Thesis for Master's Degree

Effect of *Stachys sieboldii* Miq. supplementation on gut microbiome and brain function in mice

Advisor: Prof. Sun young Lim

Eun Na

Department of Convergence Study on the Ocean Science and Technology

School of Ocean Science and Technology
Korea Maritime & Ocean University

February, 2020

본 논문을 나은의 이학석사 학위논문으로 인준함

위원장 : 문 기 환 인

위 원 : 임 선 영 인

위 원 : 서 영 완 인

2019 년 11 월 29 일

한국해양대학교 해양과학기술전문대학원

Contents

List of Tables ····· iv
List of Figures ····· v
Abstract ····· vi

1. Introduction ····· 1

2. Methods and Materials ····· 5
2.1 Animals and diets ····· 5
2.2 Measurements of fecal microorganisms ····· 7
2.2.1 Agar plate assay ····· 7
2.2.2 3M Petrifilm plate assay ····· 7
2.2.3 Next generation sequencing using 16S rRNA gene sequences 8
2.3 RNA preparation and quantative RT-PCR ····· 9
2.4 Measurements of tissue triglyceride, total cholesterol, HDL and LDL 10
2.5 Analysis of fatty acid composition using gas chromatography ···· 11
2.6 Animal behavioral assay ····· 12
2.6.1 Passive avoidance test ····· 12
2.6.2 Morris water maze test ····· 13
2.6.3 Rotarod performance test ····· 14
2.7 Statistics ····· 15

3. Result and discussion ····· 16
3.1 Effect of *S. sieboldii* Miq. supplementation on body weight food

and ratio of intake food efficiency ····· 16
3.2 Effect of *S. sieboldii* Miq. supplementation on fecal microorganism composition ····· 18
3.2.1 Changes in fecal beneficial microorganisms ····· 20
3.2.2 Changes in fecal detrimental microorganisms ····· 24
3.3 Effect of *S. sieboldii* Miq. supplementation on cytokine expression in mesenteric lymph nodes ····· 27
3.3.1 Change in interleukin-6 (IL-6) mRNA expression ····· 27
3.3.2 Change in interleukin-10 (IL-10) mRNA expression ····· 28
3.4 Effect of *S. sieboldii* Miq. supplementation on tissue lipid profiles 30
3.4.1 Change levels of triglyceride, total cholesterol, HDL and LDL in serum ····· 30
3.4.2 Change levels of triglyceride, total cholesterol, HDL and LDL in heart ····· 31
3.4.3 Change levels of triglyceride, total cholesterol, HDL and LDL in liver ····· 32
3.5 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition in several tissues and feces ····· 33
3.5.1 Change in fatty acid composition in the serum ····· 33
3.5.2 Change in fatty acid composition in the RBC ····· 35
3.5.3 Change in fatty acid composition in the liver ····· 37
3.5.4 Change in fatty acid composition in the intestine ····· 39
3.5.5 Change in fatty acid composition in the mesenteric lymph nodes ····· 41
3.5.6 Change in fatty acid composition in the heart ····· 43
3.5.7 Change in fatty acid composition in the kidney ····· 45
3.5.8 Change in fatty acid composition in the spleen ····· 47
3.5.9 Change in fatty acid composition in the cortex ····· 49
3.5.10 Change in fatty acid composition in the cerebellum ····· 51
3.5.11 Change in fatty acid composition in the retina ····· 53
3.5.12 Change in fatty acid composition in the feces ····· 55
3.6 Effect of *S. sieboldii* Miq. supplementation on memory/learning ability ····· 57

3.6.1 Effect of *S. sieboldii* Miq. supplementation on memory ability ····· 57
3.6.2 Effect of *S. sieboldii* Miq. supplementation on spatial learning performance ····· 59
3.7 Effect of *S. sieboldii* Miq. supplementation on motor coordination ability ····· 62

4. Summary and conclusion ····· 64

References ····· 67

List of Tables

Table 1 Diet compositions of the experimental groups ····· 6
Table 2 Fatty acids composition of each diet ····· 6
Table 3 Primer sequences ····· 9
Table 4 Body weight gain, food intake FER in the experimental groups 17
Table 5 Lipid profiles in serum of the experimental groups ····· 30
Table 6 Lipid profiles in heart homogenates of the experimental groups 31
Table 7 Lipid profiles in liver homogenates of the experimental groups 32
Table 8 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of serum ····· 34
Table 9 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of RBC ····· 36
Table 10 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of liver ····· 38
Table 11 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of intestine ····· 40
Table 12 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of mesenteric lymph nodes ····· 42
Table 13 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of heart ····· 44
Table 14 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of kidney ····· 46
Table 15 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition spleen ····· 48
Table 16 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition cortex ····· 50
Table 17 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of cerebellum ····· 52
Table 18 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of retina ····· 54
Table 19 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of feces ····· 56

List of Figures

Fig. 1 Roots of *Stachys sieboldii* Miq. ····· 2
Fig. 2 Weight gain per day in experimental groups ····· 17
Fig. 3 Aerobic bacteria count using LB agar plate in experimental groups 19
Fig. 4 Aerobic bacteria count using petrifilm plate in experimental groups 19
Fig. 5 Lactic acid bacteria count using LBS in experimental groups ····· 21
Fig. 6 Lactic acid bacteria count using petrifilm plate in experimental groups ····· 21
Fig. 7 Coliform count using petrifilm plate in experimental groups ····· 22
Fig. 8 Effect of S. sieboldii Miq. supplementation on composition of fecal beneficial microbiota analyzed by 16S rDNA sequences ····· 23
Fig. 9 Effect of *S. sieboldii* Miq. supplementation on composition of fecal detrimental microbiota analyzed by 16S rDNA sequences ····· 25
Fig. 10 Effect of *S. sieboldii* Miq. supplementation on IL-6 expression in mesenteric lymph nodes analyzed by qPCR ····· 27
Fig. 11 Effect of *S. sieboldii* Miq. supplementation on IL-10 expression in mesenteric lymph nodes analyzed by qPCR ····· 28
Fig. 12 Effect of *S. sieboldii* Miq. supplementation on latency in passive avoidance performance test ····· 58
Fig. 13 Effect of *S. sieboldii* Miq. supplementation on latency in Morris water maze test ····· 60
Fig. 14 Effect of *S. sieboldii* Miq. supplementation on retention in Morris water maze test ····· 61
Fig. 15 Effect of *S. sieboldii* Miq. supplementation on latency in rotarod test ····· 63

초석잠 섭취가 마우스 장내 미생물총과 뇌 기능 개선에 미치는 영향

나 은

한국해양대학교 해양과학기술전문대학원
해양과학기술융합학과

요 약

본 연구는 초석잠 섭취가 장내 미생물총 다양성에 미치는 효과와 미생물총 조성 변형이 기억학습능력 관련 뇌 기능에 미치는 효과에 대해 연구하였다. 4주령 ICR 종의 수컷 마우스 26마리를 각각 대조군과 초석잠을 섭취한 실험군으로 나뉘어 식수와 함께 자유섭취하게 하여 8주 동안 사육하였다. 체중 변화와 식이 섭취 효율에서는 두 군 간에 유의적인 차이가 없었다. 고체 한천 배지와 3M Petrifilm을 이용하여 분변의 미생물 조성 분석을 실시한 결과 초석잠 섭취군의 분변에서 일반 호기성균과 대장균군의 수가 유의적으로 낮았다($p<0.05$). 16S rDNA sequencing을 이용한 분변의 미생물 조성 분석 결과, 유해균인 Proteobacteria 문에 속하는 *Enterobacteriaceae* 과의 *E. coli* 와 *Bacteroides* sp. 가 초석잠 섭취군의 분변에서 감소됨을 알 수 있었다. qPCR을 이용하여 사이토카인 발현을 관찰한 결과 초석잠 섭취군의 장간막 림프절에서 IL-6 와 IL-10 모두 대조군에 비해 유의적으로 감소한 것을 알 수 있었다($p<0.05$). 초석잠 섭취군의 혈청에서는 총 콜레스테롤, 중성지방, LDL-콜레스테롤이 유의적으로 대조군에 의해 감소하였고($p<0.05$), 간 및 심장조직에서도 유사한 경향을 나타내는 것을 확인하였다. 초석잠 섭취군의 대장과 분변 조직에서의 지방산 조성은 단쇄지방산(6:0)의 함량이 대조군에 비해 증가하였다. 또한 신경계의 대뇌, 소뇌 및 망막의 지방산 조성은 총 n-6 지방산 함량이 초석잠 섭취군에서 감소하였으며 총 n-3 지방산

함량이 증가하는 경향을 나타내었다.

마우스의 학습 및 기억력을 측정하기 위해 수동회피테스트를 실시한 결과, 실험 첫 번째 날에는 마우스들이 전기쇼크에 대한 경험이 없었기 때문에 대조군과 초석잠 섭취군 모두 latency가 30초 이내로 매우 짧았다. 그러나 두 번째 날에는 초석잠 섭취군의 latency가 대조군에 비해 유의적으로 높았다 ($p<0.05$). 운동조정능력을 측정하기 위하여 rotarod 시험을 실행한 결과 첫 번째 날에는 유의적 차이를 보이지 않았으나, 두 번째 날과 세 번째 날에는 초석잠 섭취군의 latency가 대조군에 비해 유의적으로 높은 것을 알 수 있었다($p<0.05$). 공간 기억력을 측정하기 위해 Morris water maze를 실시한 결과 초석잠 섭취군과 대조군 사이에는 유의적 차이가 없었다.

이상의 결과들로부터, 초석잠 섭취는 염증성 사이토카인(IL-6) 발현을 감소시키고 장내 유해 미생물군을 감소시켰고 혈중 콜레스테롤, 중성지방 및 LDL-콜레스테롤 수치를 낮출 수 있는 것으로 사료된다. 이러한 장내 미생물 총 조성 변화는 기억학습능력관련 뇌 기능과 운동조정능력을 개선시켰다. 따라서 꾸준한 초석잠 섭취는 장 건강을 개선시켜 뇌 기능을 향상시키는 것으로 여겨진다.

KEY WORDS: *Stachys sieboldii* Miq., Microbiome, Pro- and anti-inflammatory cytokines, Brain function, fatty acid composition

1. Introduction

A Lamiaceae family herb, *Stachys sieboldii* Miq. native to East Asia including Korea and China. It is an annual herb, and its stem is erect, its leaves are long oval, and the flowers are pink, blooming in late August. The roots grow about 3–6 cm deep in autumn, and of shape and effects are similar to *Cordyceps sinensis* so it is called *Cordyceps sinensis* of plant (Fig. 1). In Japan, it is used as a delicacy for new year's dishes, and according to book a on Chinese herbal medicines, it is known to enhance cerebral activity and memory, or strengthen the intestines. This plant is used as an ingredient in salads and fermented foods. Traditionally, *S. sieboldii* Miq. is also known for healing inflammation and preventing dementia (Ryu & Kim, 2004; Lee et al., 2014; Kim et al., 2017). Several reports have detailed the health benefits of S. sieboldii Miq., such as its anti-inflammatory activity, ability to lower anoxia, immunosupervisive function and antinephritic activity (Hayashi et al., 1994; Yamahara et al., 1990; Zinchenko et al., 1981). In animal brain tissues, *S. sieboldii* Miq. inhibits the activation of acetylcholine esterase so that improve dementia symptoms, monoamine oxidase and xanthine oxidase so that maintain normal functioning of the brain and repress brain tissue damage by reactive oxidase species. Also extractions from *S. sieboldii* Miq. roots and leaves restrained the formation of lipid peroxidase and showed activity to scavenging nitrites (Ryu & Kim, 2004; Baek et al. 2004; Baek et al. 2003).

Fig. 1 Roots of *Stachys sieboldii* Miq.

Several studies reported that there are several bioactive polysaccharides present in *S. sieboldii* Miq. (Hayashi et al., 1994; Yin et al., 2006; Feng et al., 2015). Hayashi et al. (1994) isolated acteoside as a component of *S. sieboldii* Miq. and suggested that acteoside can mitigate renal injury by inhibiting complement activation. Later Yin et al. (2006) isolated stachyose, a tetrasaccharide consisting of one glucose, one fructose, and two galactose molecules, and showed that it pronoted the proliferation of *Bifidobacterium* in the human intestine and improved the intestinal environment by eliminating harmful substances. Feng et al. (2015) purified the polysaccharide SSP-II from an acidic fraction with rhamnose, glucuronic acid, gallacturonic acid, glucose, galactose and arabinose. Previous literature indicates that polysaccharides from plants may have anti-tumor, antioxidative and anti-inflammatory properties (Chen et al., 2011; Zhao et al., 2013; Wang et al., 2015; Wang et al., 2014). In addition, most of these polysaccharides may possess the potential to act as powerful prebiotics that can maintain and renew intestinal health. Polysaccharides are resistant to digestion in the small intestine, but are metabolized by bacteria in the colon, where they are fermented into short-chain fatty acids (SCFAs), gases and other metabolites, that can affect the intestinal environment (Schott et al., 2013). The amount and types of polysaccharides that reach the colon influence the composition of the gut microflora. Healthy gastrointestinal environments contain diverse and abundant bacteria, which interact with the mucosal epithelium and are responsible for maintaining normal substance metabolism, immune response and intestinal angiogenesis (Vandeputter et al., 2016). Nie et al. (2019) confirmed that polysaccharides from the seeds of Plantago asiatica L. could increase the number of bacteria such as *Bacteroides vulgatus*, *Lactobacillus fermentum*, *Prevotella loescheii*, and *Bacteoides ovatus* and promote the production of SCFAs. Zhao et al. (2018) found that a diet rich in fiber could optimize the gut microflora, produce more SCFAs, and help control blood glucose levels more effectively in type 2 diabetes. It is well known that an altered gut microflora is associated with human metabolic diseases, such as obesity, type 2 diabetes, and cardiovascular disease. A study found that vegans had decreased levels of *Bacteroides* sp. and *Escherichia coli* and other *Enterobacteriaceae*. compared with a control group (Zimmer et al., 2012). They also suggested that butyrate from microbiota enhanced interleukin

IL-10 and IL-4 secretion, but inhibited IL-2 and interferon-γ (IFN-γ) release in anti-CD3 stimulated monocytes, presenting an anti-inflammatory profile for butyrate.

Many studies also suggest that a disturbed gut microflora composition may affect the function of the mucosal immune system, resulting in intestinal inflammation (Tung et al., 2011; Goldsmith & Sartor, 2014; Li et al., 2017). However, the effect of *S. sieboldii* Miq. supplementation on the fecal microflora and cytokine expression has not yet been investigated. Thus, in this study, the changes in diversity and composition of microbiota in feces were analyzed in *S. sieboldii* Miq. supplemented mice. In addition, we evaluated the production of major cytokines (IL-6 and -10) and there relation to inflammation and fatty acid composition in several tissues.

The gut-brain axis consists of bidirectional communication between the central and the enteric nervous system, linking cognitive centers of the brain with peripheral intestinal function. Recent reports have indicated that this bidirection activity between the intestinal microbiota and the gut-brain axis appears to be caused through signaling from the gut-microbiota to the brain and from the brain to the gut-microbiota by means of neural, endocrine, immune, and humoral links (Carabotti et al., 2015). This may also be a route through which the gut microbiota may impact neurodevelopmental processes and brain functions. For example, dysregulation of the gut-brain axis communication is associated with metabolic diseases (Daly et al., 2011; de Lartigue et al., 2011; Grasset et al., 2017) and psychiatric and co-occuring non-psychiatric disorders (Maes et al., 2007, 2008; Cryan and O'Mahony, 2011; Grenham et al., 2011; O'Mahony et al., 2011). As a result, these disorders are also frequently associated with alterations in the gut microbiota composition or function, which could also contribute to the disruption of the molecular dialogue existing within the gut and brain. These associations could be related to dietary-induced alterations in the intestinal microbiota which in turn, may contribute to (neuro) inflammation and dysregulation of the neuroendocrine system associated with obesity comorbid with mental impairments.

In this present study, we investigated the effect of *S. sieboldii* Miq. supplementation on cognitive ability using a passive avoidance test and the Morris water maze test and to elucidate the relationship between gut microbiota and brain function.

2. Methods and Materials

2.1. Animals and diets

S. sieboldii Miq. roots were obtained from Misan Inc. (Daegu, Korea). This experimental protocol was approved by Gyeongsang National University (approval number: GNU-171116-M0051). Male Crj: CD-1 mice 4 weeks of age were obtained from Samtako Inc. (Osan, Korea). Twenty six mice were randomly divided into two groups of thirteen: The first group was the control group that was fed on 5% palm oil as a fat source (Control) (Table 1). The second group was fed on diet with 20% dried roots of *S. sieboldii* Miq. *S. sieboldii* Miq. contained 3.6% protein, 0.3% fat and 1.5 % carbohydrate. The diet composition was adjusted according to the amounts of nutrients contained in *S. sieboldii* Miq. The composition of diets followed the AIN-93M (Reeves et al., 1993). Customized diets were stored at -4℃, and fresh supplies were given to the mice once every two days. Body weights were measured once a week. Food efficiency ratio, FER was calculated by dividing the weight gain during the experiment by the dietary intake (formula below). Mice were maintained at our thermo-hygrostat facility under conventional conditions of controlled temperature (23 ± 1℃), relative humidity (65 ± 5%) and illumination (12-hours light:dark cycle). Mice were allowed to free access to food and water and maintained on these diets for 8 weeks. At the end experiments, the mice were sacrificed. Organs were removed and stored at -70° C.

$$\text{FER (\%)} = \frac{\text{Weight gain during the entire breeding period (g)}}{\text{Dietary Intake for Total Breeding Period (g)}} \times 100$$

Table 1 Diet compositions of the experimental groups

Ingredients	Diet Group (g/kg)	
	Control	*S. sieboldii* Miq.
Corn starch	488	473
Casein	200	164
Sucrose	150	150
Cellulose	50	50
Mineral	40	40
Vitamin	20	20
Methionine	2	2
Palm oil	50	47
S. sieboldii Miq. Root		200

Table 2 Fatty acids composition of each diet

	Control	*S. Sieboldii* Miq .
12:0	0.67	0.46
14:0	1.81	1.63
16:0	28.56	25.87
20:0	2.52	1.92
Total Sat.	33.56	29.89
14:1n-9	0.13	0.00
16:1n-9	0.13	2.20
20:1n-9	31.89	34.79
Total Mono.	32.15	36.99
18:2n-6	31.37	30.19
22:2n-6	2.92	2.93
Total n-6	34.28	33.12
Total fatty acids (ug/mg wet tissue)	2.72	3.30

2.2 Measurements of fecal microorganism

2.2.1 Agar plate assay

Luria-Bertani Broth (LB, Difco Laboratories, MD, USA) and Lactobacillus selective agar base (LBS, NEOGEN, MI, USA) plate were made by the pour plate according to direction. Fecal samples (0.05 g) were mixed with 100ul of PBS and vortexed to homogeneity. Using a 10-fold dilution with PBS, the sample was diluted serially to 10^{-7}. After solidification of 12 to 15 ml of the LB, LBS plate count agar, 500 ul of each sample dilution in the range of 10^{-4} to 10^{-7} was over laid with plates and incubated for 24 to 48 h at 37℃, and the total number of colonies were counted (AOAC).

2.2.2 3M Petrifilm plate assay

using the dilution method described above, Petrifilm aerobic, lactic acid bacteria and coliform count plates were inoculated with the diluted samples according to the manufacturer's instruction. For total microbial counts, all colonies staining in various shades of red were counted. For ascertaining the number of coliform and lactic acid bacteria colonies, only red colonies with one or more gas-associated bubbles (within 1 colony diameter) were counted (Park et al., 2001).

2.2.3 DNA extraction and analysis of 16S rDNA gene sequences

To investigate the change of Intestinal microbial composition, We extracted DNA from mice feces that collected right before the sacrifice using DNeasy Blood & Tissue Kit (Qiagen, CA, uSA). DNA samples were prepared according to sequencing company's guide line. NGS Microbiome Taxonomic Profiling analysis was performed by ChunLab Inc. (Seoul, Korea) using EzBioCloud data base (Yoon et al., 2017).

2.3 RNA preparation and quantitative polymerase chain reaction (q-PCR)

The total RNA from homogenized mesenteric lymph nodes tissues was isolated with the use of a Minibest universal RNA Extraction Kit (Takara, Kusatsu, Japan) and performed according to the manufacturer's protocol of the manufacturer. The total RNA set concentration to 476 ng and then synthesized with prime script 1st strand cDNA synthesis Kit (Takara, Kusatsu, Japan). qPCR was performed on cDNA samples using the SYBR™ Green PCR Master Mix (applied systems, IL, uSA) and performed according to the manufacturer's protocol of the manufacturer. Primers used were at Table 3. Cycling conditions were holding stage: 4 min at 50℃ and 15 min at 95℃, cycling stage (x50 cycles): 10 sec at 95℃, 20 sec at 50℃ and 30 sec at 72℃, melt curve stage: 20 sec at 95℃, 40 sec at 60℃, 15 sec at 95℃. Analysis used the sequence detection software supplied with the instrument (StepOne Real-Time PCR System, applied biosystems, IL, uSA). The relative quantitation value is expressed as 2^- DcT, where DcT is the difference between the mean CT value of duplicates of the sample and of the β-actin control (Kim et al., 2010).

Table 3 Primer sequences

Name	Sequence (5'-3')	Size
β-actin_F[1)]	AAGATCTGGCACCACACCTT	20 mer
β-actin_R[2)]	CCTGTGGTACGACCAGAG	18 mer
IL6_F	TCTGGGAAATCGTGGAAATG	20 mer
IL6_R	GGTACTCCAGAAGACCAGA	19 mer
IL10_F	ATAACTGCACCCACTTCCCA	20 mer
IL10_R	GGGCATCACTTCTACCAG	18 mer

[1)] Forward primer, [2)] Reverse primer

2.4 Measurements of tissue triglyceride, total cholesterol, HDL and LDL

1g of serum, liver, heart were homogenized with 3ml of homogenizing buffer (154 mM KCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4). Then centrifuged in 4° C, 13,000 rpm, 20 minutes (Combi-514R, Incheon, Korea). After centrifugation take upper layer for measurement. Total cholesterol, TG, and HDL-Cholesterol levels in serum, liver, heart were measured by using commercial enzymatic kits (Asan Pharm Co., Hwasung, Korea). At the end of the experiment, the absorbance of samples were compared with the standard solution's standard curve for the concentration of cholesterol to determine the cholesterol content of samples. LDL-Cholesterol was calculated as following Friedewald formula (Friedewald et al., 1972) using any kit reagent.

LDL-Cholesterol (mg/dl) = Total Cholesterol − (HDL-Cholesterol + triglyceride/5)

2.5 Analysis of fatty acid composition using gas chromatography

The Fatty acids extracted from the organ tissues were prepared in accordance with the method developed by Folch et al. (1957). The homogenized organ tissues were then transmethylated with 14% BF3-methanol at 100℃ for 60 min using a modified version of the method employed by Morrison and Smith (1964) that involved the addition of hexane. Gas liquid chromatography using a 100 m x 0.25 mm i.d. 0.2 um capillary column (SP-2560, Supelco, Bellefonte, uSA) was employed to separate the fatty acid methyl esters, and the latter were detected through flame ionization (Salem et al., 1996). The detector and injector temperatures were set to 250℃. The oven temperature program began at 130℃ and increased to 17 5℃ at 4/min, then increased at 1/min to 210, and finally increased at 30℃/min to 245 with a final hold for 15 min. The chromatograms were recorded and the percentage composition of individual peaks was calculated with a VARIAN CP-3380 (Varian Inc., CA, uSA). The fatty acid methyl esters were identified through a comparison with the retention times obtained using a standard mixture (462 Standard, Nu-Chek-Prep, MN, uSA). The percentages of individual and total fatty acids were obtained using an internal standard (22:3n-3 as methyl ester).

2.6 Animal behavioral assay

2.6.1 Passive avoidance test

A step-through passive avoidance apparatus (Jarvik & Kopp, 1967) (Gemini Avoidance System, San Diego Instruments Inc., San Diego, USA) was used to appraise learning and memory abilities. The box consisted of two compartments of equal size (17 x 12 x 11 cm) is separated by a sliding type door (6 x 6 cm). 20 W bulbs were used to illuminate one of the two compartments from above. The other compartment was not illuminated and had an electrifiable floor. A sliding type door was located in the center of the box to allow the mice to move freely between two compartments. The door was then closed and the mice were placed in their respective compartments. After 60 seconds of adaptation period, the compartment lit up and the sliding door opened. Once a mouse entered the dark compartment, the door closed and an electric shock of 0.4 mA was delivered to their foot for 1 second. Each mouse took one trial, and was assigned a cut-off time of 300 seconds. The initial latency time required to enter the dark compartment was recorded. After 24 hours, the latency time was measured in the same condition as during the acquisition trial, and was tested on serial days.

2.6.2 Morris water maze test

To evaluate performance in a spatial task, the Morris water maze test was performed. This method has been previously described in detail by Moriguchi et al. (2000) and Moriguchi & Salem (2003). Briefly, the swimming area was arbitrarily divided into four quadrant (regions A–D), and two starting points were arranged at the corners of a quadrant rim. After swimming training, the mice were submitted to a visible trial where a black (visible) escape platform was placed in quadrant region A. If a mouse failed to find the platform within 90 seconds, it was gently placed on the platform for 30 seconds. On the second day, a hidden platform was used in place of the black platform and each mouse received two trials per day after being randomly placed at the two different starting points. The maximal trial length was 90 seconds, with a maximal intertrial interval of 9 minute. The escape latency was then defined as the sum of the two trials on a given day. The escape latency, swimming time, swimming speed, the duration of the floating state (resting time), and swimming path were automatically digitized and recorded by computer. Sessions were repeated for four serial days for a total of eight trials. On the day following the last session, the platform was removed and the mouse was allowed to search for the platform for 90 seconds (probe trial). The number of crossings of the position where the platform had been placed (quadrant region A) and the number of crossings in the corresponding imaginary positions in the other quadrant regions (regions B–D) were recorded.

2.6.3 Rotarod performance test

When mice were 6 weeks old, Rota Rod Test was performed to assess potential effects of *S. sieboldii* Miq. on motor function (Singewald et al., 2004). Mice were tested on a device (Rotarod B1001, B.S Technolab Inc., Seoul, Korea). We set speeds of device at 2 to 20 rpm on the first day, 2 to 30 rpm on the second day and 2 to 40 rpm on third, fourth days, For two trials/day at maximum 4 min/trial. The latency times on the bar right before the first fall was recorded.

2.7 Statistics

All results were expressed as means ± the standard error of the mean (SEM), with statistical significance determined by t-test using SPSS+/WIN12.0 (Statistical Package for Social Science, version 1 2.0)

3. Result and discussion

3.1 Effect of *S. sieboldii Miq.* supplementation on the body weight food intake/ efficiency ratio

Fig. 2 shows weight gain as measured once every two days. The body weights of control and *S. sieboldii* Miq. supplemented mice were 43.9±1.31 and 40.6±1.35, respectively. Table 3 shows that Weight gain (g/day), Food intake (g/day) and Food efficiency ratio (FER) of each group fed control feed or *S. sieboldii* Miq. root mixed feed. There was no significant difference between the control group and the experimental groups.

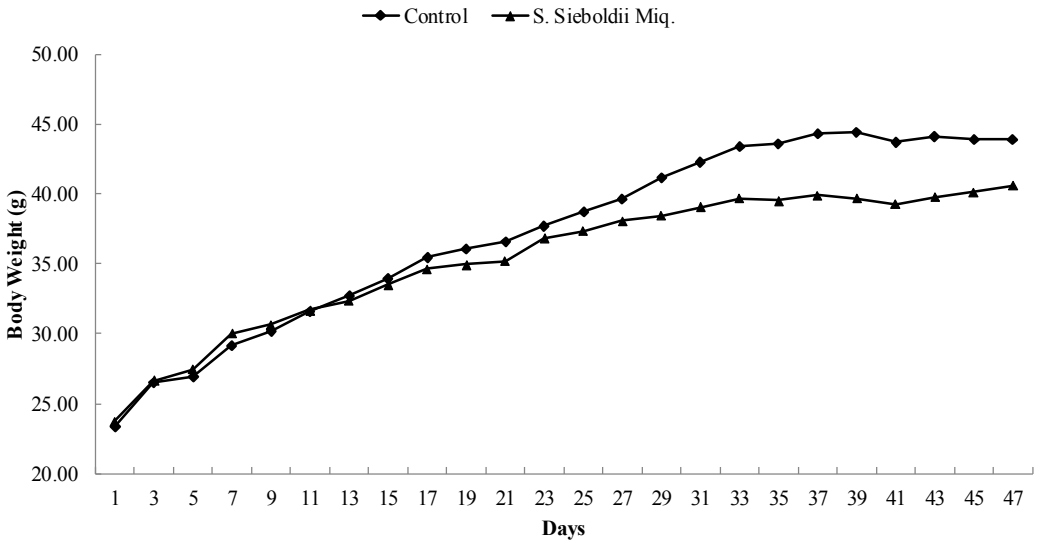

Fig. 2 Weight gain per day for the experimental groups

Table 3 Body weight gain, food intake FER for the experimental groups

	Weight gain (g/day)[1)]	Food intake (g/day)[1)]	FER[2)]
Control	0.37±0.07	7.0±0.5	0.10±0.00
S. sieboldii Miq.	0.30±0.07	6.9±0.00	0.08±0.01

[1)] Data were expressed as mean ± the standard error of the mean (SEM), n=13.

[2)] Food efficiency rate (FER) was calculated as [total body weight gain (g)/total food intake (g)]*100

3.2 Effect of *S. sieboldii* Miq. supplementation on fecal microorganism composition

LB agar plate and petrifilm aerobic plate were used to measure the total amount of aerobic bacteria in feces and the results are shown below (Fig. 3-4). There was significant difference in the amount of aerobic bacteria between the control group and the experimental group.

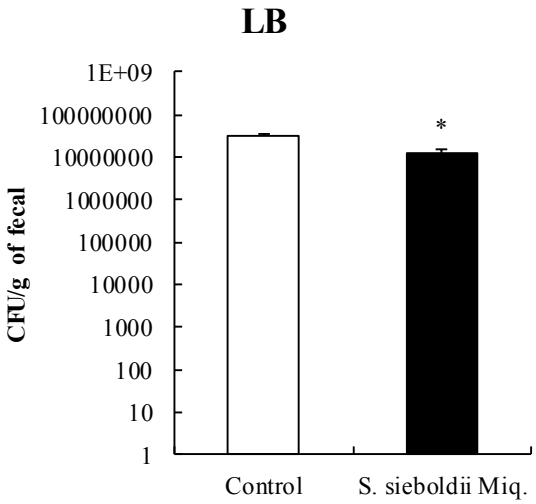

Fig. 3 Aerobic bacteria count using LB agar plate in experimental groups. Values with different letters are significantly different at $p<0.05$.

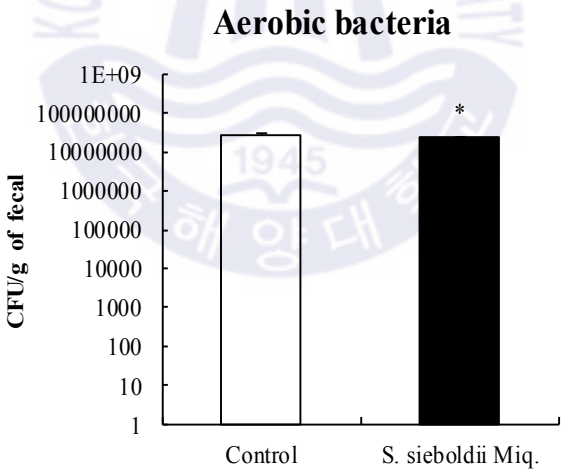

Fig. 4 Aerobic bacteria count using petrifilm plate in experimental groups. Values with different letters are significantly different at $p<0.05$.

3.2.1 Changes in beneficial microorganisms from feces

Lactic acid bacteria, which are commonly known as a beneficial microorganism, was measured by agar plate and petrifilm plates (Fig. 5-6). The content of lactic acid bacteria was 8.32%, 11.46% lower in *S. sieboldii* Miq. intake mice' s feces respectively and the results are significant, suggesting that *S. sieboldii* Miq. might not contain enough polysaccharides to enhance proliferation of lactic acid bacteria.

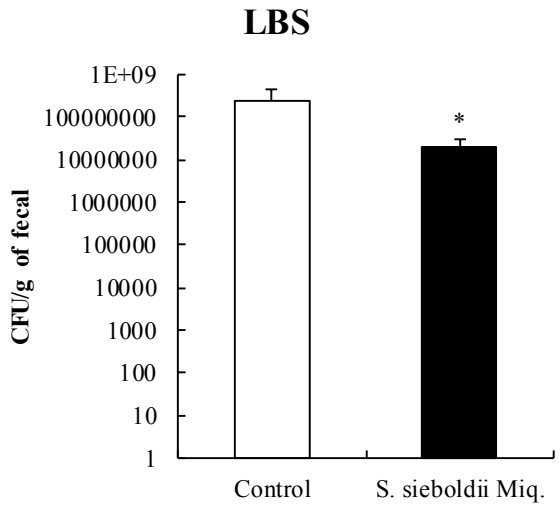

Fig. 5 Lactic acid bacteria count using LBS in the experimental groups. Values with different letters are significantly different at $p<0.05$.

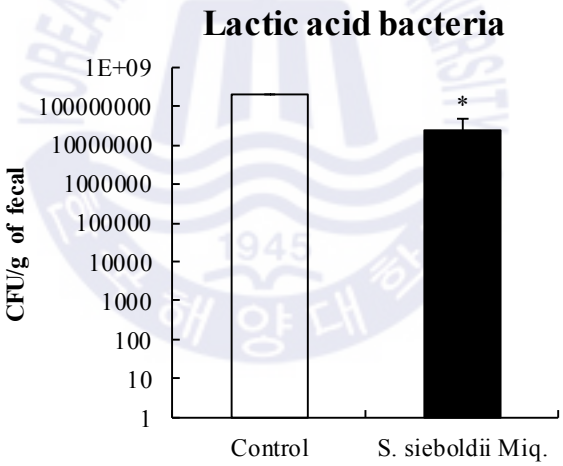

Fig. 6 Lactic acid bacteria count using petrifilm plate in the experimental groups. Values with different letters are significantly different at $p<0.05$.

In 3M Petrifilm coliform assay, the supplementation with *S. sieboldii* Miq. significantly reduced the counts Coliform batecria in the feces compared to the control group (Fig. 7).

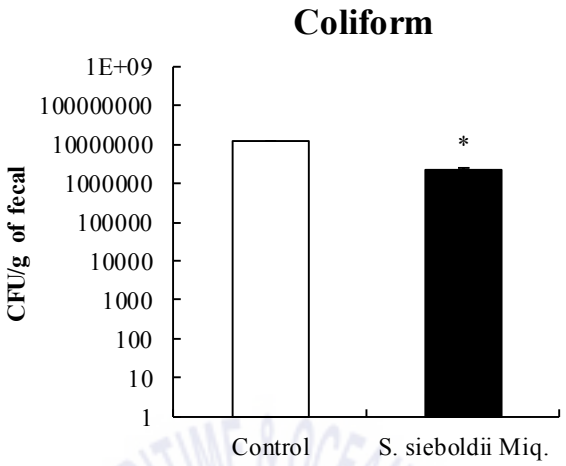

Fig. 7 Coliform count using petrifilm plate in the experimental groups. Values with different letters are significantly different at $p<0.05$.

16S rDNA sequencing data showed that the supplementation with *S. sieboldii* Miq. increased beneficial gut microflora including *Ruminococcaceae* and *Akkermansia mucinphila* in feces compare to the control (Fig. 8).

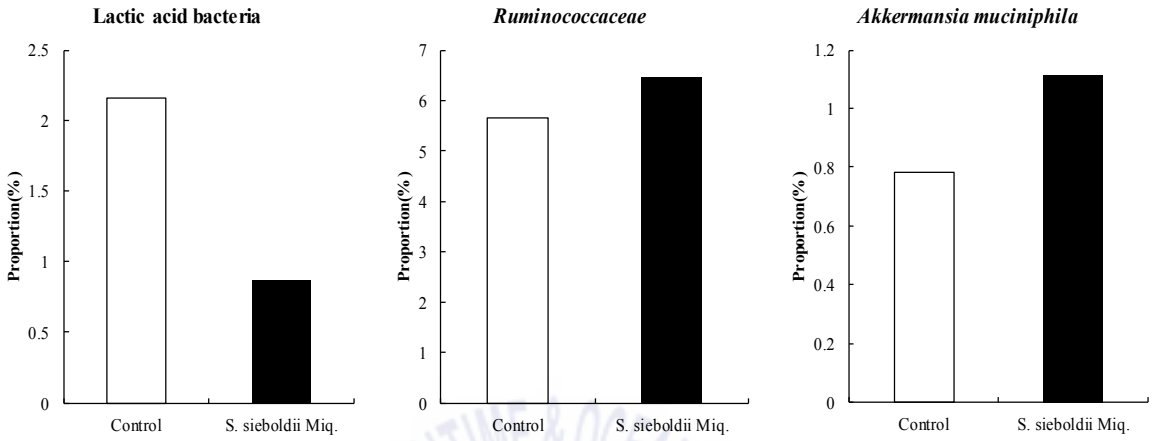

Fig. 8 Effect of *S. sieboldii* Miq. supplementation on composition of fecal beneficial microbiota analyzed by 16S rDNA sequences. Values with different letters are significantly different at $p<0.05$.

3.2.2 Changes in fecal detrimental microorganisms

16S rDNA sequencing data also showed, that supplementation with *S. sieboldii* Miq. greatly decreased the community of harmful microflora (*Enterobacteriaceae* including *E. coli*, and *Bacteroides* sp.) in feces compared to the control (Fig. 9).

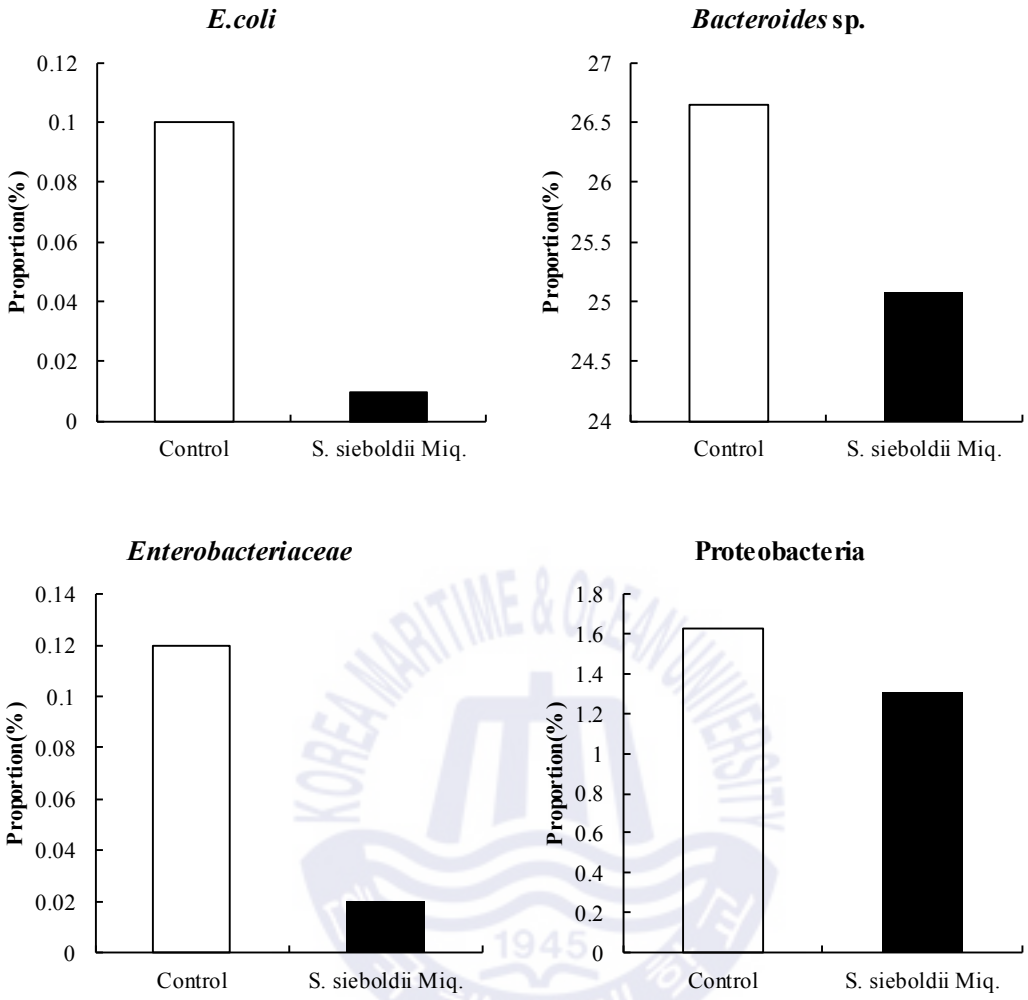

Fig. 9 Effect of *S. sieboldii* Miq. supplementation on the composition of fecal detrimental microbiota analyzed by 16S rDNA sequences. Values with different letters are significantly different at $p<0.05$.

Healthy gastrointestinal microflora are characterized by high numbers and diversity of bacteria (Vandeputter et al., 2016), which interact with the mucosal epithelium and are responsible for normal substance metabolism, immune response, and intestinal angiogenesis (Candela et al., 2014). In the current study, we found that supplementation with *S. sieboldii* Miq. deceased the harmful microbiota and increased beneficial microbiota in feces. Hong et al. showed that *Stachys* greatly decreased the community richness of microbiota in feces compared with that in the control (Li et al., 2017). Based on 16S rDNA gene sequencing of gut microbiota, the four major phyla in the feces were identified to be Bacteroidetes, Verrucomicrobia, Firmicutes and Proteobacteria, which are consistent with our current results. Lactic acid bacteria are known probiotics with many health benefits, including improvement of normal microflora, inhibition of infectious diseases, reduction of serum cholesterol, and alleviation of intestinal bowel disease symptoms (Maldonado et al., 2007). Several studies have indicated that stachyose, widely distributed in *Stachys* and legumes, can influence gut microbiota such as *Bifidobacterium* and *Lactobacillus* (Li et al., 2017). However, in the present study, supplementation with *S. sieboldii* Miq. did not increase the population of lactic acid bacteria.

Previous more specific studies about the microflora that we shown above, have noted that patients with cirrhosis have a decrease in nonpathogenic bacteria including *Lachnospiraceae*, *Ruminococcaceae*, and Clostridiales XIV and an increase in pathogenic bacteria including *Enterococcus*, *Enterobacteriaceae*, and *Bacteroidaceae* (Chen et al., 2011; Bajaj et al., 2012). And also, Dao et al., (2016) reported that *A. muciniphila* is associated with a healthier metabolic status and better clinical outcomes after calorie restriction in overweight/obese adults.

3.3 Effect of *S. sieboldii* Miq. supplementation on cytokine expression in mesenteric lymph nodes

3.3.1 Changes in Interleukin-6 (IL-6) mRNA expression

Mice supplemented with *S. sieboldii* Miq. had an IL-6 expression level that was significant lower than control group ($p<0.05$) (Fig. 10).

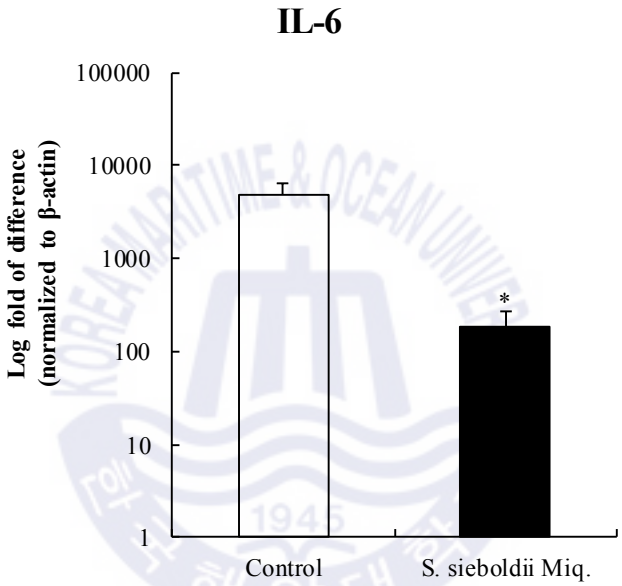

Fig. 10 Effect of *S. sieboldii* Miq. supplementation on IL-6 expression in mesenteric lymph nodes analyzed by qPCR. Values with different letters are significantly different at $p<0.05$.

3.3.2 Changes in Interleukin-10 (IL-10) mRNA expression

Mice supplemented with *S. sieboldii* Miq. had an IL-10 expression level that was significant lower than control group ($p<0.05$). (Fig. 11)

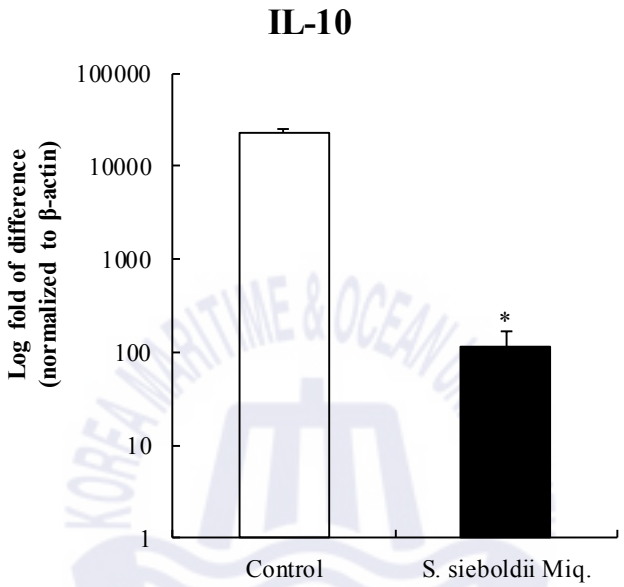

Fig. 11 Effect of *S. sieboldii* Miq. supplementation on IL-10 expression in mesenteric lymph nodes analyzed by qPCR. Values with different letters are significantly different at $p<0.05$.

Cytokines can act not only locally to amplify the cellular immune response, but also systemically to change behavior, metabolism, and neuroendocrine secretions (Johnson, 1997). T cells and macrophages secrete IL-6 to stimulate the immune response, particularly in tissue damages leading to numerous types of inflammatory processes (McCurry et al., 1993). IL-10 inhibits the production of pro-inflammatory and Th1 cytokines such as tumor necrosis factor (TNF)-α and IFN-γ (Mosmann, 1994). Liu et al. found that intake of stachyose decreased the mRNA expression of IL-6, and TNF-α, Akt and PI3K in pancreas type 2 diabetes animal model (Liu et al., 2018). In addition, they reported a negative correlation between the communities of Christensenellaceae and Bacteriodales and levels of IL-6 and TNF-α. Qian et al. demonstrated that resistant starch as a carrier for stachyose reduced the expression of serum IL-6 and TNF-α in dextran sulfate sodium-induced colitis in mice (Qian et al., 2013). Bogert et al. suggested that some pathogenic bacteria including Streptococcus and Veillonella increased levels of cytokines IL-8, IL-6, IL-10, and TNF-α (Bogert et al., 2014). In the present study, we also observed lower mRNA expression of IL-6 in mice supplemented with *S. sieboldii* Miq., suggesting that lower levels of IL-6 are indicative of improved anti-inflammatory effects. We also observed that higher levels of pathogenic bacteria (*Enterobacteriaceae* including *E. coli*, *Bacteroides* sp.) might be associate with increased IL-6 mRNA expression in our system. However, this study demonstrated a reduced mRNA expression of IL-10 in mice. A limitation of this study is that it could not explain the causal factor; therefore, the possibility that the resident microbes influenced the IL-10 levels remains.

3.4 Effect of *S. sieboldii* Miq. supplementation on tissue lipid profiles

3.4.1 Changes levels of triglyceride, total cholesterol, HDL and LDL in the serum

After feeding normal feed and normal feed mixed with *S. sieboldii* Miq. root for 8 weeks, Total cholesterol, Triglyceride (TG), HDL-cholesterol (HDL), LDL-cholesterol (LDL) for each mice' s liver, heart, serum were measured. In mice supplemented with *S. sieboldii* Miq. Serum Total cholesterol (Table 5), TG, LDL were 16.17%, 26.08%, 22.67% respectively significantly lower than the control group ($p<0.05$). Kang et al. (2018) reported that long term intake of sunsik added *S. sieboldii* Miq. decreased in serum total cholesterol and LDL content. And these result correspond to above results.

Table 5 Lipid profiles in serum of the experimental groups

		Triglyceride (mg/dL)	cholesterol (mg/dL)		
			Total	LDL	HDL
Serum	Control	196.24 ± 0.23[1)]	341.23 ± 1.52	184.01 ± 3.98	117.97 ± 4.36
	S. sieboldii Miq.	145.07 ± 0.70*	289.47 ± 0.88*	142.30 ± 10.80*	118.16 ± 11.28

[1)] Each variable represents the mean ± SEM, n=5

* $p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.4.2 Changes levels of triglyceride, total cholesterol, HDL and LDL in the heart

In the case of mice supplemented with *S. sieboldii* Miq., the concentration of Total cholesterol, Triglyceride TG, LDL in the heart were slightly lower and HDL was slightly higher than control group, but there was no significant difference (Table 6).

Table 6 Lipid profiles in heart homogenates of the experimental groups

		Triglyceride (mg/dL)	cholesterol (mg/dL)		
			Total	LDL	HDL
Heart	Control	169.72 ± 36.74	95.61 ± 9.16	21.38 ± 3.01	40.29 ± 4.39
	S. sieboldii Miq.	142.25 ± 14.46	94.74 ± 8.15	20.88 ± 6.63	45.41 ± 1.08

[1] Each variable represents the mean ± SEM, n=5

* $p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.4.3 Changes levels of triglyceride, total cholesterol, HDL and LDL in liver

In the case of mice supplemented with *S. sieboldii* Miq., the concentration of Total cholesterol, Triglyceride TG, LDL in the liver were slightly lower and HDL was slightly higher than control group, but there was no significant difference (Table 7).

Table 7 Lipid profiles in liver homogenates of the experimental groups

		Triglyceride (mg/dL)	cholesterol (mg/dL)		
			Total	LDL	HDL
Liver	Control	311.74 ± 36.09	99.71 ± 2.79	9.27 ± 3.34	27.05 ± 0.70
	S. sieboldii Miq.	296.48 ± 26.62	95.61 ± 3.08	8.37 ± 4.88	28.99 ± 0.44

[1] Each variable represents the mean ± SEM, n=5

* $p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition in several tissues and feces

3.5.1 Change in fatty acid composition in the serum

In the serum, the percentages of 16:0, 21:0 and 20:5n-3 (EPA) were found to be significantly higher in the *S. sieboldii* Miq. group than in the control group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids (Table 8).

Calder (2004) reported that long-chain n-3 PuFAs including EPA have been shown to decrease blood triacylglycerol (triglyceride) concentrations, to decrease production of chemoattractants, growth factors, adhesion molecules, inflammatory eicosanoids and inflammatory cytokines, to lower blood pressure, to increase nitric oxide production, endothelial relaxation and vascular compliance, to decrease thrombosis and cardiac arrhythmias and to increase heart rate variability. And these results may related to the serum lipid profiles that are shown in this study.

Table 8 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of serum

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
4:0	0.04 ± 0.04[1)]	0.11 ± 0.06
6:0	0.30 ± 0.07	0.30 ± 0.10
14:0	0.07 ± 0.07	0.00 ± 0.00
15:0	0.12 ± 0.06	0.04 ± 0.04
16:0	2.31 ± 0.26	4.78 ± 0.48*
17:0	0.24 ± 0.06	0.29 ± 0.06
18:0	0.46 ± 0.12	0.71 ± 0.35
20:0	1.28 ± 0.61	0.86 ± 0.67
21:0	0.00 ± 0.00	3.98 ± 0.25*
22:0	4.06 ± 1.20	2.71 ± 1.10
23:0	5.33 ± 2.00	3.76 ± 2.22
24:0	9.37 ± 2.15	7.44 ± 3.72
Total Sat.[2)]	23.58 ± 1.17	24.99 ± 3.04
Total Mono.[3)]	14.14 ± 2.76	20.27 ± 4.11
18:2n-6	3.53 ± 0.72	4.93 ± 3.44
18:3n-6	2.90 ± 1.25	2.35 ± 1.07
20:2n-6	2.47 ± 0.38	2.74 ± 0.77
20:3n-6	1.98 ± 0.78	3.22 ± 1.30
20:4n-6	4.73 ± 2.20	9.48 ± 4.07
22:2n-6	15.90 ± 5.33	6.33 ± 6.33
Total n-6[4)]	31.50 ± 5.46	29.06 ± 6.37
18:3n-3	5.36 ± 2.62	7.78 ± 3.28
20:3n-3	1.09 ± 0.49	2.24 ± 0.68
20:5n-3	0.83 ± 0.20	1.28 ± 0.31*
22:6n-3	3.68 ± 0.26	3.65 ± 0.52
Total n-3[5)]	11.81 ± 2.84	14.97 ± 4.70

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.2 Change in fatty acid composition in the RBC

In the red blood cells (RBC), the percentages of 16:0, total saturated, 22:2n-6 and 20:5n-3 (EPA) fatty acids in the *S. sieboldii* Miq. group were found to be significantly higher than the control group. However, the percentages of 6:0, 14:0, total n-6 including 20:4n-6 were found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of monounsaturated, n-3 polyunsaturated fatty acids found in the liver (Table 9).

Table 9 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of RBC

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
6:0	0.36 ± 0.04[1)]	0.00 ± 0.00*
14:0	0.22 ± 0.06	0.00 ± 0.00*
15:0	0.18 ± 0.09	0.04 ± 0.04
16:0	2.83 ± 0.03	7.54 ± 0.65*
17:0	0.24 ± 0.15	0.07 ± 0.04
18:0	0.98 ± 0.13	0.70 ± 0.07
20:0	3.54 ± 0.77	3.42 ± 0.66
21:0	6.05 ± 0.56	8.24 ± 0.66
22:0	6.21 ± 0.30	6.39 ± 2.17
23:0	3.96 ± 0.47	4.11 ± 1.12
24:0	11.19 ± 2.29	6.46 ± 1.47
Total Sat.[2)]	35.78 ± 1.03	36.97 ± 0.64
Total Mono.[3)]	12.81 ± 1.08	19.06 ± 0.30*
18:2n-6	2.83 ± 0.25	3.45 ± 0.52
18:3n-6	1.81 ± 0.27	2.07 ± 0.20
20:2n-6	3.62 ± 0.43	3.88 ± 0.35
20:3n-6	4.52 ± 1.67	4.38 ± 0.10
20:4n-6	17.73 ± 0.71	5.33 ± 0.89*
22:2n-6	0.00 ± 0.00	3.60 ± 1.87*
Total n-6[4)]	30.51 ± 1.11	22.70 ± 0.84*
18:3n-3	2.82 ± 0.30	3.02 ± 0.52
20:3n-3	5.39 ± 1.59	6.57 ± 1.53
20:5n-3	1.58 ± 0.11	3.10 ± 0.53*
22:6n-3	9.80 ± 0.10	8.58 ± 0.75
Total n-3[5)]	19.59 ± 1.75	21.27 ± 1.08

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.3 Change in fatty acid composition in the liver

In the liver, the percentages of 13:0 fatty acids in the *S. sieboldii* Miq. group was found to be significantly lower than the control group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the liver (Table 10).

Table 10 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of liver

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
4:0	0.01 ± 0.01[1)]	0.04 ± 0.03
6:0	0.08 ± 0.02	0.12 ± 0.07
8:0	0.03 ± 0.01	0.06 ± 0.01
10:0	0.00 ± 0.00	0.01 ± 0.01
11:0	0.02 ± 0.01	0.02 ± 0.01
12:0	0.02 ± 0.01	0.02 ± 0.01
13:0	0.09 ± 0.01	0.02 ± 0.01*
14:0	0.62 ± 0.06	0.61 ± 0.05
15:0	0.52 ± 0.27	0.86 ± 0.28
16:0	0.09 ± 0.03	0.04 ± 0.03
17:0	5.86 ± 0.48	4.12 ± 1.15
18:0	0.08 ± 0.02	0.15 ± 0.05
20:0	0.25 ± 0.07	0.15 ± 0.09
21:0	1.92 ± 0.48	1.59 ± 0.11
22:0	5.21 ± 0.25	5.05 ± 0.64
23:0	0.42 ± 0.19	0.67 ± 0.32
24:0	4.11 ± 0.83	3.84 ± 0.47
Total Sat.[2)]	19.23 ± 0.25	17.16 ± 2.12
Total Mono.[3)]	28.86 ± 0.55	31.40 ± 1.70
18:2n-6	39.11 ± 0.72	32.75 ± 3.06
18:3n-6	0.37 ± 0.06	0.32 ± 0.12
20:2n-6	0.50 ± 0.38	0.81 ± 0.68
20:3n-6	0.46 ± 0.25	0.61 ± 0.12
20:4n-6	2.25 ± 0.23	1.85 ± 0.63
Total n-6[4)]	42.68 ± 0.84	36.34 ± 3.02
18:3n-3	0.31 ± 0.06	1.69 ± 0.72
20:3n-3	0.91 ± 0.21	0.65 ± 0.21
20:5n-3	2.06 ± 0.22	2.01 ± 0.29
22:6n-3	5.61 ± 1.33	5.43 ± 1.24
Total n-3[5)]	8.90 ± 1.13	9.78 ± 1.63

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.4 Change in fatty acid composition in the intestine

In the intestine, the percentages of 6:0 and 20:3n-6 fatty acids in the *S. sieboldii* Miq. group were found to be significantly higher than the control group. while the percentages of 16:0 and 18:0 fatty acids in the *S. sieboldii* Miq. group were lower compared with that in the control group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the intestine (Table 11).

Under the activation of prebiotics including oligosaccharides, bacteria produce a large amount of SCFAs, which reduce intestinal pH value, prevent the growth of harmful bacteria and promote intestinal peristalsis to accelerate the excretion of pathogenic bacteria and toxins (Bogert et al., 2014). As SCFAs, 6:0 fatty acid was significantly increased in the intestine of mice with *S. sieboldii* Miq.

Table 11 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of intestine

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
4:0	0.01 ± 0.0[1)]	0.02 ± 0.02
6:0	0.05 ± 0.03	0.44 ± 0.08*
8:0	0.01 ± 0.01	0.01 ± 0.01
10:0	0.00 ± 0.00	0.01 ± 0.01
11:0	0.01 ± 0.01	0.04 ± 0.04
12:0	0.05 ± 0.01	0.24 ± 0.02
13:0	0.01 ± 0.01	0.05 ± 0.03
14:0	1.28 ± 0.09	1.80 ± 0.13
15:0	0.13 ± 0.01	0.41 ± 0.26
16:0	23.88 ± 3.44	19.49 ± 0.00*
17:0	0.17 ± 0.07	0.35 ± 2.51
18:0	7.29 ± 1.24	4.89 ± 0.02*
20:0	0.30 ± 0.19	0.12 ± 0.19
21:0	1.16 ± 0.20	1.42 ± 1.85
22:0	2.64 ± 0.92	3.65 ± 0.64
23:0	3.33 ± 1.25	0.33 ± 1.86
24:0	0.84 ± 0.26	0.95 ± 1.69
Total Sat.[2)]	41.17 ± 6.15	39.14 ± 3.42
Total Mono.[3)]	43.38 ± 7.69	41.37 ± 7.05
18:2n-6	5.92 ± 1.26	7.85 ± 0.62
18:3n-6	0.29 ± 0.27	0.29 ± 0.15
20:2n-6	1.04 ± 0.91	0.69 ± 0.59
20:3n-6	0.54 ± 0.13	2.57 ± 0.11*
20:4n-6	1.37 ± 1.37	1.30 ± 0.77
22:2n-6	0.21 ± 0.21	1.00 ± 0.73
Total n-6[4)]	9.38 ± 1.89	13.70 ± 2.00
18:3n-3	1.59 ± 0.82	1.13 ± 0.72
20:3n-3	0.26 ± 0.26	0.41 ± 0.35
20:5n-3	1.57 ± 0.71	1.13 ± 0.47
22:5n-3	0.00 ± 0.00	0.42 ± 0.42
22:6n-3	2.65 ± 0.40	2.86 ± 1.32
Total n-3[5)]	6.07 ± 1.16	5.96 ± 1.60

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.

[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids

*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.5 Change in fatty acid composition in the mesenteric lymph nodes

In the mesenteric lymph nodes, a higher percentages of 20:2n-6 and 20:3n-3 fatty acids in the *S. sieboldii* Miq. group were found compared to those of the control group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the mesenteric lymph nodes (Table 12).

Table 12 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of mesenteric lymph nodes

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
12:0	0.07 ± 0.00[1)]	0.07 ± 0.01
14:0	1.35 ± 0.01	1.26 ± 0.04
15:0	0.13 ± 0.02	0.16 ± 0.02
16:0	0.01 ± 0.01	0.03 ± 0.02
17:0	11.86 ± 1.87	10.84 ± 1.09
18:0	0.07 ± 0.02	0.07 ± 0.01
21:0	6.80 ± 3.15	4.21 ± 1.41
22:0	0.23 ± 0.04	0.75 ± 0.32
23:0	0.60 ± 0.33	0.24 ± 0.17
24:0	1.28 ± 0.46	1.21 ± 0.32
Total Sat.[2)]	22.39 ± 2.04	18.83 ± 0.87
Total Mono.[3)]	26.20 ± 1.74	28.19 ± 0.92
18:2n-6	45.75 ± 5.33	44.51 ± 3.02
18:3n-6	0.23 ± 0.05	0.47 ± 0.08
20:2n-6	0.16 ± 0.04	0.73 ± 0.19*
20:3n-6	0.10 ± 0.03	0.24 ± 0.06
20:4n-6	1.64 ± 1.15	0.89 ± 0.56
22:2n-6	0.23 ± 0.08	0.65 ± 0.26
Total n-6[4)]	48.12 ± 4.24	47.49 ± 2.89
18:3n-3	0.19 ± 0.05	0.26 ± 0.06
20:3n-3	0.13 ± 0.06	1.30 ± 0.42*
20:5n-3	0.66 ± 0.56	1.55 ± 0.47
22:6n-3	2.31 ± 0.69	2.30 ± 0.67
Total n-3[5)]	3.30 ± 1.18	5.40 ± 1.60

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.6 Change in fatty acid composition in the heart

In the heart, the percentages of 15:0, total n-6 poly unsaturated fatty acids including 20:4n-6 (Arachidonic acid, AA) fatty acids in the *S. sieboldii* Miq. group were found to be significantly lower than the control group. ($p<0.05$). Meanwhile, the percentage of the total monounsaturated fatty acids including 14:0, 16:0 and 18:2n-6 were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated and n-3 polyunsaturated fatty acids found (Table 13).

Table 13 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of heart

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
6:0	0.00 ± 0.00[1)]	0.02 ± 0.01
14:0	0.12 ± 0.02	0.42 ± 0.09*
15:0	0.13 ± 0.02	0.07 ± 0.01*
16:0	9.36 ± 0.92	11.79 ± 0.47*
17:0	0.17 ± 0.03	0.18 ± 0.03
18:0	0.84 ± 0.28	0.36 ± 0.09
20:0	1.87 ± 0.54	1.33 ± 0.03
21:0	5.94 ± 0.99	4.63 ± 0.77
22:0	6.71 ± 1.35	8.12 ± 0.44
23:0	2.76 ± 1.12	1.42 ± 0.22
24:0	5.89 ± 1.81	7.26 ± 0.67
Total Sat.[2)]	33.79 ± 0.83	35.60 ± 0.97
Total Mono.[3)]	26.98 ± 1.13	32.29 ± 1.83*
18:2n-6	3.57 ± 0.44	5.41 ± 0.26*
18:3n-6	1.27 ± 0.23	1.25 ± 0.09
20:2n-6	2.52 ± 0.25	2.19 ± 0.19
20:3n-6	1.61 ± 0.75	1.93 ± 0.85
20:4n-6	13.11 ± 1.43	7.53 ± 1.82*
22:2n-6	5.80 ± 0.61	5.38 ± 0.92
Total n-6[4)]	27.88 ± 0.83	23.69 ± 0.73*
18:3n-3	1.27 ± 0.14	1.13 ± 0.08
20:3n-3	1.78 ± 0.52	1.16 ± 0.23
20:5n-3	1.96 ± 0.79	1.65 ± 0.27
22:6n-3	6.34 ± 0.57	4.39 ± 0.75
Total n-3[5)]	11.36 ± 1.39	8.32 ± 1.02

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.7 Change in fatty acid composition in the kidney

In the kidney, the percentages of 20:4n-6 and 22:2n-6 unsaturated fatty acids in the *S. sieboldii* Miq. group were found to be significantly higher than the control group ($p<0.05$). Meanwhile, the percentage of 18:2n-6 was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the kidney (Table 14).

Table 14 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of kidney

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
4:0	0.04 ± 0.00[1)]	0.03 ± 0.03
6:0	0.12 ± 0.01	0.15 ± 0.08
11:0	0.01 ± 0.01	0.02 ± 0.02
12:0	0.10 ± 0.02	0.04 ± 0.02
13:0	0.25 ± 0.17	0.03 ± 0.03
14:0	1.08 ± 0.22	0.55 ± 0.19
15:0	0.13 ± 0.02	0.09 ± 0.02
16:0	17.79 ± 2.13	15.21 ± 1.67
17:0	0.07 ± 0.01	0.16 ± 0.06
18:0	0.06 ± 0.01	0.37 ± 0.15
20:0	0.97 ± 0.32	1.51 ± 0.23
21:0	1.75 ± 0.40	2.81 ± 0.45
22:0	0.00 ± 0.00	1.58 ± 1.58
23:0	0.99 ± 0.41	1.61 ± 0.28
24:0	5.64 ± 0.53	3.30 ± 1.02
Total Sat.[2)]	29.00 ± 1.22	27.46 ± 1.38
Total Mono.[3)]	36.95 ± 2.65	27.46 ± 4.69
18:2n-6	5.86 ± 0.31	4.20 ± 0.19*
18:3n-6	0.69 ± 0.20	0.92 ± 0.21
20:2n-6	6.90 ± 1.23	7.89 ± 1.19
20:3n-6	0.81 ± 0.11	1.14 ± 0.14
20:4n-6	3.99 ± 0.73	7.16 ± 0.43*
22:2n-6	2.28 ± 0.60	4.89 ± 0.63*
Total n-6[4)]	20.54 ± 2.60	26.20 ± 1.83
18:3n-3	4.05 ± 1.63	7.36 ± 0.29
20:3n-3	0.63 ± 0.03	2.03 ± 0.74
20:5n-3	0.68 ± 0.68	2.65 ± 0.41
22:6n-3	3.20 ± 0.53	6.83 ± 2.23
Total n-3[5)]	8.56 ± 2.44	18.87 ± 3.04

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.8 Change in fatty acid composition in the spleen

In the spleen, the percentages of 18:3n-3 fatty acid in the *S. sieboldii* Miq. group was found to be significantly higher than the control group ($p<0.05$). Meanwhile, the percentage of 23:0 fatty acid was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the spleen (Table 15).

Table 15 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of spleen

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
4:0	0.24 ± 0.02[1)]	0.22 ± 0.06
6:0	0.88 ± 0.12	0.72 ± 0.23
10:0	0.26 ± 0.02	0.21 ± 0.05
14:0	1.34 ± 0.09	1.37 ± 0.08
16:0	26.52 ± 0.46	28.71 ± 1.42
18:0	4.49 ± 0.38	5.07 ± 1.02
20:0	0.21 ± 0.12	0.68 ± 0.13
21:0	0.25 ± 0.15	0.17 ± 0.09
22:0	0.66 ± 0.23	0.79 ± 0.30
23:0	5.25 ± 0.88	1.58 ± 0.17*
24:0	0.30 ± 0.05	0.18 ± 0.11
Total Sat.[2)]	40.41 ± 1.17	39.80 ± 2.36
Total Mono.[3)]	39.37 ± 4.28	32.42 ± 4.49
18:2n-6	7.46 ± 0.55	7.79 ± 0.41
18:3n-6	1.46 ± 0.82	1.17 ± 0.59
20:2n-6	8.67 ± 4.40	16.00 ± 1.79
20:3n-6	0.48 ± 0.09	0.33 ± 0.06
22:2n-6	0.39 ± 0.12	0.66 ± 0.16
Total n-6[4)]	18.46 ± 4.34	25.95 ± 1.85
18:3n-3	0.24 ± 0.01	0.51 ± 0.06*
22:6n-3	0.89 ± 0.08	1.09 ± 0.41
Total n-3[5)]	**1.76 ± 0.09**	**1.80 ± 0.56**

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.9 Change in fatty acid composition in the cortex

In the cortex, the percentages of 20:0, 22:6n-3 (DHA) and total n-3 polyunsaturated fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 polyunsaturated fatty acids found in the cortex (Table 16).

Lim and Suzuki (2002) reported that a Dietary n-3 fatty acid deficiency is associated with biochemical changes such as decreased brain DHA (Bourre et al., 1984; Moriguchi et al., 2000) and monoaminergic neurotransmitter levels (Delion et al., 1994). In addition, behavioral and physiological alterations occur, such as disturbed electroretinographic measurements and other vision-related parameters (Neuringer et al., 1994), reduced learning ability (Yamamoto et al., 1988; Frances et al., 1996; Greiner et al., 1999; Nakashima et al., 1993). Such studies have suggested that dietary n-3 polyunsaturated fatty acids (PUFAs) play an important role in normal cerebral development.

Table 16 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of cortex

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
6:0	0.08 ± 0.01[1)]	0.06 ± 0.01
14:0	0.12 ± 0.05	0.00 ± 0.00
15:0	0.03 ± 0.03	0.02 ± 0.02
16:0	17.27 ± 0.70	14.38 ± 1.05
17:0	0.09 ± 0.01	0.09 ± 0.02
18:0	13.13 ± 1.11	11.81 ± 0.37
20:0	1.31 ± 0.11	2.17 ± 0.07*
21:0	4.81 ± 1.71	3.60 ± 0.67
22:0	7.77 ± 0.17	11.12 ± 1.21
24:0	2.98 ± 0.13	3.76 ± 0.49
Total Sat.[2)]	47.58 ± 0.15	47.00 ± 1.00
Total Mono.[3)]	28.61 ± 1.07	28.94 ± 0.68
18:2n-6	0.32 ± 0.15	0.14 ± 0.01
18:3n-6	1.47 ± 0.11	1.49 ± 0.87
20:3n-6	2.25 ± 0.71	1.23 ± 0.80
20:4n-6	3.15 ± 0.44	1.96 ± 0.34
Total n-6[4)]	7.18 ± 1.31	5.27 ± 1.16
18:3n-3	0.96 ± 0.48	0.86 ± 0.18
20:5n-3	0.55 ± 0.09	0.67 ± 0.07
22:6n-3	15.11 ± 0.69	17.87 ± 0.42*
Total n-3[5)]	16.63 ± 0.21	19.39 ± 0.41*

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.10 Change in fatty acid composition in the cerebellum

In the cerebellum, the percentages of 15:0 and 22:0 saturated fatty acids were found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). Meanwhile, the percentages of 22:6n-3 and total n-3 polyunsaturated fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 polyunsaturated fatty acids found in the cerebellum (Table 17).

Table 17 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of cerebellum

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
12:0	0.97 ± 0.38[1)]	0.69 ± 0.14
13:0	1.84 ± 0.71	0.34 ± 0.08
14:0	0.57 ± 0.17	0.12 ± 0.01
15:0	0.11 ± 0.01	0.02 ± 0.02*
16:0	11.23 ± 1.42	12.68 ± 0.22
17:0	0.06 ± 0.03	0.12 ± 0.03
18:0	9.32 ± 0.99	11.09 ± 0.30
20:0	1.00 ± 0.52	2.03 ± 0.28
21:0	1.49 ± 0.08	0.52 ± 0.52
22:0	7.19 ± 0.11	6.67 ± 0.11*
Total Sat.[2)]	33.90 ± 1.00	34.33 ± 0.58
Total Mono.[3)]	33.36 ± 0.43	35.19 ± 0.58
18:2n-6	1.62 ± 0.23	0.87 ± 0.68
18:3n-6	1.74 ± 0.41	1.85 ± 0.25
20:3n-6	4.21 ± 0.10	3.89 ± 0.37
20:4n-6	4.28 ± 0.39	4.68 ± 0.91
22:2n-6	3.28 ± 0.20	1.62 ± 0.81
Total n-6[4)]	15.14 ± 0.95	12.90 ± 1.28
18:3n-3	0.39 ± 0.39	0.89 ± 0.44
20:5n-3	0.71 ± 0.05	0.81 ± 0.13
22:6n-3	15.40 ± 0.37	18.98 ± 0.00*
Total n-3[5)]	16.26 ± 0.35	20.41 ± 0.46*

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.11 Change in fatty acid composition in the retina

In the retina, the percentages of 20:0, 22:0, 20:3n-6 and 22:6n-3 (DHA) fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$) and 20:4n-6 unsaturated fatty acid was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). No significant differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids found in the retina (Table 18).

Table 18 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of retina

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
14:0	0.00 ± 0.00[1)]	0.08 ± 0.08
15:0	0.00 ± 0.00	0.04 ± 0.04
16:0	1.6 ± 0.68	0.13 ± 0.07
17:0	0.00 ± 0.00	0.72 ± 0.50
18:0	0.00 ± 0.00	1.65 ± 0.92
20:0	0.08 ± 0.08	1.35 ± 0.50*
21:0	1.18 ± 0.29	2.54 ± 0.74
22:0	0.24 ± 0.14	3.11 ± 1.11*
23:0	2.93 ± 1.39	6.34 ± 2.36
24:0	14.74 ± 1.10	9.35 ± 3.12
Total Sat.[2)]	20.76 ± 1.14	31.11 ± 5.00
Total Mono.[3)]	14.24 ± 2.64	16.95 ± 2.47
18:2n-6	1.23 ± 0.32	1.26 ± 0.62
18:3n-6	0.00 ± 0.00	3.01 ± 2.01
20:2n-6	0.20 ± 0.20	1.47 ± 0.70
20:3n-6	1.10 ± 0.74	5.43 ± 4.50*
20:4n-6	31.18 ± 3.20	11.99 ± 6.19*
22:2n-6	0.85 ± 0.85	0.00 ± 0.00
Total n-6[4)]	34.56 ± 3.08	23.18 ± 7.29
18:3n-3	0.74 ± 0.41	2.68 ± 1.51
20:3n-3	7.39 ± 3.50	2.49 ± 1.33
20:5n-3	1.51 ± 0.71	10.20 ± 3.64
22:6n-3	26.69 ± 0.69	29.81 ± 0.00*
Total n-3[5)]	36.44 ± 4.04	45.18 ± 9.13

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.5.12 Change in fatty acid composition in the feces

In the feces, the *S. sieboldii* Miq. group showed higher percentage of 6:0 and 18:2n-6 fatty acids compared with that in the control group ($p<0.05$). No differences were uncovered between the two groups in terms of the percentages of total saturated, monounsaturated and n-3 polyunsaturated fatty acids found in feces. However, about 3.18% higher levels of total n-6 polyunsaturated fatty acid were found in the *S. sieboldii* Miq. group (Table 19). Alsp SCFAs, fatty acid of 6:0 was significantly increased in feces of mice with *S. sieboldii* Miq.

Table 19 Effect of *S. sieboldii* Miq. supplementation on fatty acid composition of feces

	Control	*Stachys sieboldii* Miq.
Fatty Acids		
6:0	0.19 ± 0.05[1)]	2.52 ± 2.36*
12:0	0.08 ± 0.04	0.17 ± 0.01
13:0	0.00 ± 0.00	0.03 ± 0.03
14:0	0.64 ± 0.05	0.60 ± 0.24
15:0	0.29 ± 0.02	0.53 ± 0.24
16:0	0.03 ± 0.03	0.22 ± 0.07
17:0	0.00 ± 0.00	0.12 ± 0.12
18:0	3.72 ± 0.45	2.44 ± 1.15
20:0	0.46 ± 0.15	0.71 ± 0.03
21:0	11.61 ± 0.84	12.71 ± 1.51
22:0	1.12 ± 0.17	1.38 ± 0.31
23:0	2.47 ± 1.17	3.88 ± 0.72
24:0	8.59 ± 2.32	6.85 ± 0.28
Total Sat.[2)]	29.02 ± 0.98	29.64 ± 2.19
Total Mono.[3)]	38.97 ± 1.42	37.88 ± 0.61
18:2n-6	1.05 ± 0.52	2.86 ± 0.56*
18:3n-6	1.89 ± 0.45	2.52 ± 0.23
20:2n-6	3.21 ± 0.94	1.75 ± 0.34
20:3n-6	1.92 ± 0.51	2.17 ± 0.91
20:4n-6	0.97 ± 0.38	1.83 ± 0.17
22:2n-6	0.96 ± 0.42	2.05 ± 0.23
Total n-6[4)]	10.00 ± 0.94	13.18 ± 0.54*
18:3n-3	1.60 ± 0.49	1.27 ± 0.06
20:3n-3	1.26 ± 0.55	2.24 ± 0.09
20:5n-3	3.86 ± 1.93	6.18 ± 0.89
22:6n-3	14.83 ± 5.09	7.90 ± 0.81
Total n-3[5)]	21.54 ± 3.16	17.59 ± 1.63

[1)]Data were expressed as mean ± the standard error of the mean (SEM), n=5.
[2)]Sat. means saturated fatty acids; [3)]Mono. means monounsaturated fatty acids; [4)]n-6 means n-6 poly unsaturated fatty acids; [5)]n-3 means n-3 poly unsaturated fatty acids
*$p<0.05$, significant effect between the control and *S. sieboldii* Miq. groups

3.6 Effect of *S. sieboldii* Miq. supplementation on memory/learning ability

3.6.1 Effect of *S. sieboldii* Miq. supplementation on memory ability

Passive avoidance through a learning trial and memory acquisition test means measuring each latency time that it makes mice to move on from light room to a dark room (Fig. 12). When the latency time exceeds 300 sec, it is assured that a memory is established and the measurements are stopped. This experiment is conducted for two days. On the first day of the experiment during the learning trial there was no big difference between the two groups. The control group's average latency was 16.50 ± 3.28 sec and for the *S. sieboldii* Miq. group the average latency was 19.36 ± 4.91 sec. After 24 hours, on the second day of the experiment, The memory acquisition test was conducted. Both the control group and *S. sieboldii* Miq. group's average latencies were significantly increased compared to the learning trial (36.31 ± 9.10, 71.31 ± 27.12 sec, respectively) ($p<0.05$). We found that mice fed with a diet of *S. sieboldii* Miq. had a higher average latency time, than the control group. It is widely assumed that changes in nervous system function associated with dietary n-3 fatty acid deficiencies are directly related to the loss of brain and retinal DHA content (Fedorova & Salem, 2006). Lee et al. (2013) also reported that mice injected with ethanol extracts from *S. sieboldii* Miq. (500 mg/kg) showed a 93% memory improvement compared to the positive control (10 mg/kg of Tacrine injected) group. Putting the above result together, *Stachys sieboldii* Miq. showed anti-amnestic and cognitive-enhancing activities related to the memory processes, and these activities were dependent on the to treatment duration and the learning models.

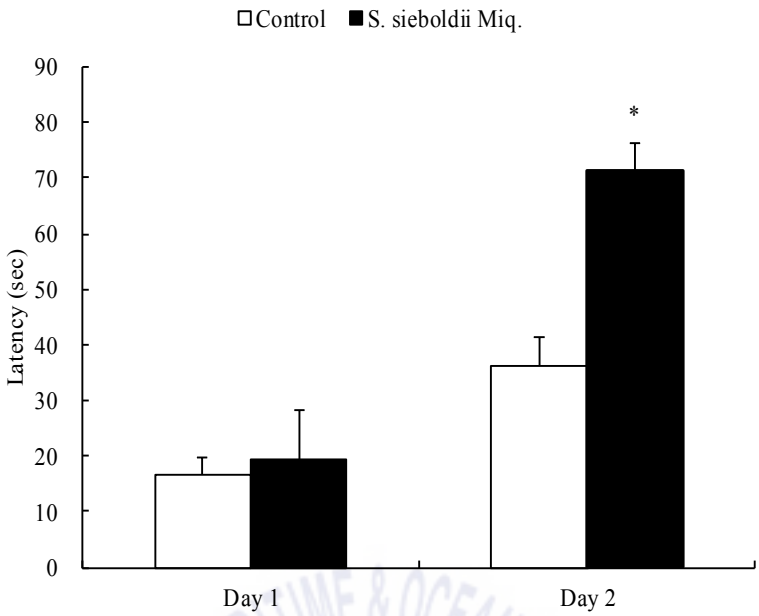

Fig. 12 Effect of *S. sieboldii* Miq. supplementation on latency in passive avoidance performance test Values with different letters are significantly different at $p<0.05$.

3.6.2 Effect of *S. sieboldii* Miq. supplementation on spatial learning performance

In the Morris water maze test for evaluating the spatial perception ability, there was no significant difference between two groups (Fig. 13). However In the probe trial day, *S. sieboldii* Miq. group significantly remembered the location of the platform (region A) compared to the control group (Fig. 14). The number of crossings of the position where the platform had been placed (quadrant region A) and the number of crossings in the corresponding imaginary positions in the other quadrant regions (regions B–D) were statistically significant ($p<0.05$).

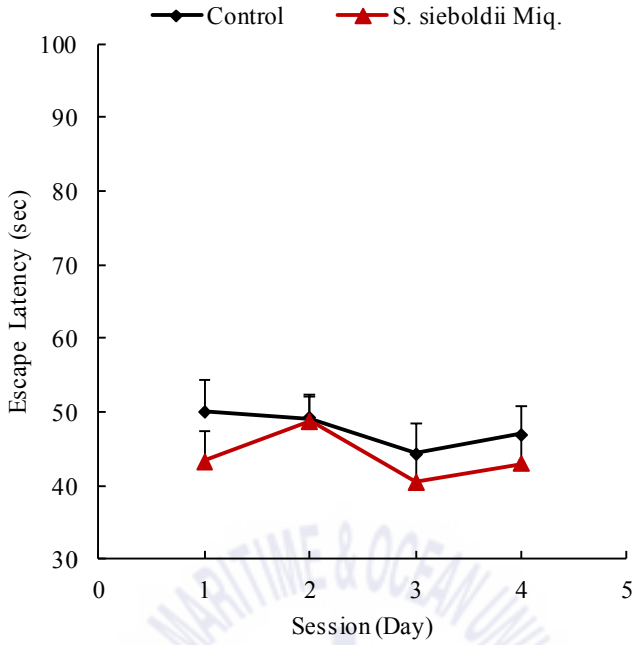

Fig. 13 Effect of *S. sieboldii* Miq. supplementation on latency in Morris water maze test Values with different letters are significantly different at $p<0.05$.

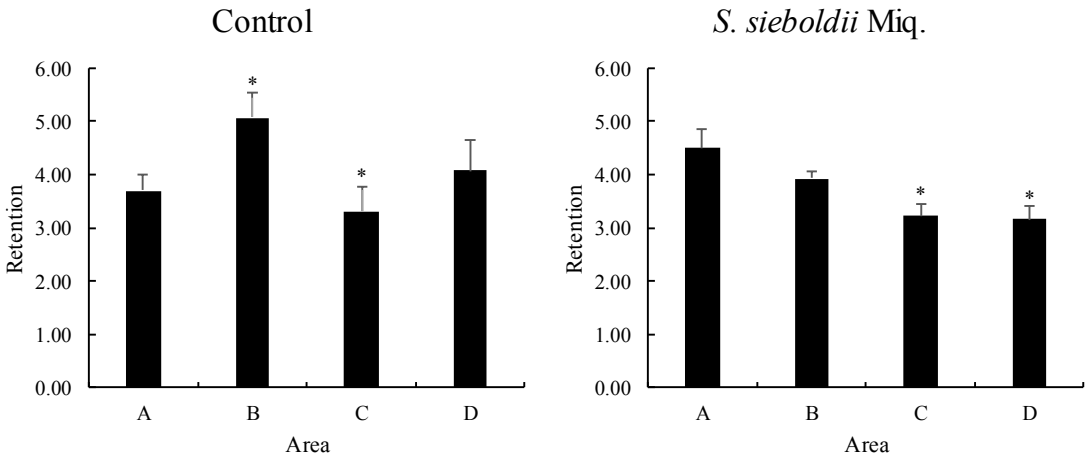

Fig. 14 Effect of *S. sieboldii* Miq. supplementation on retention in Morris water maze test Values with different letters are significantly different at $p<0.05$.

3.7 Effect of *S. sieboldii* Miq. supplementation on motor coordination ability

Latencies to fall off a rotating and accelerating rod were used as parameter of motor function and coordination (Singewald et al., 2004). Fig. 15 Shows that on the first day of the experiment there was no big difference between two groups (control group's average latency was 45.42 ± 6.91 sec and *S. sieboldii* Miq. group's average latency was 56.32 ± 6.27 sec), however from the second day onward there was a significant difference between two groups ($p<0.05$). Mice fed with a diet of *S. sieboldii* Miq. had higher average latency time than the control group. On the fourth day of experiment, the latency has no significant difference between and this is regarded to learned how to acclimate through repeated trials. Caston et al. (1995) and Lalonde et al. (1995) reported that among several behavioral tests that measure motor performance, the rotarod is a suitable test for the evaluation of cerebellar deficiences in rodents. Janssen et al. (2015) reported that mice groups who had a significantly lower AA and higher DHA in the brain, including the cortex and cerebellum had a longer latency in the rotarod test. These results seem to be similar to our results. A pattern similar to that of brain fatty acid composition was also uncovered in the case of the retina.

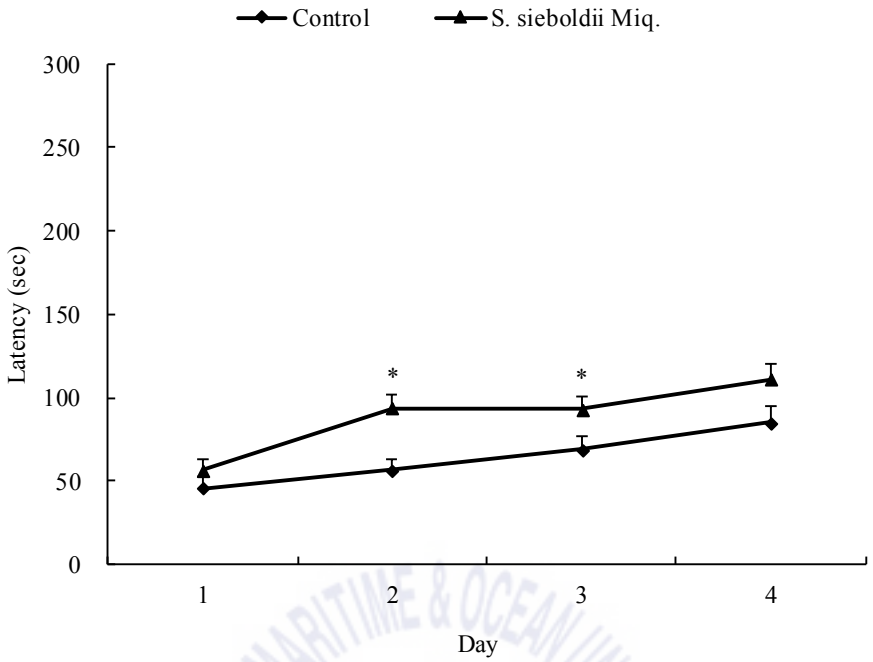

Fig. 15 Effect of *S. sieboldii* Miq. supplementation on latency in rotarod test Values with different letters are significantly different at $p<0.05$.

4. Summary and Conclusion

In this study, we looked at the relationship between the expression of inflammatory cytokines and the diversity of the microflora in the gut can be useful as a novel way to evaluate gut function or help to design new clinical treatments. The present study is the first report to clarify the effect of *S. sieboldii* Miq. supplementation on the 16S rDNA gene expression of the fecal microflora and cytokine expression. First, body weight and FER in each mouse group showed no significant difference. Our results show that supplementation with *S. sieboldii* Miq. increased beneficial gut microbiota and greatly decreased the presence of harmful microbiota including *E. coli* and *Bacteroides* sp. The level of IL-6 mRNA expression in mesenteric lymph nodes of mice with *S. sieboldii* Miq. was significantly lower than the control group. We suggest that this might be due to the lower abundances of harmful microbiota in gut.

Also, we analyzed lipid profiles and fatty acid compositions in several tissues in order to investigate the effect of *S. sieboldii* Miq. on motor learning/memory ability improvement. In Serum lipid profiles, the levels of total cholesterol, triglyceride and LDL-cholesterol, showed a significant decrease in the *S. sieboldii* Miq. intake group' s serum compared to the control group. The HDL-cholesterol content was slightly higher, but there was no significant indication that this trend is similar in either liver or heart lipid profiles.

Serum, RBC, liver, intestine, mesenteric lymph nodes, heart, kidney, cortex, cerebellum, retina tissues and feces matter were taken from control and *S. sieboldii* Miq. intake mice to investigate fatty acid compositions. In case of serum, the fatty acid composition, the percentages of 16:0, 21:0 and 20:5n-3 (EPA) were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). For RBC fatty acid composition, the percentages of 6:0, 14:0, 20:4n-6, 20:5n-3 (EPA) fatty acids and total n-6 polyunsaturated fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). However, the percentages of 6:0, 14:0, total n-6 including 20:4n-6 were found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). In the liver fatty acid composition, the percentages of 13:0 was found to be significantly lower than in the *S. sieboldii* Miq. group

($p<0.05$). In the intestine fatty acid composition, the percentages of 6:0 and 20:3n-6 were found to be significantly higher than in the *S. sieboldii* Miq. group, while the percentages of 16:0 and 18:0 were lower compared with those in the control group ($p<0.05$). In the mesenteric lymph nodes fatty acid composition, a higher percentage of 20:2n-6 and 20:3n-3 were found in the *S. sieboldii* Miq. group compared to that of the control group ($p<0.05$). In the heart fatty acid composition, the percentages of 15:0, total n-6 poly unsaturated fatty acids including 20:4n-6 were found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). Meanwhile, the percentage of total monounsaturated fatty acids including 14:0, 16:0 and 18:2n-6 were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). In the kidney fatty acid composition, the percentages of 20:4n-6 and 22:2n-6 were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). Meanwhile, the percentage of 18:2n-6 was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). In the spleen, the percentages of 18:3n-3 fatty acid in the *S. sieboldii* Miq. group was found to be significantly higher than the control group ($p<0.05$). Meanwhile, the percentage of 23:0 fatty acid was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). In the cortex, the percentage of 20:0, 22:6n-3 (DHA) and total n-3 polyunsaturated fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). In the cerebellum fatty acid composition, the percentage of 15:0 and 22:0 saturated fatty acids were found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$). Meanwhile, the percentage of 22:6n-3 and total n-3 polyunsaturated fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). In the retina fatty acid composition, the percentage of 20:0, 22:0, 20:3n-6 and 22:6n-3 fatty acids were found to be significantly higher in the *S. sieboldii* Miq. group ($p<0.05$). and 20:4n-6 unsaturated fatty acid was found to be significantly lower in the *S. sieboldii* Miq. group ($p<0.05$).

In the *S. sieboldii* Miq. intake mice group' s intestine tissues and feces, the overall fatty acid composition shows similar results regarding the higher SCFAs compositions than in the control group.

In the *S. sieboldii* Miq. intake mice group' s retina and brain tissues, fatty acid compositions show similar results regarding the lower n-6 fatty acids compositions and higher n-3 fatty acids compositions than in the control group. In addition, in

those tissues of the *S. sieboldii* Miq. group, the percentage of 22:6n-3 fatty acid was significantly higher than in the control group.

The results of the learning and memory test of mice administerd by the passive avoidance test, the latency was very short in both the control group and the *S. sieboldii* Miq. group within 30 seconds because the mice have had no arior exposure of any electric shocks. However, on the second day, the *S. sieboldii* Miq. intake mice group showed a significantly longer latency than the control group. In the Morris water maze test, the number of crossings of the position where the platform had been placed and the number of crossings in the corresponding imaginary positions in the other quadrant regions were statistically significant ($p<0.05$). In the motor activity assessed by the rotarod test, there was no significant difference between two groups on the first day of the experiment. However from the second day onwards mice fed on a diet of *S. sieboldii* Miq. had a higher average latency time than the control group ($p<0.05$). On the final day there was no significant difference in the latency between the groups. This is likely due to repetitive adaptation during the repeated trials.

In summary, these above results show that an adequate intake of *S. sieboldii* Miq. might be helpful to lower inflammatory cytokines due to the increase of beneficial intestinal microflora, lower serum cholesterol, triglyceride, LDL-cholesterol and improve motor performance, memory and cognitive ability.

References

Agustí, A., García-Pardo, M. P., López-Almela, I., Campillo, I., Maes, M., Romaní-Pérez, M., & Sanz, Y. 2018. Interplay between the gut-brain axis, obesity and cognitive function. Frontiers in neuroscience, 12, pp.155-171

Baek, H.S., Na, Y.S., Kim, D.H., Lee, C.H., Ryu, B.H., and Song, S.K., 2004. Antioxidant Activities of *Stachys sieboldii* Miq. Roots. Journal of Life Science, 14(1), pp.1-7.

Baek, H.S., Na, Y.S., Ryu, B.H., and Song, S.K., 2003. Antioxidant Activities of *Stachys sieboldii* Miq. stalks. Korean J Biotechnol Bioeng, 18(4), pp.266-271.

Bajaj, J. S., Hylemon, P. B., Ridlon, J. M., Heuman, D. M., Daita, K., White, M. B., and Gillevet, P. M. 2012. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. American Journal of Physiology-Gastrointestinal and Liver Physiology, 303(6), pp.675-685.

Bourre, J. M., Francois, M., Youyou, A., Dumont, O., Piciotti, M., Pascal, G. and Durand, G., 1989. The effects of dietary α-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. The Journal of nutrition, 119(12), pp.1880-1892.

Bourre, J. M., Pascal, G., Durand, G., Masson, M., Dumont, O., and Piciotti, M. 1984. Alterations in the fatty acid composition of rat brain cells (neurons, astrocytes, and oligodendrocytes) and of subcellular fractions (myelin and synaptosomes) induced by a diet devoid of n-3 fatty acids. Journal of neurochemistry, 43(2), pp.342-348.

Calder, P. C. 2004. n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clinical science, 107(1), pp.1-11.

Candela, M., Turroni, S., Biagi, E., Carbonero, F., Rampelli, S., Fiorentini, C. and Brigidi, P., 2014. Inflammation and colorectal cancer, when microbiota-host mutualism breaks. World journal of gastroenterology: WJG, 20(4), pp.908-922.

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. 2015. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Annals of gastroenterology: quarterly publication of the Hellenic Society of Gastroenterology, 28(2), pp.203-209.

Caston, J., Jones, N. and Stelz, T., 1995. Role of preoperative and postoperative sensorimotor training on restoration of the equilibrium behavior in adult mice following cerebellectomy. Neurobiology of learning and memory, 64(3), pp.195-202.

Chen, R., Liu, Z., Zhao, J., Chen, R., Meng, F., Zhang, M. and Ge, W., 2011. Antioxidant and immunobiological activity of water-soluble polysaccharide fractions purified from Acanthopanax senticosu. Food chemistry, 127(2), pp.434-440.

Chen, Y., Yang, F., Lu, H., Wang, B., Chen, Y., Lei, D., and Li, L. 2011. Characterization of fecal microbial communities in patients with liver cirrhosis.

Hepatology, 54(2), pp.562-572.

Cryan, J. F., & O' mahony, S. M. 2011. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterology & Motility, 23(3), pp.187-192.

Daly, D. M., Park, S. J., Valinsky, W. C., & Beyak, M. J. 2011. Impaired intestinal afferent nerve satiety signalling and vagal afferent excitability in diet induced obesity in the mouse. The Journal of physiology, 589(11), pp.2857-2870.

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., and Dumas, M. E. 2016. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. Gut, 65(3), pp.426-436.

De Lartigue, G., de La Serre, C. B., & Raybould, H. E. 2011. Vagal afferent neurons in high fat diet-induced obesity; intestinal microflora, gut inflammation and cholecystokinin. Physiology & behavior, 105(1), pp.100-105.

Delion, S., Chalon, S., Hérault, J., Guilloteau, D., Besnard, J. C., and Durand, G. 1994. Chronic dietary α-linolenic acid deficiency alters dopaminergic and serotoninergic neurotransmission in rats. The Journal of nutrition, 124(12), pp.2466-2476.

Fedorova, I. and Salem Jr, N., 2006. Omega-3 fatty acids and rodent behavior. Prostaglandins, leukotrienes and essential fatty acids, 75(4-5), pp.271-289.

Feng, K., Chen, W., Sun, L., Liu, J., Zhao, Y., Li, L., Wang, Y. and Zhang, W., 2015. Optimization extraction, preliminary characterization and antioxidant activity in vitro of polysaccharides from *Stachys sieboldii* Miq. tubers. Carbohydrate polymers, 125, pp.45-52.

Folch, J., Lees, M. and Stanley, G.S., 1957. A simple method for the isolation and purification of total lipides from animal tissues. Journal of biological chemistry, 226(1), pp.497-509.

Francès, H., Coudereau, J. P., Sandouk, P., Clément, M., Monier, C., and Bourre, J. M. 1996. Influence of a dietary α-linolenic acid deficiency on learning in the Morris water maze and on the effects of morphine. European journal of pharmacology, 298(3), pp.217-225.

Friedewald, W.T., Levy, R.I. and Fredrickson, D.S., 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clinical chemistry, 18(6), pp.499-502.

Frøyland, L., Vaagenes, H., Asiedu, D.K., Garras, A., Lie, Ø. and Berge, R.K., 1996. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs. Lipids, 31(2), pp.169-178.

Galdeano, C.M., De Leblanc, A.D.M., Vinderola, G., Bonet, M.B. and Perdigon, G., 2007. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. Clin. Vaccine Immunol., 14(5), pp.485-492.

Goldsmith, J.R. and Sartor, R.B., 2014. The role of diet on intestinal microbiota metabolism: downstream impacts on host immune function and health, and

therapeutic implications. Journal of gastroenterology, 49(5), pp.785-798.

Grasset, E., Puel, A., Charpentier, J., Collet, X., Christensen, J. E., Tercé, F., & Burcelin, R. 2017. A specific gut microbiota dysbiosis of type 2 diabetic mice induces GLP-1 resistance through an enteric NO-dependent and gut-brain axis mechanism. Cell metabolism, 25(5), pp.1075-1090.

Greiner, R. S., Moriguchi, T., Hutton, A., Slotnick, B. M., & Salem Jr, N. 1999. Rats with low levels of brain docosahexaenoic acid show impaired performance in olfactory-based and spatial learning tasks. Lipids, 34, pp.S239-S243.

Greiner, R.S., Moriguchi, T., Slotnick, B.M., Hutton, A. and Salem Jr, N., 2001. Olfactory discrimination deficits in n-3 fatty acid-deficient rats. Physiology & behavior, 72(3), pp.379-385.

Grenham, S., Clarke, G., Cryan, J. F., & Dinan, T. G. 2011. Brain-gut-microbe communication in health and disease. Frontiers in physiology, 2, pp.94.

Hayashi, K., Nagamatsu, T., Ito, M., Hattori, T. and Suzuki, Y., 1994. Acteoside, a component of *Stachys sieboldii* Miq, may be a promising antinephritic agent: Effect of acteoside on crescentic-type anti-GBM nephritis in rats. The Japanese Journal of Pharmacology, 65(2), pp.143-151.

Hayashi, K., Nagamatsu, T., Ito, M., Hattori, T., and Suzuki, Y. 1994. Acteoside, a component of Stachys sieboldii MIQ, may be a promising antinephritic agent (2): Effect of acteoside on leukocyte accumulation in the glomeruli of nephritic rats. The Japanese Journal of Pharmacology, 66(1), pp.47-52.

Janssen, C.I., Zerbi, V., Mutsaers, M.P., de Jong, B.S., Wiesmann, M., Arnoldussen, I.A., Geenen, B., Heerschap, A., Muskiet, F.A., Jouni, Z.E. and van Tol, E.A., 2015. Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills and hippocampal neurogenesis in developing C57BL/6J mice. The Journal of nutritional biochemistry, 26(1), pp.24-35.

Jarvik, M.E. and Kopp, R., 1967. An improved one-trial passive avoidance learning situation. Psychological reports, 21(1), pp.221-224.

Jeon, K.S., and Park, S.I. 2015. Antioxidative properties of Chinese artichoke (*Stachys sieboldii Miq*) added white bread. Culinary science and hospitality research, 21(6), pp.120-132.

Johnson, R.W., 1997. Inhibition of growth by pro-inflammatory cytokines: an integrated view. Journal of animal science, 75(5), pp.1244-1255.

Kang, J.R., Kang, M.J., Byun, H.W., Park, J.H., Kim, D.I., Chung, S.Y. and Shin, J.H., 2018. Effects of Sunsik Added *Stachys sieboldii* Miq. on Lipid Improvement in Rats Fed a High Fat-Cholesterol Diet. Journal of The Korean Society of Food Science and Nutrition. 47(6), pp.657-663.

Kim, J.Y., Cho, M.K., Choi, S.H., Lee, K.H., Ahn, S.C., Kim, D.H. and Yu, H.S., 2010. Inhibition of dextran sulfate sodium (DSS)-induced intestinal inflammation via enhanced IL-10 and TGF-β production by galectin-9 homologues isolated from intestinal parasites. Molecular and biochemical parasitology, 174(1), pp.53-61.

Kim, Y.K., Son, H.K. and Lee, J.J., 2017. Nutritional components and antioxidant

activities of various *Stachys sieboldii* Miq parts. The Korean Journal of Community Living Science, 28(2), pp.203-215.

Lalonde, R., Bensoula, A.N. and Filali, M., 1995. Rotorod sensorimotor learning in cerebellar mutant mice. Neuroscience research, 22(4), pp.423-426.

Lee, A.Y., Jeong, E.J. and Kim, Y. S. 2016. The Physicochemical Quality and Antioxidant Activity of Fermented beverage Added with *Stachys sieboldii.* MIQ Methanol Extracts. Korea J Food And Nutrition, pp.361-361.

Lee, J.E., Jin, S.Y. and Han, Y.S., 2014. Antioxidant activities and quality characteristics of tofu supplemented with Chinese artichoke powder. The Korean Journal of Food And Nutrition, 27(1), pp.10-21.

Lee, S.W., Jung, T.H. and Shin, Y.W., 2013. A comparative study of memory improving effects of stachys rhizome and lycopi rhizome on scopolamine-induced amensia in mice. The Korea Journal of Herbology, 28(5), pp.69-77.

Li, H.L., Lu, L., Wang, X.S., Qin, L.Y., Wang, P., Qiu, S.P., Wu, H., Huang, F., Zhang, B.B., Shi, H.L. and Wu, X.J., 2017. Alteration of gut microbiota and inflammatory cytokine/chemokine profiles in 5-fluorouracil induced intestinal mucositis. Frontiers in cellular and infection microbiology, 7, p.455.

Li, T., Lu, X. and Yang, X., 2017. Evaluation of clinical safety and beneficial effects of stachyose-enriched α-galacto-oligosaccharides on gut microbiota and bowel function in humans. Food & function, 8(1), pp.262-269.

Lim, S. Y., and Suzuki, H. 2002. Dose-response effect of docosahexaenoic acid ethyl ester on maze behavior and brain fatty acid composition in adult mice. International journal for vitamin and nutrition research, 72(2), pp.77-84.

Liu, G., Bei, J., Liang, L., Yu, G., Li, L. and Li, Q., 2018. Stachyose Improves Inflammation through Modulating Gut Microbiota of High-Fat Diet/Streptozotocin-Induced Type 2 Diabetes in Rats. Molecular nutrition & food research, 62(6), p.1700954.

Maes, M., Mihaylova, I., & Leunis, J. C. 2007. Increased serum IgA and IgM against LPS of enterobacteria in chronic fatigue syndrome (CFS): indication for the involvement of gram-negative enterobacteria in the etiology of CFS and for the presence of an increased gut-intestinal permeability. Journal of affective disorders, 99(1-3), pp.237-240.

Maturin, L. J., and J. T. Peeler. 1995. Aerobic plate count, p. 17. In Food and Drug Administration (ed.), Bacteriological analytical manual. AOAC International, Gaithersburg, Md.

McCurry, K.R., Campbell Jr, D.A., Scales, W.E., Warren, J.S. and Remick, D.G., 1993. Tumor necrosis factor, interleukin 6, and the acute phase response following hepatic ischemia/reperfusion. Journal of Surgical Research, 55(1), pp.49-54.

Moriguchi, T. and Salem Jr, N., 2003. Recovery of brain docosahexaenoate leads to recovery of spatial task performance. Journal of neurochemistry, 87(2), pp.297-309.

Moriguchi, T., Greiner, R.S. and Salem Jr, N., 2000. Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. Journal of neurochemistry, 75(6), pp.2563-2573.

Morrison, W.R. and Smith, L.M., 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. Journal of lipid research, 5(4), pp.600-608.

Mosman, T.R., 1994. Properties and functions of interleukin-10. In Advances in immunology (Vol. 56, pp. 1-26). Academic Press.

Nakashima, Y., Yuasa, S., Hukamizu, Y., Okuyama, H., Ohhara, T., Kameyama, T., and Nabeshima, T. 1993. Effect of a high linoleate and a high alpha-linolenate diet on general behavior and drug sensitivity in mice. Journal of Lipid Research, 34(2), pp.239-247.

Neuringer, M. and Connor, W.E., 1986. n-3 fatty acids in the brain and retina: evidence for their essentiality. Nutrition Reviews, 44(9), pp.285-294.

Neuringer, M., Reisbick, S., and Janowsky, J. 1994. The role of n-3 fatty acids in visual and cognitive development; Current evidence and methods of assessment. The Journal of pediatrics, 125(5), pp.S39-S47.

Nie, Q., Hu, J., Gao, H., Fan, L., Chen, H. and Nie, S., 2019. Polysaccharide from Plantago asiatica L. attenuates hyperglycemia, hyperlipidemia and affects colon microbiota in type 2 diabetic rats. Food hydrocolloids, 86, pp.34-42.

O' Mahony, S. M., Hyland, N. P., Dinan, T. G., & Cryan, J. F. 2011. Maternal separation as a model of brain-gut axis dysfunction. Psychopharmacology, 214(1), pp.71-88.

Park, Y.H., Seo, K.S., Ahn, J.S., Yoo, H.S. and Kim, S.P., 2001. Evaluation of the Petrifilm plate method for the enumeration of aerobic microorganisms and coliforms in retailed meat samples. Journal of food protection, 64(11), pp.1841-1843.

Qian, Y., Zhao, X., Song, J.L., Zhu, K., Sun, P., Li, G.J., Wang, R. and Kan, J.Q., 2013. Inhibitory effects of resistant starch (RS3) as a carrier for stachyose on dextran sulfate sodium-induced ulcerative colitis in C57BL/6 mice. Experimental and therapeutic medicine, 6(5), pp.1312-1316.

Reeves, P.G., Nielsen, F.H. and Fahey Jr, G.C., 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet.

Ryu, B.H. and Kim, S.O., 2004. Effects of methanol extract of *Stachys sieboldii* Miq on acetylcholine esterase and monoamine oxidase in rat brain. The Korean Journal of Food And Nutrition, 17(4), pp.347-355.

Salem Jr, N., Litman, B., Kim, H.Y. and Gawrisch, K., 2001. Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids, 36(9), pp.945-959.

Salem Jr, N., Reyzer, M. and Karanian, J., 1996. Losses of arachidonic acid in rat liver after alcohol inhalation. Lipids, 31(1Part2), pp.S153-S156.

Scott, K.P., Gratz, S.W., Sheridan, P.O., Flint, H.J. and Duncan, S.H., 2013. The influence of diet on the gut microbiota. Pharmacological research, 69(1), pp.52-60.

Singewald, N., Sinner, C., Hetzenauer, A., Sartori, S.B. and Murck, H., 2004. Magnesium-deficient diet alters depression-and anxiety-related behavior in mice-influence of desipramine and Hypericum perforatum extract. Neuropharmacology, 47(8), pp.1189-1197.

Singh, G. and Chandra, R.K., 1988. Biochemical and cellular effects of fish and fish oils. Progress in food & nutrition science, 12(4), pp.371-419.

Tinoco, J., 1982. Dietary requirements and functions of α-linolenic acid in animals. Progress in lipid research, 21(1), pp.1-45.

Tinoco, J., Babcock, R., Hincenbergs, I., Medwadowski, B. and Miljanich, P., 1978. Linolenic acid deficiency: Changes in fatty acid patterns in female and male rats raised on a linolenic acid-deficient diet for two generations. Lipids, 13(1), pp.6-17.

Tung, D., Cheung, P.H., Tudor, G., Booth, C. and Saha, S., 2011. In vivo effects of immunomodulators in a murine model of Fluorouracil-induced mucositis. Current Therapeutic Research, 72(6), pp.262-272.

van den Bogert, B., Meijerink, M., Zoetendal, E.G., Wells, J.M. and Kleerebezem, M., 2014. Immunomodulatory properties of Streptococcus and Veillonella isolates from the human small intestine microbiota. PloS one, 9(12), p.e114277.

Vandeputte, D., Falony, G., Vieira-Silva, S., Tito, R.Y., Joossens, M. and Raes, J., 2016. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. Gut, 65(1), pp.57-62.

Wang, Z., Wang, C. and Quan, Y., 2014. Extraction of polysaccharides from Phellinus nigricans mycelia and their antioxidant activities in vitro. Carbohydrate polymers, 99, pp.110-115.

Wang, Z., Wang, C., Su, T. and Zhang, J., 2014. Antioxidant and immunological activities of polysaccharides from Gentiana scabra Bunge roots. Carbohydrate polymers, 112, pp.114-118.

Weisinger, H.S., Vingrys, A.J. and Sinclair, A.J., 1996. Effect of dietary n-3 deficiency on the electroretinogram in the guinea pig. Annals of nutrition and metabolism, 40(2), pp.91-98.

Yamada, N., Shimizu, J., Wada, M., TAKITA, T. and INNAMI, S., 1998. Changes in platelet aggregation and lipid metabolism in rats given dietary lipids containing different n-3 polyunsaturated fatty acids. Journal of nutritional science and vitaminology, 44(2), pp.279-289.

Yamahara, J., Kitani, T., Kobayashi, H., and Kawahara, Y. 1990. Studies on Stachys sieboldii MIQ. II. Anti-anoxia action and the active constituents. Yakugaku Zasshi: Journal of the Pharmaceutical Society of Japan, 110(12), pp.932-935.

Yamamoto, N., Hashimoto, A., Takemoto, Y., Okuyama, H., Nomura, M., Kitajima, R., ... and Tamai, Y. 1988. Effect of the dietary alpha-linolenate/linoleate balance on lipid compositions and learning ability of rats. II. Discrimination process, extinction process, and glycolipid compositions. Journal of lipid research, 29(8),

pp.1013-1021.

Yin, J., Yang, G., Wang, S. and Chen, Y., 2006. Purification and determination of stachyose in Chinese artichoke (*Stachys Sieboldii Miq.*) by high-performance liquid chromatography with evaporative light scattering detection. Talanta, 70(1), pp.208-212.

Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J., 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. International journal of systematic and evolutionary microbiology, 67(5), p.1613.

Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y.Y., Wang, X., Fu, H., Xue, X., Lu, C., Ma, J. and Yu, L., 2018. Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. Science, 359(6380), pp.1151-1156.

Zhao, T., Mao, G., Mao, R., Zou, Y., Zheng, D., Feng, W., Ren, Y., Wang, W., Zheng, W., Song, J. and Chen, Y., 2013. Antitumor and immunomodulatory activity of a water-soluble low molecular weight polysaccharide from *Schisandra chinensis* (Turcz.) Baill. Food and chemical toxicology, 55, pp.609-616.

Zimmer, J., Lange, B., Frick, J.S., Sauer, H., Zimmermann, K., Schwiertz, A., Rusch, K., Klosterhalfen, S. and Enck, P., 2012. A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. European journal of clinical nutrition, 66(1), pp.53-60.

Zinchenko, T. V., Voitenko, G. N., and Lipkan, G. N. 1981. Anti-inflammatory, antitoxic, and hypoazotemic effect of a Stachys recta preparation, stachyrene. Farmakol Toksikol, 44(2), pp.191-194.

본 논문을 나은의 이학석사 학위논문으로 인준함

위원장 : 문 기 환 인

위 원 : 임 선 영 인

위 원 : 서 영 완 인

2019 년 11 월 29 일

한국해양대학교 해양과학기술전문대학원