

Preventive Effect of Oral Administration of *Pantoea agglomerans*-derived LPS in a Hypertensive Rat Model Upon Salt Loading

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Abstract. *Background/Aim:* Oral administration of *Pantoea agglomerans*-derived lipopolysaccharide (LPSp) has been reported to have a preventive effect against various lifestyle-related diseases. Therefore, we examined the preventive effect on high blood pressure, which is a kind of reserve arm for lifestyle-related diseases. *Materials and Methods:* Spontaneous hypertensive rat (SHR) and WKY rat were bred from 6 to 16 weeks of age. SHR were orally administered 100 µg/kg LPSp and 0.1% NaCl, and blood pressure was measured at 6, 10, 13 and 16 weeks. Furthermore, at 16 weeks of age, blood biochemical markers were measured and microbial community composition was analyzed. *Results:* SHRs developed hypertension with age, which was exacerbated by salt loading. Although there was almost no reduction in blood pressure in SHRs that received LPSp. It was suppressed at 13-16 weeks of age in those with salt loading. *Conclusion:* Oral administration of LPSp showed a preventive effect on salt-loaded hypertension.

Hypertension is the most prevalent disease globally. Patients with hypertension represent approximately 30% of the global population (1). In Japan, approximately 43 million people are estimated to have hypertension (2). In recent years, in addition to waist circumference (males ≥ 85 cm and females ≥ 90 cm), systolic blood pressure (maximum value) ≥ 130 mmHg and/or diastolic blood pressure (minimum value) ≥ 85 mmHg have also been included in the diagnostic standards of metabolic

syndrome (3). Hypertension is considered as one indicator of metabolic syndrome. Hypertension is a risk factor for several diseases, such as vascular endothelial dysfunction, ischemic heart disease, stroke, and renal failure; the higher the blood pressure, the higher the risk of morbidity and mortality (4-7).

At present, the main treatments for hypertension include drug treatment with a diuretic agent, Ca antagonist, angiotensin-converting enzyme inhibitor, angiotensin II receptor antagonist, or a sympathetic agent. However, side-effects are common in drug therapy; therefore, it is desirable to improve lifestyle habits prior to drug therapy, unless urgent treatment is required. Particularly for normotensives and mild hypertensives, non-drug dependent prevention is considered important.

Hypertension is a complex trait determined by both genetic and environmental factors and is a major public health problem due to its high prevalence and concomitant increase in the risk of cardiovascular disease (8). Among environmental factors associated with hypertension, dietary salt intake is the most common and important risk factor (9). In addition, the alleviation of hypertension due to high salt intake is related to the production of vascular endothelial growth factor c (VEGFc) by macrophages and their removal of salt from the body through lymphatic vessels. Conversely, macrophage hypofunction leads to hypertension upon high salt intake (10).

In this study, lipopolysaccharide (LPS) derived from the plant symbiotic gram-negative bacteria *Pantoea agglomerans* (LPSp), and its preventive effects on various diseases were examined (11-14). LPS is a component of the outer cell membrane of gram-negative bacteria and comprises lipids and polysaccharides. The physiological action of LPS is exerted through Toll-like receptor 4 present on the cell surface of host cells (14). LPSp is produced by a type of gram-negative bacteria which supply nitrogen and phosphorus to a wide

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range of plants during symbiosis (15-16). Moreover, in animals, oral administration of LPSp improves lifestyle-related diseases, including hyperlipidemia in rabbits, diabetes in obese mice, and cognitive function in mice with Alzheimer's disease, and suppresses arteriosclerotic lesions in mice (17-20). It is thus, recognized to have various preventive effects in different diseases. The oral administration of LPSp also improves parameters related to glucose and lipid metabolism. The efficacy of oral administration of LPSp may involve macrophage activation (21). Hypertension due to high salt intake may be improved by macrophage activation. In this study, the antihypertensive effect of oral administration of LPSp was examined in a spontaneously hypertensive rat model received high salt.

Materials and Methods

Animals and treatments. Male 6-week-old SHR/NCrIj (SHR) and WKY/NCrIj (WKY) were obtained from Charles River Laboratories Japan (Yokohama, Japan). Care and handling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kagawa University and were approved by Kagawa University Institutional Animal Care and Use Committee. Rats were housed under conditions of controlled temperature and humidity with a 12-h light/dark cycle and unrestricted access to food and water. Low-fat diet (LFD; 16.1 kJ/g, 10% of energy as fat, D12450B) was purchased from Research Diets, Inc. (New Brunswick, NJ, USA). After 1 week of prefeeding, rats were divided into the following groups (n=2-4 per group): WKY-DW group, SHR (DW) group, SHR-LPSp (LPS) group, SHR-0.1% NaCl (Na) group, and SHR-0.1% NaCl+LPSp (Na+LPS) group. Purified LPS derived from *P. agglomerans* (LPSp; Macrophix Inc., Kagawa, Japan) was dissolved in drinking water and applied at 0.1 mg/kg body weight/day (21-22). NaCl was adjusted to 0.1% with tap water (9). Each rat was then fed a low-fat diet for 10 weeks. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP) were monitored using tail-cuff methods (BP-98A; Softron Co., Tokyo, Japan) at 6, 10, 13, and 16 weeks of age. At 16 weeks of age, blood was collected from the heart of these rats, and plasma was obtained by centrifugation. All surgeries were performed under diethyl ether anesthesia, and rats were euthanized via diethyl ether inhalation.

Measurement of biochemical markers. Levels of plasma biochemical markers [triglyceride (TG), total cholesterol (T-CHO), low-density-lipoprotein (LDL-C) and high-density-lipoprotein (HDL-C) cholesterol, aspartate transaminase (AST), alanine aminotransferase (ALT), and total-bilirubin (T-BIL) and oxidized LDL/LDL (OxLDL/LDL)] were commissioned to Oriental Yeast Co. Ltd (Japan).

Microbial community analysis. Microbial DNA was extracted from freeze-dried rat fecal samples using the MPure Bacterial DNA Extraction Kit (Qiagen, Hilden, Germany). Amplification, sequencing, and analysis of extracted DNA were commissioned to Bioengineering Lab (Chiba, Japan). Sequencing was performed using MiSeq under conditions of 2x300 bp. Analysis of alpha and beta diversity was performed using QIIME script. The database was

97% OTU of Greengene attached to the bacterial flora analysis pipeline QIIME, and all sequences not judged to be chimeras were extracted and used for the subsequent analysis.

Statistical analysis. All results are expressed as mean±SEM. If a difference based on one- or two-way analysis of variance was significant, then Bonferroni's multiple comparison test was used for paired comparisons using GraphPad Prism for Windows (GraphPad Software, Inc., San Diego, CA, USA). Results were considered significant at $p < 0.05$.

Results

Changes in blood pressure over time between WKY-DW group, DW group and LPS group. Compared with the WKY-DW group, SBP, DBP and MBP in the DW group and LPS group were significantly increased, but no significant difference was found between the DW group and LPS group (Figure 1).

Changes in blood pressure over time between WKY-DW group, Na group and Na+LPS group. Compared with WKY-DW group, SBP, DBP and MBP of Na group and Na+LPS group increased significantly. In DBP, there was a significant difference between DW group and Na+LPS group, but in SBP and MBP, there was no significant difference between DW group and LPS group. In addition, SBP, DBP and MBP in the Na+LPS group decreased compared to the Na group, but no significant difference was observed (SBP: $p=0.0587$, DBP: $p=0.1421$, MBP: $p=0.1546$, respectively) (Figure 2).

Analysis of biochemical markers between DW group, LPS group, Na group and Na+LPS group. Several biochemical markers were evaluated in the DW group, LPS group, Na group, and Na+LPS group. TP, ALT, GLU, and AMY, did not differ significantly between the four groups. Regarding TG, the LPS group and Na group showed significant decreases compared with the DW group. Regarding AST, the Na+LPS group showed a decrease, whereas the Na group showed an increase, compared with the LPS group, but these differences were not significant. Regarding HDL-C, the Na+LPS group showed a tendency to increase compared with the Na group, but this difference also was not significant (Figure 3).

Gut microbiota community analysis. Gut microbiota composition was analyzed by extracting microbial DNA from stool samples. As shown in Figure 4A, relative abundance of bacteria was calculated at the phylum level. There was no difference in this regard between the DW group and LPS group, but the Na group showed an increase in the abundance compared with these two groups. Compared with the Na group, the Na+LPS group showed a decrease in the relative abundance of Firmicutes/

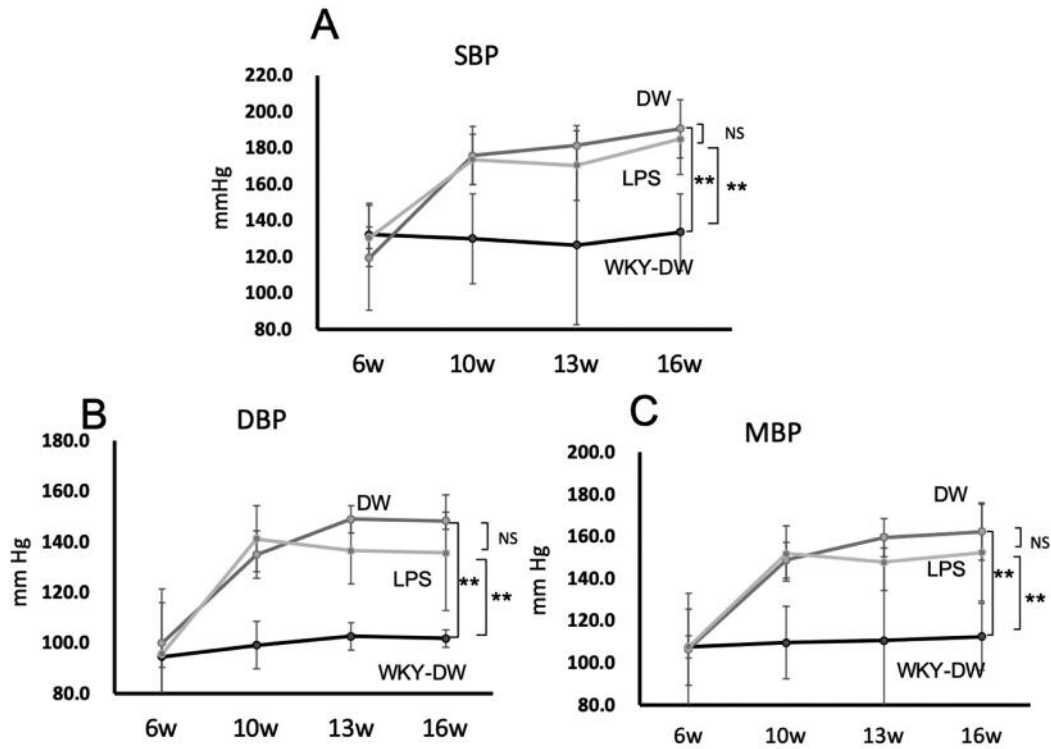


Figure 1. Changes in blood pressure over time in the WKY-DW group, DW group and LPS group. SBP (A), DBP (B) and MBP (C) were measured at 6 weeks old, 10 weeks old, 13 weeks old, 16 weeks old rats. Black: WKY-DW group, gray: DW group, light gray: LPS group. The data are presented as mean \pm SEM and were obtained from 2-4 rats per group. * p <0.05, ** p <0.01, *** p <0.001 compared to the WKY-DW group.

Bacteroidetes; however, the difference was not significant (Figure 4B). Furthermore, weighted UniFrac analyses were used to calculate distances between the fecal samples from the DW group and LPS group (Figure 4C), Na group and Na+LPS group (Figure 4D). 3-dimensional (3D) scatterplots were generated by principal coordinate analysis (PCoA) to visualize whether the experimental groups in the input phylogenetic tree have significantly different microbial communities. This method allows us to present dissimilarities of the data in terms of distance. The composition of the fecal microbial communities of the DW group and LPS Group were not found to be distinct (Figure 4C). However, the Na group and Na+LPS group were found to be distinct (Figure 4D). A clear separation was observed in the PCoA between the 2 clusters, representing the microbial compositions of the Na group and Na+LPS group, indicating 2 extremely different gut environments.

Discussion

Epidemiological studies have shown that high blood pressure is a risk factor for stroke and cardiovascular disease, diabetes, aging, and dyslipidemia. The commonly used SHR hypertensive model rat also has abnormal lipid metabolism

as well as spontaneous hypertension (4-7). In this study, although SBP, MBP, and DBP increased over time, oral administration of LPSp at 0.1 mg/kg, improved slightly blood pressure (Figure 1). However, when the Na group was administered 0.1 mg/kg LPSp, there was a suppression of the increase in blood pressure compared with that in the non-treated Na group (Figure 2).

High salt intake is associated with high blood pressure and increased risk of cardiovascular disease (9). Numerous basic and clinical studies have shown that excessive salt intake can increase blood pressure. Salt intake acts not only on hemodynamics but also on macrophages, which play a role in regulating intradermal sodium concentration (10).

When salt intake exceeds the ability of salt excretion in the kidney, it mainly accumulates subcutaneously. This subcutaneously accumulated sodium is then excreted from the lymphatic vessels in a manner controlled by macrophages via VEGF_C production (10). As such, if the function of macrophages decreases, then it may lead to high blood pressure. In this study, oral administration of LPSp was effective at preventing hypertension upon salt intake. It is hypothesized that LPSp acting on maintenance and enhancement of macrophage function, promotes elimination of salt to suppress hypertension.

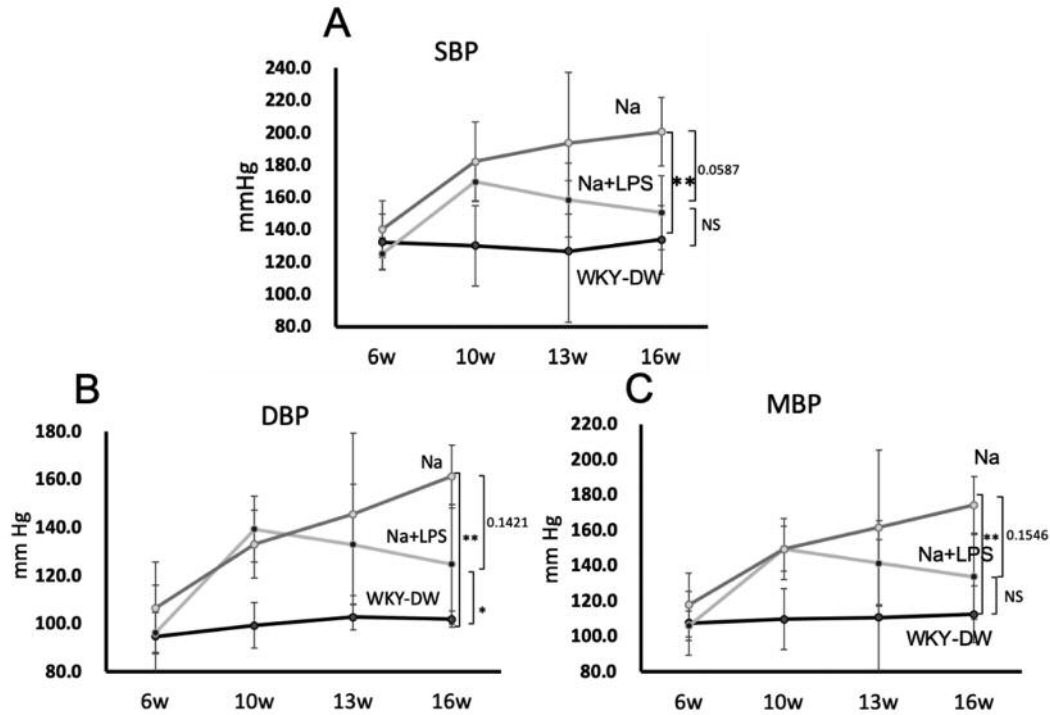


Figure 2. Changes in blood pressure over time in the WKY-DW group, Na group and Na+LPS group. SBP (A), DBP (B) and MBP (C) were measure at 6 weeks old, 10 weeks old, 13 weeks old, 16 weeks old rats. Black: WKY-DW group, gray: Na group, light gray: Na+LPS group. The data are presented as mean±SEM and were obtained from 2-4 rats per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the WKY-DW group.

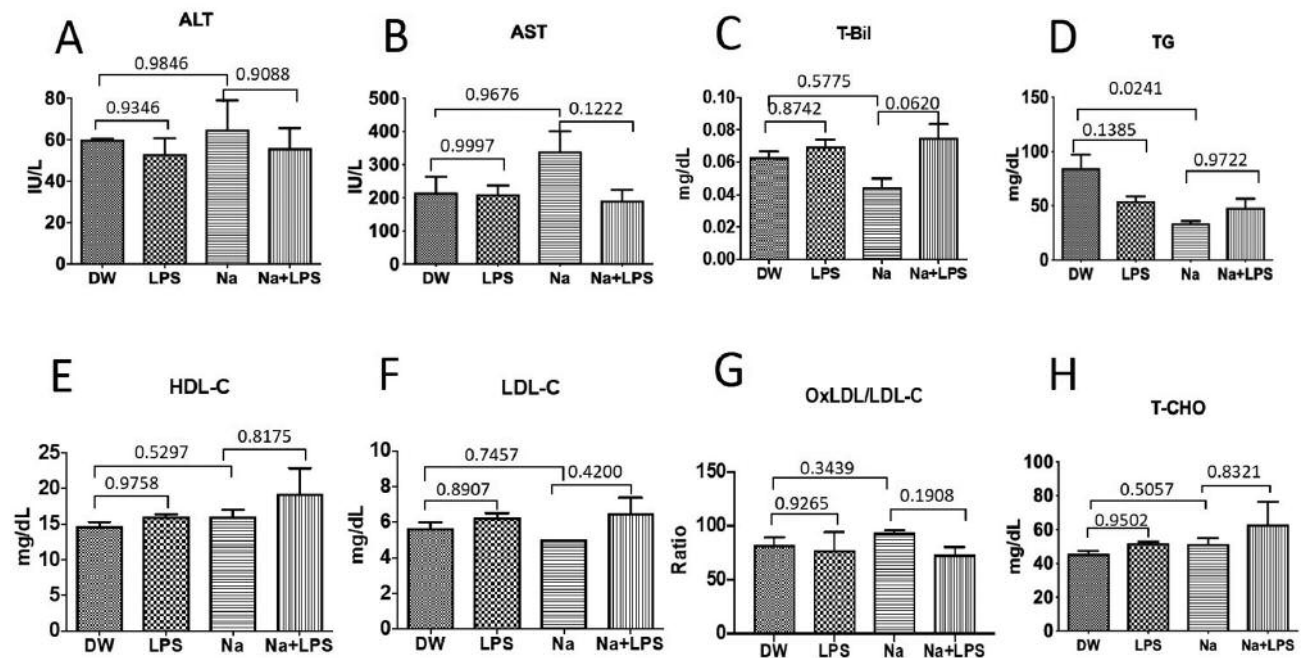


Figure 3. The levels of plasma biochemical markers in the DW group, LPS group, Na group and Na+LPS group. ALT: alanine aminotransferase (A), AST: Aspartate transaminase (B), T-BIL: total bilirubin (C), TG: triacylglycerol (D), HDL-C: high density lipoprotein cholesterol (E), LDL-C: low density lipoprotein cholesterol (F), OxLDL /LDL: oxidized LDL/LDL (G), T-CHO: total cholesterol (H). The data are presented as mean±SEM and were obtained from 2-4 rats per group.

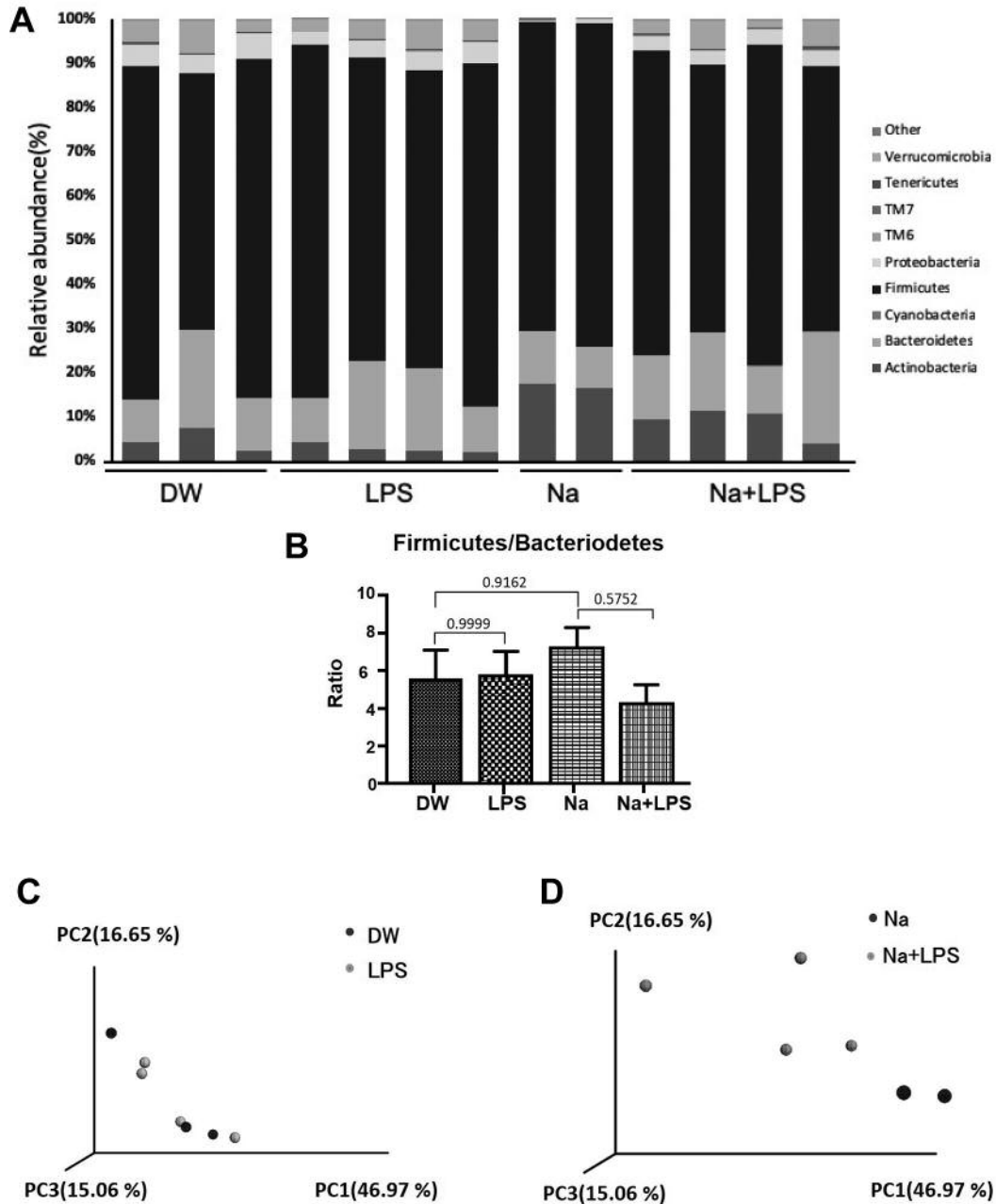


Figure 4. Gut microbiota composition in the DW group, LPS group, Na group and Na+LPS group. Variations in bacterial community compositions in stool samples at the phylum levels. The ratio of Bacteroidetes to Firmicutes (B). Principal component analysis results from stool samples of DW and LPS group (C). C: Black circle: DW group; Gray circle: LPS group. Principal component analysis results from stool samples of Na and Na+LPS group (D). D: Black circle: Na group; Gray circle: Na+LPS group. The data were obtained from 2-4 rats per group.

In recent years, many studies related to intestinal microflora and disease onset have been reported (22). According to the results of a study on intestinal flora and obesity, the intestinal bacterial composition differs between obese and normal mice kept in the same environment. In obese mice, the abundance of Firmicutes is increased and that of Bacteroidetes is decreased; thus, the ratio of

Firmicutes/Bacteroidetes (F/B) is increased (22). In addition, in a hypertensive rat model, the F/B ratio has been reported to be increased as in obesity, and the diversity of intestinal bacterial flora is decreased (23). However, LPSp intake brought the F/B ratio to the same level as in DW group. The level of Bacteroidetes was decreased by NaCl, but it was normalized by oral administration of LPSp (Figure 4B). Oral

administration of LPSp to ApoE-deficient mice in an arteriosclerosis model resulted in a significant decrease in enteric flora compared to that in the control group, along with an increase in the level of Bacteroidetes (19). This study produced similar results.

Hypertension is mainly caused by obesity and kidney injury. However, in contrast to DW group and Na group, the administration of LPSp did not result in significant changes in biochemical markers, such as lipid and liver metabolism levels (Figure 3). Although the mechanism of improvement of salt intake-induced hypertension by LPSp is unclear, macrophages may be involved in promoting sodium excretion. Based on the above data, we plan to analyze the role of macrophages in salt excretion.

Conflicts of Interest

The Authors have no financial conflicts of interest.

Authors' Contributions

R. Z. and H. I. were responsible for the study concept and design; R. Z. and S.U. was responsible for acquisition of data; T. M. and H. K. were responsible for analysis and interpretation of data; R. Z. was responsible for drafting the manuscript; and G. S. was responsible for study supervision.

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