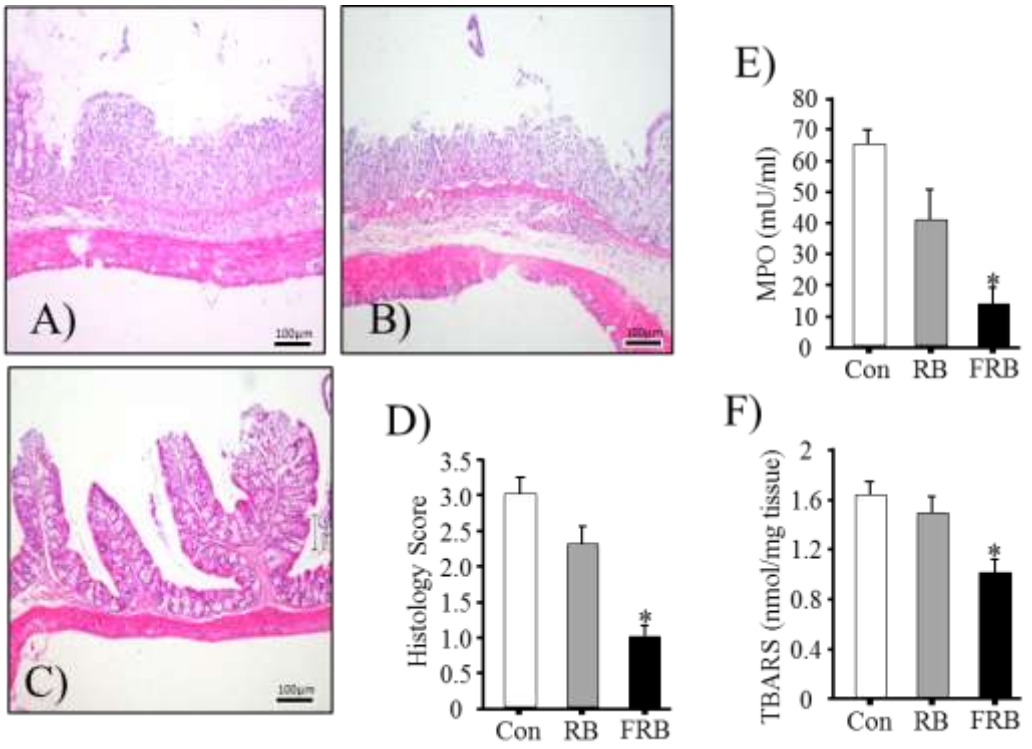


Figure 4.3: Histological analysis and colitis marker assessment. Images of H&E-stained colons (A) control, (B) RB, (C) FRB, (D) Histological score. Histological score determined by following way (I) epithelial loss (0, no; 1, mild; 2, moderate; 3, severe; 4, massive), (II) crypt cell damage (0, no; 1, mild; 2, moderate; 3, severe; 4, massive), (III) infiltration of inflammatory cells in the mucosa (0, no; 1, mild; 2, moderate; 3, severe; 4, massive). The sum of the scores of three parameters was defined as the histopathological score. (E) Colonic MPO activity, (F) TBARS level. Values are expressed as mean ± SEM (n=4). *p< 0.05 compared to control.

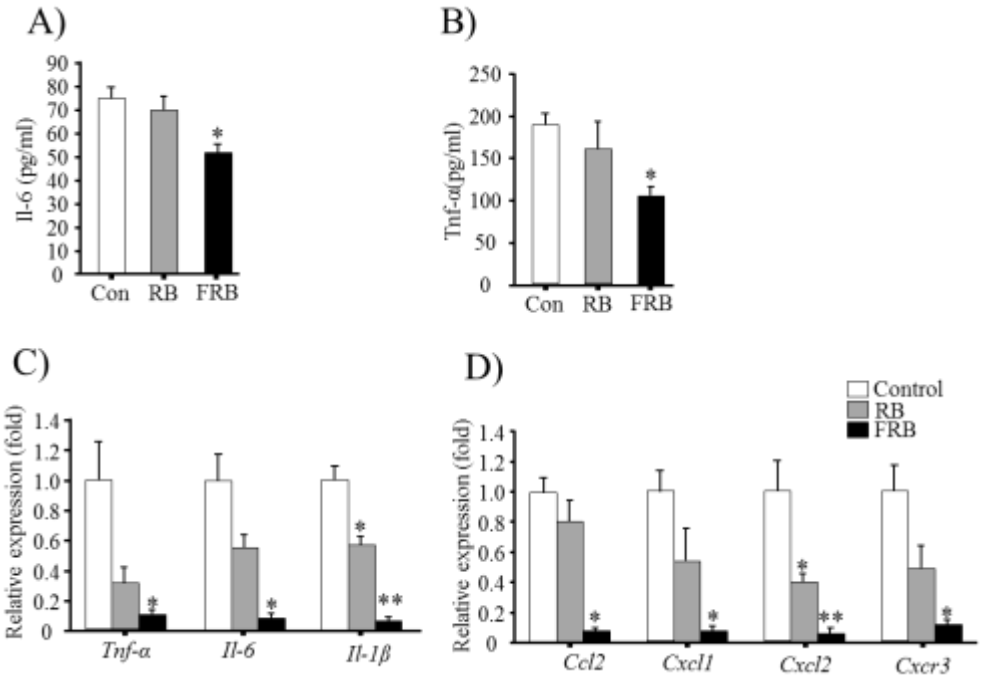


Figure 4.4: Inflammatory mediators in colitis. Proinflammtory cytokines in serum (A) Tnf-α, (B) Il-6, (C) mRNA expression of pro-inflammatory cytokines and (D) chemokines and chemokine receptor in colon. Values are expressed as mean ± SEM (n=4). *p< 0.05, **p< 0.01 compared to control.

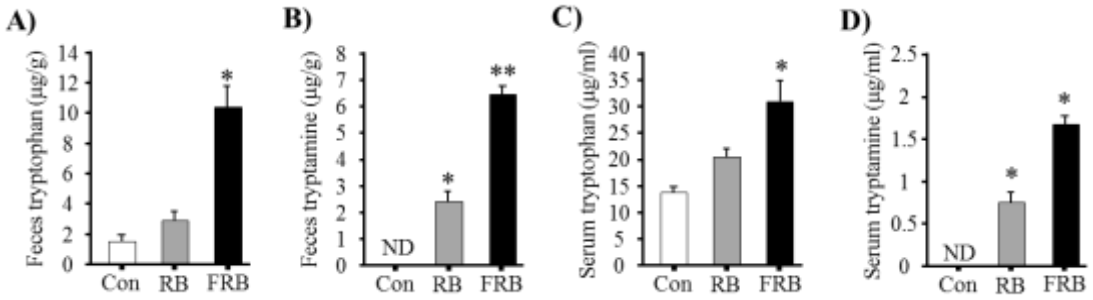


Figure 4.5: Tryptophan and tryptamine levels determination. Feces (A) tryptophan, (B) tryptamine levels before DSS administration, serum tryptophan (C), tryptamine (D) levels after DSS administration. Values are expressed as mean ± SEM (n=4). *p< 0.05 compared to control.

CHAPTER 5

Measurement of serotonin distribution and 5-hydroxyindoleacetic acid excretion after oral administration of serotonin using HPLC fluorescence detection

5.1. Introduction

Serotonin (5-HT) is an important indole compound, widely renowned as a monoamine neurotransmitter with numerous functions in the central nervous system (Nocito *et al*., 2007). 5-HT is synthesized from essential amino acid tryptophan in the enterochromaffin cells of the gastrointestinal tract and in serotonergic neurons in the central nervous system (Brenner *et al*., 2007). Centrally and peripherally synthesized 5-HT functions independently due to the inability of 5-HT to cross the blood-brain barrier and about 95% of all serotonin found in blood is stored in platelets (Lesurtel *et al*., 2006). Approximately 2% of dietary tryptophan is converted to 5-HT (de Jong *et al*., 2010). Peripheral 5-HT is metabolized primarily in the liver and lung via enzymatic conversion by monoamine oxidase-A (MAO-A; EC 1.4.3.4), resulting in 5-hydroxyindole-3-acetic acid (5-HIAA) (de Jong *et al*., 2010; Diaz *et al*., 2008). 5-HIAA is the predominant metabolite of 5-HT and is subsequently excreted in the urine (Joy *et al*., 2008; Liu *et al*., 2008). Serotonin has also multiple functions in peripheral organs acting via 15 transmembrane serotonin receptors (SERT) (Cloëz-Tayarani and Changeux, 2007).

Peripheral 5-HT has been studied because it is emerged as a mediator and activator of various biological responses in the peripheral organ, such as vasoconstriction and intestinal motility, as well as functioning as an intrinsic cofactor for the T-cell mediated immune system, cell proliferation and differentiation (Leon-Ponte *et al*., 2007). 5-HTalso played an important role in liver regeneration and minimizing oxidative stress in the liver (Lesurtel *et al*., 2006; Zhang *et al*., 2015).

In recent years, 5-HT has gained attention for its peripheral effects, prevention of metabolic disorders, for example reducing weight gain, hyperglycemia, insulin resistance, and the expansion of intra-abdominal adipocytes without affecting food intake in mice fed a high-fat diet (Watanabe *et al*., 2016). However, most research regarding 5-HT has been conducted using intraperitoneal injections (Watanabe *et al*., 2016; Weissbach *et al*., 1961), and there is little information regarding orally administered 5-HT. Several fruits and vegetables, such as the cherry tomato, contain high levels of 5-HT (Islam *et al*., 2016; Ly *et al*., 2008), suggesting that the use of these foods has potential as a therapeutic strategy for the treatment of metabolic disorders in the future.

5.2.1 Materials and Methods

5.2.2. Chemicals and reagents

HPLC-grade acetonitrile and water were obtained from Kanto Kagaku (Tokyo, Japan). 5-HT, ammonium formate, and formic acid were purchased from Wako Pure Chemicals

(Osaka, Japan). 5-HIAA and 5-hydroxytryptophan (5-HW) were purchased from Sigma-Aldrich (Tokyo, Japan).

5.2.3. Animals

Seventy-two male ICR mice (6-week-old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were kept in a pathogen-free mouse colony (temperature, 23±3°C; humidity, 55±10%; lighting cycle, 12 h/d) and had free access to standard diet and drinking water.

5.2.4. Experimental design

Here, a single oral dose of 5-HT was administered to mice at different concentrations by gavage after dividing them into 4 treatment groups: (1) control group: vehicle only (0.3 mL of 0.4% cellulose), (2) low group: 0.3 mL of 0.1 mg/mL of 5-HT, (3) medium group: 0.3 mL of 1 mg/mL of 5-HT, and 4) high group: 0.3 mL of 10 mg/mL of 5-HT. Mice were fasted overnight before administration of 5-HT at 10 AM. Three mice per group were sacrificed at every time point (0.5, 1, 3, 6, 12, and 24 h), and serum as well as liver and lung tissues were collected in every time interval. In addition, feces and urine were collected at 24 h to determine overall 5-HT excretion. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80°C.

5.2.5 Collection of feces and urine

Mice were singly housed in metabolic cages and chow diet and drinking water were provided in each cage ad libitum. Total feces were collected for 24 h after gavage, weighed, and stored at −30ºC. The metabolic cages were thoroughly examined to identify any food ingredients attached to the cage wall. In order to determine creatinine excretion in urine for 24 h, cage was thoroughly washed with 10 mL distilled water. All the diluted urine samples were collected and then filtered using filter paper and preserved at −30ºC for further analysis. Creatinine levels were determined using a commercial assay (Lab Assay Creatinine, Wako Pure Chemicals). Excretion percentages of 5-HT were determined with the following equation: (excreted amount of 5-HT/oral dose) x 100. Group values were estimated by subtracting the control group value.

5.2.6 Serotonin and 5-HIAA extraction procedure and HPLC figure

Described in method and materials in chapter 2

5.2.7 Statistical analyses

Results are presented as means ± standard deviation (SD). Differences between the time intervals were analyzed for significance with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using SigmaPlot version 12.5 (San Jose, CA, USA). Significance was set at $P < 0.05$.

5.3. Results

5.3.1. Serum levels of 5-HT after oral administration of 5-HT

Serum 5-HT levels increased within 30 min after oral gavage as shown in **Figure 5.1**. In control group, 5-HT levels were found consistent until 12 h after, which may be due to the effects of circadian rhythms (Mateos *et al*., 2009). There were no apparent differences in the measured 5-HT levels between the control and low dose groups. In medium dose group (10 mg/kg), the concentration of 5-HT reached its maximum level 12 h after administration and then began to decline. In high dose group (100 mg/kg), the 5-HT level also reached its maximum level 12 h after administration as shown in **Figure 5.1**. Serum 5-HT levels in the control group were similar to the results reported in another study (Liu *et al*., 2008). Once ingested, 5-HT is taken up by different receptors present in various tissues and blood platelets. Exogenous 5-HT affects the uptake capacity of the serotonin transporter (SERT) (Brenner *et al*., 2007).

5.3.2. Determination of 5-HT in the liver and lung after oral administration of 5-HT

In the liver, significant increases in 5-HT levels were found 1 h after treatment in the high dose group and reached maximum levels 3 h as shown in **Figure 5.2**. For medium group, 5-HT concentration was significantly higher 3 h than that of the control group. While 5-HT concentrations in the liver of mice in the low dose group and control group did not reach significance difference. All values returned to baseline levels 6 h after gavage in the low dose group, 12 h after gavage in the medium dose group, and 24 h after gavage in the high dose group. In lung, 5-HT concentrations were found to quickly

increase within 30 min in high dose group as shown in **Figure 5.3**. Overall, 5-HT levels increased in a dose- and time-dependent manner. Maximum 5-HT levels were observed in the high dose groups during the 6- to12-h time intervals. Lung 5-HT levels declined to baseline levels by 24 h after administration in all treatment groups except the high dose group. In the blood, virtually all of the 5-HT is contained in the platelets and is quickly removed during systemic circulation through the liver and lung. Here, 5-HT accumulation in the lung was more extensive than that in the liver as because higher concentration of platelets found in the lung (Gershon *et al*., 1966). Lung 5-HT levels of the control group were found to be similar to that reported in another study (Sherwin *et al*., 1986).

5.3.3. 5-HT and 5-HIAA excretion in feces and urine after oral administration of 5-HT

Administration of 5-HT resulted in increased excretion of 5-HIAA in the urine. Urinary creatinine ranged from 8-10 mg/24 h in the different groups ($P > 0.05$, data not shown). **Table 5.1** illustrates the total 5-HT and 5-HIAA levels measured in the urine. Total excretion of 5-HT and 5-HIAA from feces is described in **Table 5.1**. We determined that approximately 8.46, 2.42, and 0.42% of 5-HT was excreted in the urine, whereas 9.18, 1.99, and 11.11% of 5-HT was excreted in the feces for the low, medium, and high dose treatment groups, respectively. The metabolic pathway that converts 5-HT to 5-HIAA has been extensively investigated by other researchers (Liu *et al*., 2008; Helander *et al*., 1992). In our study, significant amounts of 5-HIAA excreted in the urine, but not 5-HT (**Table 5.1**). Dietary 5-HT increases the urinary excretion of 5-HIAA (Tohmola *et*

al., 2015); however, there is a paucity of data regarding excretion of 5-HT and 5-HIAA in the feces. In this study, we included fecal analysis of 5-HT and 5-HIAA levels in order to determine the extent of 5-HT that escapes metabolism. 5-HT and 5-HIAA were eliminated via the urine in a dose-dependent manner after oral administration; these phenomena were similar to that reported in another study wherein high levels of 5-HT were administered by intraperitoneal injection (Weissbach *et al*., 1961).

5.4. Discussion

Here, this study described that oral administration of 5-HT results in a rapid and prolonged increase in serum 5-HT levels in a dose- and time-dependent manner. These results provide new information on the importance of foods and supplements that contain high levels of 5-HT for the prevention of metabolic syndrome. As it is well known that peripheral 5-HT is involved in metabolic homeostasis and may offer new approaches for developing therapeutic drugs for hyperlipidemia, diabetes, and obesity treatment (Watanabe *et al*., 2011; Zhang *et al*., 2015) when administered via intraperitoneal injection. Commonly prescribed medications such as selective serotonin reuptake inhibitors (SSRIs) influence 5-HT availability by interfering with its metabolic clearance and uptake capacity and blocking serotonin transporter; however, they have side effects when used long term (Oosting *et al*., 2016). Our results indicate that orally administered 5-HT rapidly increases serum 5-HT levels and prolongs bioavailability. Mice in the high dose group excreted 5-HT in high amounts and showed extensive metabolism of 5-HT to 5-HIAA, which may be attributable to the high dose being above

the pharmacological range. Overall, these data suggest that orally administered 5-HT or dietary 5-HT may be as efficacious as intraperitoneal injections of 5-HT.

This study also describes a simplified method for 5-HT and 5-HIAA detection using HPLC-fluorescence analysis as a selective, specific, and sensitive assay. Finally, this study reports important findings about the accumulation, metabolism, and excretion of 5-HT after oral administration that have significant implications for future studies of lifestyle-related diseases.

Table 5.1: Twenty-four h urinary 5-HT and 5-HIAA excretion analysis

	Urinary excretion		Feces excretion	
Group	5-HT (µg)	5-HIAA (µg)	5-HT (µg)	5-HIAA (µg)
Control	6.36 ± 2.81	411.69 ± 85.34	0.64 ± 0.49	2.60 ± 1.37
Low	8.90 ± 3.98	423.11 ± 98.04	3.59 ± 2.55	7.71 ± 3.39
Medium	13.63 ± 7.04	604.41 ± 10.89	6.63 ± 3.47	21.56 ± 14.38
High	18.97 ± 2.06	1739.08 ± 635.16*	334.12 ± 54.24*	52.80 ± 17.32*

Table 5.1: Effects of oral administration of serotonin. Data are expressed as means ± SD (n=3). One-way ANOVA was performed followed by Dunnett's multiple comparison test. *P < 0.05 indicates significant difference from control.

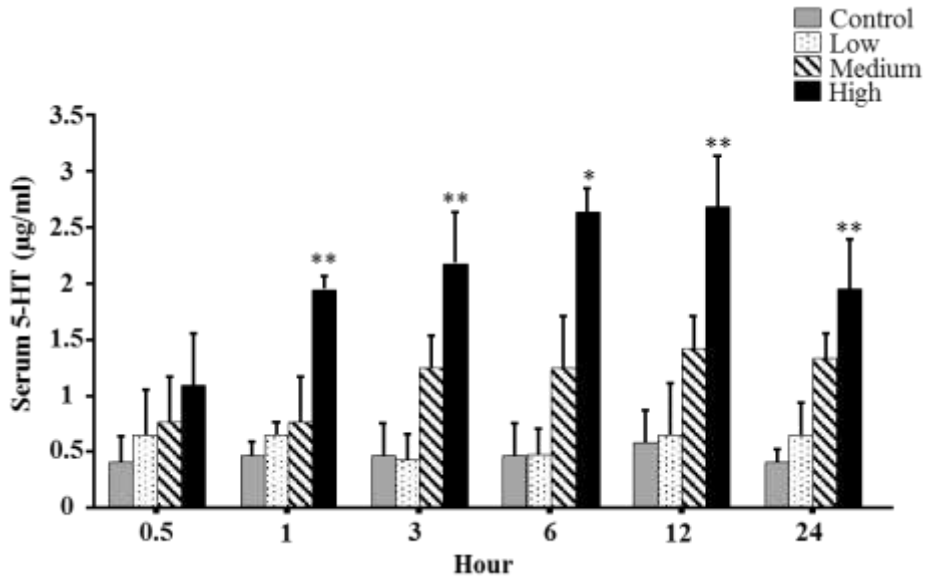


Figure 5.1: Serum 5-HT levels after 5-HT oral administration. Data are expressed as means ± SD (n=3). One-way ANOVA was performed followed by Dunnett's multiple comparison test. *P < 0.05 and ** P < 0.01 indicate a significant difference from control.

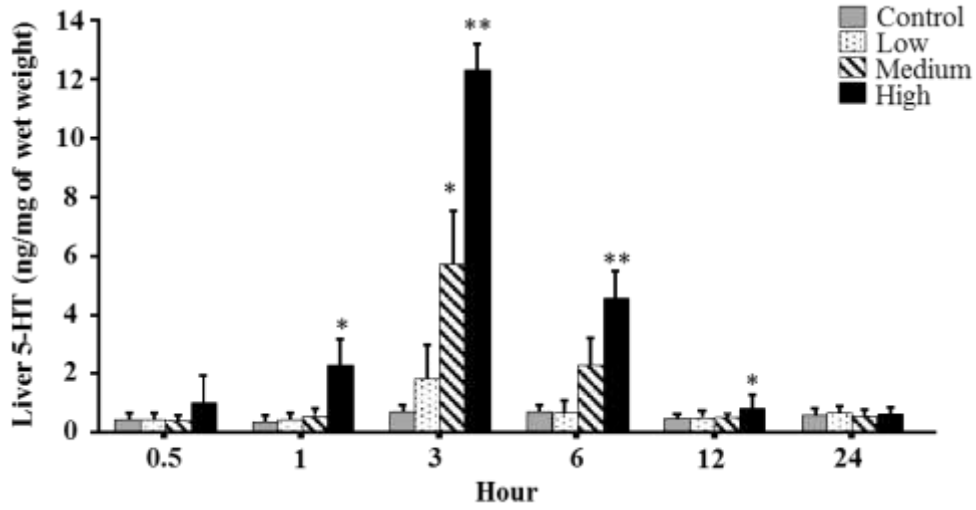


Figure 5.2: Liver 5-HT levels after 5-HT oral administration. Data are expressed as means ± SD (n=3). One -way ANOVA was performed followed by Dunnett's multiple comparison test. *P < 0.05 and ** P < 0.01 indicate a significant difference from control.

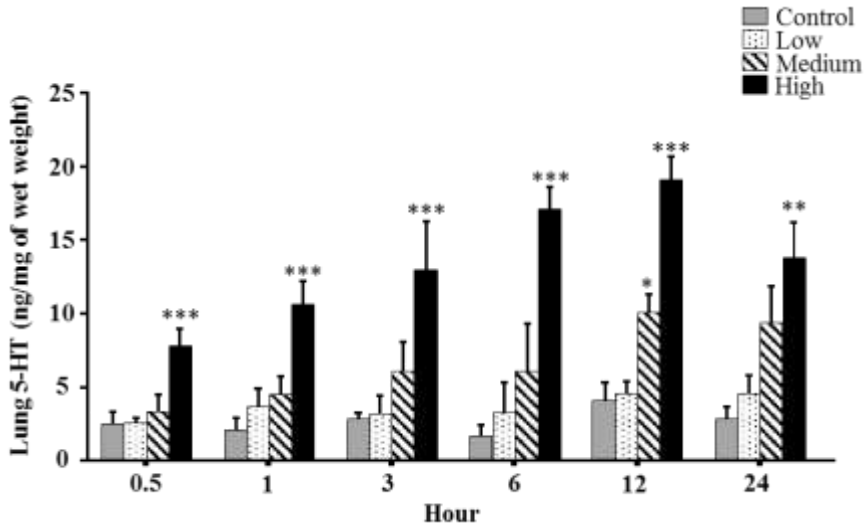


Figure 5.3: Lung 5-HT levels after5-HT oral administration. Data are expressed as means ± SD (n=3). One-way ANOVA was performed followed by Dunnett's multiple comparison test. *P < 0.05, ** P < 0.01, and *** P < 0.001 indicate a significant difference from control.

Conclusion

This study may contribute to our understanding of the origin and nutritional, physiological and medical consequences of ingesting naturally occurring indole compounds. Indole compounds tryptophan, serotonin and tryptamine comprise a diverse class of plant metabolites useful to human by modulating oxidative stress, intestinal inflammation, and hormone secretion. Many of these compounds are produced in minor quantities, and extraction and purification are often uneconomical. Producing large amounts of indole compounds in common foods may provide a better alternative. For example, considering the effects of serotonin in humans, diets enriched with serotonin-rich fruits and vegetables may prevent certain diseases such as metabolic syndrome. Therefore, the results of this study may be used to further understand the nutritional value of our food, thereby allowing us to make food choices that maximize our health benefits and may be used to make informed food choices that are good sources of both serotonin and tryptophan, such as cherry tomato, tomato, kiwi, banana and potato.

UC is a chronic intestinal illness affecting millions across the globe, mainly originated by life style practices and autoimmunity. Although many medical treatments for IBD exist, many have risky side effect profiles. An interaction between dietary tryptophan elements and the Ahr is established, which is finally emerged as a regulator of the gastrointestinal immune system and these studies delineate a mechanism in where inflammatory cytokines and other colitis marker were inhibited in the gut through the activation of Ahr by a 0.5% tryptophan diet and the subsequent induction of the *Il-22-Stat3* pathway. This study highlights advancements our understanding of Ahr activity

and tryptamine in regulation of UC, finally tryptamine may be regarded as "nutriceuticals", which also emphasized that tryptophan supplementation could be considered an optimizing candidate for IBD prevention and treatment.

The anti-colitis effects of FRB have been summarized in this study by introducing experimental colitis in mice models. The inhibitory effects of pro-inflammatory cytokines and lowering MPO activity have been identified as the major mechanisms underlying these anti-colitis effects, which were ensured by the fermented rice bran diet supplementation. Moreover, the improving effects of dysbiosis may be related to the anti-colitis effects, as FRB increases the SCFAs production both in normal and disease condition. However, the active components in dietary factors derived from fermented rice have not yet been identified, here we have found 10 times higher tryptamine in fermented rice bran. Since FRB is a just food expanding in many countries, it can be consumed by a large number of people and give them its health-promoting function. This present study demonstrates a possibility that the consumption of FRB as a dietary supplementation to prevent intestinal inflammatory disorders. Finally, this study describes the bioavailability of orally administered 5-HT and subsequent increases in serum 5-HT levels. Although only a very small amount of tryptophan is converted into serotonin, orally administered serotonin may be treated as an excellent source for increasing its bioavailability and might be contribute to maintain a healthy life and pathogenesis of disease and these results may offer new strategies for preventing life style related disease.

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