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Effect of *ad libitum* ingestion of magnesium-rich mineral hard water on development of depressive-like phenotypes in mice exposed to chronic social defeat stress

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Abstract

Inflammatory alteration of the feature of gut microbiota is one of causative factors for depressive disorders. Here, we examined whether ingestion of magnesium-rich (212 ppm) mineral hard water (mineral water), which prevents gut inflammation, had prophylactic effect on development of depressive-like phenotypes in 10-days social defeat stress (SDS)-subjected C57BL/6J mice. SDS-susceptible mice with social disability exhibited increased body weight gain, and ingestion of mineral water ameliorated the increased body weight gain without any effects on daily food consumption or water intake. Relative abundance of *Bacteroides* spp. in colonic microbiota was decreased in water- and mineral water-ingesting SDS-susceptible mice, the level of reduction in the former being greater than in the latter, and was correlated inversely with body weight gain, and positively with sociability of mice. Overall, it is suggested that *ad libitum* magnesium-rich mineral water ingestion ameliorates development of psychosocial stress-induced depressive-like phenotypes partially *via* preventing decrease of colonic *Bacteroides* spp.

Keywords: depressive phenotype; magnesium-rich mineral water; colonic microbiota; sociability; body weight.

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Psychosocial stress-induced mood disorders such as major depressive disorder, anxiety disorder, anthropophobia, *etc.* cause not only reduction of QOL of the patients but also socio-economical loss. Currently available pharmacotherapy involving anti-depressants ameliorates the symptoms in only a limited number of patients frequently with severe adverse effects [1, 2].

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Thus, in addition to an increase of the number of patients with mood disorders, no available curative therapy/preventive approach for mood disorders is a serious problem worldwide [3].

Recently, as a causative factor for depressive disorder, the gut microbiota-mediated dysregulation of the gut-brain axis is widely noticed [4-9]. With this scenario, psychological stress induces a leaky gut through induction of a gut inflammatory response and/or dysbiosis, resulting in central inflammation through microglial activation. Previously, we demonstrated that in C57BL/6J mice, which are resilient to chronic unpredictable mild stress, experimental colitis increased susceptibility to the stress, and they develop depressive-like behavior with an altered composition of the gut microbiota involved in regulation of the gut mucus barrier such as *Bacteroides* spp. [10]. On the other hand, it is suggested that a psychological stress-induced leaky gut is caused by low grade inflammation based on dysbiosis [6]. Thus, prophylactic approaches to gut inflammation and/or dysbiosis are expected to prevent the development of depressive disorders.

Previously, we found that using magnesium chloride solution (200 ppm as magnesium) and commercially available magnesium-rich (200 ppm) refined deep-sea water (RDSW), prophylactic *ad libitum* ingestion of magnesium, an antagonist for the P2X7 receptor [11], of which activation in colonic resident mast cells is the first step in the developmental cascade of colitis [12], prevented activation and accumulation of mast cells in the colon, lessening development of experimental colitis in mice [13]. Therefore, *ad libitum* ingestion of magnesium might exert any preventive effect on development of depressive-like phenotypes in psychological stress-exposed mice.

In this study, we examined the effect of prophylactic *ad libitum* ingestion of RDSW on development of depressive-like phenotypes in C57BL/6J mice exposed to social defeat stress (SDS), a widely used experimental paradigm well-reflecting psychosocial stress occurring in modern human society.

Materials and methods

Materials

RDSW (Amami's Water®/Water Hardness 1000, Ako Kasei Co., Ltd., Ako, Japan) was used, its ingredients being as follows: magnesium (212 ppm), calcium (73.8 ppm), sodium (60.1 ppm), and potassium (57.5 ppm).

Animals

Five-week-old male C57BL/6JJmsSlc (C57BL/6J; total, 26 mice) mice, and breeder-retired male ICR mice (total, 23 mice) were purchased from Japan SLC (Hamamatsu, Japan), and used for experiments after 1-week habituation to the animal facility. The mice were housed in cages individually with food (AIN-93G for C57BL/6J mice, and MF for ICR ones) and water (ultra-pure water, resistivity: 18 or more MΩ) available *ad libitum* in a controlled environment at 22±1°C with a 12 h/12 h light/dark cycle in a specific pathogen-free facility. All experiments were performed in strict accordance with ARRIVE guidelines and were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (authorization numbers: DEB-15-001 and DEB-20-001, 2015 to 2024). The number of animals was kept to the minimum necessary for meaningful interpretation of the data, and animal discomfort was minimized.

SDS exposure

Experimental C57BL/6J mice were divided into four groups, water (ultra-pure water)-ingesting control (N=5) and SDS (N=8), and RDSW-ingesting control (N=5) and SDS (N=8) groups. Exposure to SDS was performed from Day 0 to 10 based on the modified SDS-protocol reported previously [14]. During the SDS-exposure period including behavioral tests, body weight, food consumption and water intake were recorded daily.

Social interaction (SI) test

To assess the sociability of mice quantitatively, we performed the SI test on Day 10 after the last SDS exposure using an SI test apparatus with a 40 cm (W) × 40 cm (D) × 50 cm (H) arena containing a perforated plastic interaction box, which was placed one side of the arena, as reported previously [14]. In the SI test, the behavior

of the experimental mice in an arena with or without an unfamiliar ICR mouse as a social target in an interaction box surrounded by an interaction zone (a 7 cm-wide area) was monitored for 150 s with video tracking system EthoVision XT version 11.5 (Nordus Information Technology, Wageningen, Netherland), and SI ratios (%) were calculated as 100 × (time spent in the interaction zone in the presence of a social target)/(time spent in the interaction zone in the absence of a social target). The mice with SI ratios below and over 100% were defined as being susceptible and resilient, respectively, to SDS. Exploratory and escape behaviors were calculated using the total time spent in and the numbers entering the interaction and escape (two corner regions (8 cm × 8 cm) on the side opposite the interaction box in the arena) zones, respectively, during the SI test.

16S rRNA metagenome analysis

Total DNA was extracted from mouse colonic feces obtained on Day 10, and 16S rRNA metagenome analysis was performed following the previously reported protocol [14]. In brief, mouse colonic feces were obtained under deep anesthesia with intraperitoneal injection of a mixture of medetomidine (0.75 mg/kg), midazolam (4 mg/kg) and butophanol (5 mg/kg), and using their genome DNA obtained with a DNeasy® PowerSoil® kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, the V3-V4 hypervariable regions of 16S rRNA were amplified using primers, forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'), and the amplicons were purified using an Agencourt AMPure XP system (Beckman Coulter Genomics, MN, USA), and sequenced on an Illumina Miseq platform (Illumina, CA). Bioinformatic analyses were performed with a QIIME II pipeline (version 2023.5) with SILVA data base release 132.

Statistical analysis

All data are expressed as means ± SD. To detect significant differences among the groups, one-way (**Fig. 1a**, and **Table 2**) and two-way repeated measures (**Fig. 1b, c, d,** and **Table 1**) ANOVA followed by Tukey-Kramer post hoc multiple comparisons were performed. A p-value of 0.05 or less was considered statistically significant. In **Fig. 2** (**b, c**), the area under the curve (AUC) derived from body weight gain was calculated using the body weight change *vs* day curves in each mouse by the trapezoidal rule.

Results

Depressive-like phenotypes

Reduction of sociability in mice is a critical index for their successful exposure to psychosocial stress in the chronic SDS paradigm [15, 16]. In the SI test (**Fig. 1a**), 5 mice each of 6 and 8 ones exposed to 10-days SDS in water and RDSW-ingested groups, respectively, exhibited decreased SI ratios ($p<0.001$ and $p<0.01$, respectively), and we judged these 5 mice in both groups as susceptible ones to SDS. On the other hand, the SI ratios of 1 mouse and 3 mice in water and RDSW groups, respectively, were over 100%, meaning these mice were resilient to SDS, and thus 1 resilient mouse in water-ingested group was excluded in the following examinations. There was no difference in the SI ratio between the water- and RDSW-ingesting SDS-susceptible groups.

As shown in **Fig. 1b**, control mice in both the water and RDSW-ingesting groups exhibited a daily gradual increase in body weight during the experimental period, and there was no detectable effect of RDSW ingestion on the body weight gain of control mice. The body weight gain of water-ingesting susceptible mice was significantly ($p<0.001$) greater than that in water-ingesting control ones. RDSW-ingesting susceptible mice exhibited a similar profile in body weight gain, but the level of increase was significantly ($p<0.001$) lower than in the case of water-ingesting susceptible mice. In contrast, the body weight gain of RDSW-ingesting resilient mice was almost equal with that of RDSW-ingesting control ones, while was significantly ($p<0.001$) lower than that of RDSW-ingesting susceptible ones.

During the experimental period, the average food intake was approximately 2.5 g/day in both water- and RDSW-ingesting control mice, and thus, there seemed to be no or negligible effect of RDSW-ingestion on food consumption of mice (**Fig. 1c**). As the previous findings, food consumption in water-ingesting mice was increased

by SDS-exposure, while there was no significant alteration in RDSW-ingesting mice with susceptibility and resiliency to SDS exposure comparing to the RDSW-control ones.

As depicted in **Fig. 1d**, daily water intake was increased by SDS-exposure in both water- and RDSW-ingesting mice, and there was no significant difference in daily water intake between water-susceptible and RDSW-susceptible ones, and RDSW-susceptible and RDSW-resilient ones.

During the SI test under blind conditions, we seemed that some mice might exhibit different behavior, especially exploratory behavior as to target ICR mice. Therefore, we analyzed the mouse behavior in the SI test using the video tracking system in more detail. **Table 1** shows the effect of RDSW ingestion on SDS-induced alteration of sociability-related behavior in mice. There was no apparent alteration in exploratory and escape behavior of control mice caused by RDSW ingestion. In water-ingesting susceptible mice, there was a significant ($p<0.01$) decrease in the numbers of interest behavior under the presence of a target ICR mouse (9.8±3.0 numbers/150 sec) comparing to those under the absence of one (21.0±4.9 numbers/150 sec) (54% decrease), while RDSW-ingesting susceptible mice exhibited a tendency of decrease of the interest behavior (10.8±2.2 and 17.4±2.9 numbers/150 sec under the presence and absence of a target ICR mouse, respectively, 38% decrease). In addition, the duration of an escape behavior under the presence of an ICR mouse was significantly ($p<0.01$) greater in water-ingesting susceptible mice (8.23±5.40 sec/number) than in water-ingesting control ones (2.57±1.00 sec/number), but there was no significant alteration in the duration of an escape behavior between RDSW-control and -susceptible mice (1.96±0.56 and 6.32±3.76 sec/number, respectively).

Feature of gut microbiota

On analysis of the gut microbiota (**Fig. 2a**), the plot profiles of β-diversity were different between control and susceptible mice in both water- and RDSW-ingesting groups (circles and triangles, respectively), while there was no apparent difference in the profiles between water- and RDSW-control groups, and water- and RDSW-susceptible groups (open and closed symbols, respectively). These results indicated that water-ingesting susceptible mice exhibited altered diversity compared with in water-ingesting control ones, while ingestion of RDSW had no detectable effect on the diversity in either control or susceptible mice.

Table 2 shows a comparison of the abundance of bacterial genera in the colonic microbiota in control and susceptible mice with or without RDSW ingestion. The relative abundance of *Bacteroides* spp. was significantly lower in susceptible mice than in control ones in both the water- and RDSW-ingesting groups, while the abundance of *Bacteroides* spp. in RDSW-susceptible mice was significantly higher than in water-susceptible ones ($p<0.001$), and there was no difference in its abundance between RDSW-ingesting control and resilient mice. The abundance of *Bacteroides* spp. was significantly correlated positively with the SI ratios and negatively with the AUC of body weight gain of mice (**Figs. 2b** and **2c**). The relative abundance of *Candidatus Saccharimonas* spp. and *Dubosiella* spp. was significantly greater in both water- and RDSW-ingesting susceptible mice, but the increases in their levels on SDS exposure in RDSW-ingesting mice were less and greater, respectively, than those in water-ingesting ones, and there was no difference in the abundance between RDSW-control and -resilient groups. There was no correlation between the abundance of these bacteria genera and the SI ratios or body weight gain (*data not shown*).

Discussion

Here, we found that (1) *ad libitum* ingestion of RDSW prevented partially, but significantly, the increase of body weight gain and decrease of sociability in SDS-susceptible mice, (2) these alterations in development of depressive-like phenotypes were correlated with those of the abundance of *Bacteroides* spp. in the colonic microbiota, and (3) relative abundance of *Bacteroides* spp. in colonic microbiota was decreased in water- and RDSW-ingesting SDS-susceptible mice, and the level of reduction in the former was greater than in the latter. Collectively, it is suggested that development of depressive-like phenotypes in mice subjected to psychosocial stress depends, at least in part, on abundance of *Bacteroides* spp. in colonic microbiota, and *ad libitum* ingestion of RDSW might ameliorate the development of depressive-like phenotypes in mice due, at least in part, to preventing reduction

of colonic *Bacteroides* spp.

SDS-susceptible mice in the water and RDSW groups exhibited an increase of body weight gain compared to the control groups, this phenotype being a representative characteristic exhibited by SDS-susceptible C57BL/6J mice [14, 17-19]. However, the body weight gain of RDSW-susceptible mice was less than that of water-susceptible ones, despite there being no differences in the daily food consumption and water intake between the two groups. Furthermore, the body weight gain of RDSW-ingested susceptible mice was greater than that of RDSW-ingested resilient ones, nevertheless their food consumption and water intake were almost equal, implying alteration of absorption and metabolism of nutrients *via* gut microbiota in SDS-susceptible mice. *Bacteroides* spp. is a prominent genus of gut microbiota, and is reported to prevent obesity by promoting the catabolism of branched chain amino acid in the brown adipose tissue [20], and we also found that the body weight gain of mice was significantly inversely correlated with the relative abundance of colonic *Bacteroides* spp. In this study, the relative abundance of *Bacteroides* spp. in RDSW-ingesting susceptible mice was significantly lower than in RDSW-ingesting control ones, but was significantly greater than in water-ingesting susceptible ones. Together with no alteration of the relative abundance of *Bacteroides* spp. in RDSW-ingesting resilient mice, *ad libitum* ingestion of RDSW might ameliorate SDS-induced increase of body weight gain through, at least in part, preventing decrease of colonic *Bacteroides* spp.

It is reported that *Bacteroides* spp. play roles in maintenance of gut barrier integrity by regulating the metabolism of complex carbohydrates such as glycans of mucin, a mucus barrier component [21] and inhibition of the generation of proinflammatory cytokines due to low grade inflammation [22]. In this study, we found that there was significant correlation between the relative abundance of *Bacteroides* spp. and SI ratios. Therefore, it is indicated that colonic *Bacteroides* spp. might play a preventive role in development of social disability under conditions of psychosocial stress exposure. In addition, RDSW-ingesting susceptible mice exhibited alleviated decrease of interest behavior and increase of escape behavior comparing to the cases of water-ingesting susceptible ones. These findings suggested the possibility that ingredients of RDSW might have preventive effects on low grade inflammation in the gut. We previously demonstrated that magnesium played a major role in the prophylactic effect of RDSW on the development of experimental colitis, and a postulated underlying mechanism was the blockade of activation of the P2X7 receptor expressed by colonic resident mast cells [13]. In the preliminary examination, we examined the colonic expression levels of CD63, a mast cell marker, CD11b, a macrophage marker, P2X7 receptor, IL-1β and TNF-α, but there were no apparent differences in their levels between control and SDS-susceptible mice (*unpublished observation*), implying that no detectable inflammatory response might occur in the colons of SDS-susceptible mice. Taken together with the aforementioned scenario that undetectable low grade inflammation might be occurred in a psychological stress-induced leaky gut [6], it is speculated that inhibition of P2X7 receptors expressed by gut resident immune cells might be involved in the preventive effect of magnesium as a major constituent in RDSW on the development of social disability, although there is no available information on the mechanisms underlying the psychological stress-induced gut inflammation. RDSW also contains concentrated calcium, sodium and potassium at the levels of 73.8, 60.1 and 57.5 ppm, respectively, while we previously demonstrated that they had no or extremely weak inhibitory effects on P2X7 receptors [11], implying their no or negligible contribution to preventive effects of RDSW on development of depressive-like phenotypes in CSDS-susceptible mice.

The amount of magnesium oxide as an ingredient of the diet AIG-93G is 2.4% (Clea Japan, Inc.), and thus, the mouse ingesting amount of magnesium is calculated to be 72 mg/day, when average daily food consumption of a mouse is 3 g/day (**Fig. 1c**). From RDSW, mice are estimated to ingest 1 mg/day of magnesium, when their average daily water intake is 5 mL/day (**Fig. 1d**), indicating that the magnesium amounts ingested daily by mice are less from RDSW than from the diet. On the other hand, magnesium in RDSW is considered to be a soluble ionic form, while magnesium oxide in the diet is practically insoluble, a little amount of which might be dissolved in gastric acid. Taken together with the finding that magnesium ions exert inhibitory effects on P2X7 receptors [23, 24], and 10-days daily *ad libitum* ingestion of RDSW increased the colonic magnesium levels from 1.5 μg/g wet feces under control conditions to 2.5 μg/g wet feces [13], ingestion of magnesium ions from RDSW is suggested to play major roles in the preventive effects of RDSW on development of depressive-like phenotypes in CSDS-susceptible mice.

It is well-known that ingestion high concentrations of magnesium induce diarrhea, but in the present study, we detected no loose stool and/or diarrhea in RDSW-ingested control and CSDS-subjected mice (*data not shown*), this being considered to be reasonable based on the low magnesium contents in RDSW comparing to those in the diet described above. In addition, magnesium deficiency is potential to associate with development of depression in humans and animals [25], and administration of magnesium supplement with selective serotonin reuptake inhibitors is reported to improve depressive symptoms in patients with major depression disorder [26]. Thus, there is possible involvement of RDSW-containing magnesium in its preventive effects on development of depressive-like phenotypes found in this study, but this possibility seems to be denied, because it is considered that *ad libitum* ingestion of RDSW with low magnesium contents could not restore blood and/or tissue magnesium deficiency under development of depressive-like phenotypes.

Candidatus Saccharimonas spp. exhibited the same alteration profiles in the relative abundance as the case of *Bacteroides* spp. Gu *et al.*, reported that in valproic acid-induced autism rats, the abundance of *Candidatus Saccharimonas* spp. was significantly correlated with autism-like behavior [27]. Since dysbiosis also plays a critical role in the pathogenesis of autism [28-30] and a leaky gut is found in autism patients [31-33], *Candidatus Saccharimonas* spp. might be harmful bacteria in brain psychological disorders *via* dysfunction of microbiota-gut-brain axis.

The diversity of some bacterial genera of colonic microbiota were altered in SDS-susceptible mice, while there were no detectable differences in the features between water- and RDSW-control mice, or between water- and RDSW-susceptible ones. Thus, although there were some alterations in relative composition of bacterial genera, it is indicated that *ad libitum* ingestion of RDSW had no or only a negligible effect on the diversity of colonic microbiota of C57BL/6J mice. As for *Dubosiella* spp., SDS-susceptible mice in both water- and RDSW-ingesting groups had higher abundance of them, but there is no available information on *Dubosiella* spp. in brain psychological disorders, further investigations being necessary on the potential roles of this bacterial genus in the pathogeneses of depressive disorders.

In conclusion, it is suggested that development of depressive-like phenotypes such as increase of body weight gain and decrease of social disability in mice subjected to psychosocial stress depends, at least in part, on abundance of *Bacteroides* spp. in colonic microbiota, and *ad libitum* daily ingestion of ionic forms of magnesium with foods, supplements, mineral water, *etc*. might alleviate development of psychosocial stress-induced depressive-like phenotypes partially through preventing the decrease of colonic *Bacteroides* spp.

Table 1. | Effect of RDSW administration on CSDS-induced decrease of sociability in mice

	Water		RDSW		
	Control (N=5)	Susceptible (N=5)	Control (N=5)	Susceptible (N=5)	Resilient (N=3)
Interest behavior (sec/number)					
under absence of ICR	3.11±1.36	3.35±0.513	3.42±0.85	3.88±0.80	4.57±1.30
under presence of ICR	5.16±1.40[a]	3.54±2.18	4.55±0.54	4.43±1.30	4.72±2.02
Interest behavior (sec/150 sec)					
under absence of ICR	57.8±12.0	69.0±13.0	55.6±14.9	66.8±13.3	67.9±15.9
under presence of ICR	76.2±6.5[a]	31.7±15.1[c,***]	80.5±5.8[b]	46.2±7.8[a,**]	74.9±25.7[†]
Interest behavior (numbers/150 sec)					
under absence of ICR	21.8±9.3	21.0±4.9	16.4±2.6	17.4±2.9	16.0±7.2
under presence of ICR	15.4±3.4	9.8±3.0[b]	17.8±1.6	10.8±2.2	19.0±13.2
Escape behavior (sec/number)					
under absence of ICR	2.04±0.76	2.16±0.53	1.69±0.48	2.47±0.31	2.21±0.75
under presence of ICR	2.57±1.00	8.23±5.40[c,**]	1.96±0.56	6.32±3.76[a]	4.94±4.13
Escape behavior (sec/150 sec)					
under absence of ICR	32.1±5.1	28.4±8.4	35.1±4.7	30.8±6.2	30.0±14.7
under presence of ICR	29.5±5.6	72.2±14.6[c,***]	28.3±7.4	58.4±10.3[c,***]	18.9±17.3[†††]
Escape behavior (numbers/150 sec)					
under absence of ICR	17.4±6.3	13.4±3.7	22.4±7.7	12.8±4.1	14.0±7.9
under presence of ICR	13.4±7.1	12.6±8.8	15.2±4.82	11.6±5.3	3.67±0.58[a]

Interest and escape behaviors were determined based on entering numbers and staying time of mice in interaction and escape zones, respectively, during the SI test. Each value represents the mean±SD (water-control and -susceptible, and RDSW-control and -susceptible: N=5, RDSW-resilient: N=3). [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ (*vs* under absence of ICR in respective control or susceptible group). *$p<0.05$, **$p<0.01$, ***$p<0.001$ (*vs* respective control in control or RDSW group). [†]$p<0.05$, [†††]$p<0.001$ (*vs* RDSW-susceptible group). In interest behavior (sec/number), there was no significant main effect of phenotypes ($F_{(4,72)}=0.892$, $p=0.479$), a significant main effect of target mice ($F_{(1,18)}=4.37$, $p=0.0436$) and no significant interaction between phenotypes and target mice ($F_{(4,72)}=0.883$, $p=0.484$). In interest behavior (sec), there was a significant main effect of phenotypes ($F_{(4,72)}=4.11$, $p=0.0076$), no significant main effect of target mice ($F_{(1,18)}=0.154$, $p=0.697$) and a significant interaction between phenotypes and target mice ($F_{(4,72)}=10.2$, $p<0.001$). In interest behavior (number), there was no significant main effect of phenotypes ($F_{(4,72)}=1.03$, $p=0.403$), a significant main effect of target mice ($F_{(1,18)}=5.80$, $p=0.0212$) and no significant interaction between phenotypes and target mice ($F_{(4,72)}=2.55$, $p=0.0560$). In escape behavior (sec/number), there was a significant main effect of phenotypes ($F_{(4,72)}=3.26$, $p=0.0223$), a significant main effect of target mice ($F_{(1,18)}=13.2$, $p<0.001$) and no significant interaction between phenotypes and target mice ($F_{(4,72)}=2.41$, $p=0.0675$). In escape behavior (sec), there was a significant main effect of phenotypes ($F_{(4,72)}=10.8$, $p<0.001$), a significant main effect of target mice ($F_{(1,18)}=12.5$, $p=0.0011$) and a significant interaction between phenotypes and target mice ($F_{(4,72)}=14.7$, $p<0.001$). In escape behavior (number), there was a significant main effect of phenotypes ($F_{(4,72)}=3.00$, $p=0.0310$), a significant main effect of target mice ($F_{(1,18)}=6.48$, $p=0.0153$) and no significant interaction between phenotypes and target mice ($F_{(4,72)}=0.865$, $p=0.494$).

Table 2. | Comparison of relative abundance of bacterial genera in colonic microbiota in CSDS-exposed mice

	Water		RDSW		
Bacterial genera	Control (N=5)	Susceptible (N=5)	Control (N=5)	Susceptible (N=5)	Resilient (N=3)
Bacteriodes	16.5±2.1	5.02±1.35***	17.5±2.4	8.16±4.32^{***,c}	16.2±0.5††
unidentified Desulfovibrionaceae	12.6±4.3	8.99±4.89	9.47±4.21	7.07±1.95	17.9±5.4†
Faecalibaculum	9.54±8.04	8.72±3.54	12.7±9.0	3.80±2.64	2.54±2.85
Lachnospiraceae NK4A136 group	7.07±1.97	5.71±2.53	7.46±2.16	5.95±1.12	10.2±5.5
Parabacteroides	6.54±1.96	5.81±4.17	5.86±2.72	5.74±4.28	6.05±2.14
unidentified Lachnospiraceae	5.98±1.60	3.98±2.20	4.97±1.32	4.72±1.44	7.51±3.60
unidentified Muribaculaceae	5.49±2.14	7.00±1.91	5.68±1.18	9.11±5.92	3.06±1.78
Mucispirillum	3.99±4.91	2.08±2.76	2.59±2.33	0.853±0.673	1.71±2.80
Lactobacillus	3.92±4.91	3.83±6.53	7.33±5.49	7.42±9.39	3.14±4.46
Bifidobacterium	3.55±3.80	11.1±1.8*	3.60±4.45	10.1±3.8	4.70±3.70
Candidatus Saccharimonas	0.0146±0.0219	11.6±11.6*	0.00408±0.00626	0.704±0.567^{a}	0.129±0.040
Dubosiella	0.00252±0.00564	4.36±3.45	0±0	11.1±6.2^{***,a}	1.92±1.21†

Each value represents the mean±SD (water-control and -susceptible, and RDSW-control and -susceptible: N=5, RDSW-resilient: N=3). *p<0.05, **p<0.01 (*vs* control or RDSW group). ^{a} p<0.05 and ^{c} p<0.001 (*vs* water-susceptible group). $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 (*vs* RDSW-susceptible group). The results of one-way ANOVA were as follows: *Bacteroides*: $F_{(4,72)}$=22.5, p<0.001, significant differences were detected between water-control and -susceptible (p<0.001), RDSW-control and -susceptible (p<0.001), water-control and RDSW-susceptible (p<0.001), and water-susceptible and RDSW-control (p<0.001), *unidentified Desulfovibrionaceae*: $F_{(4,72)}$=3.71, p=0.0227, significant differences were detected between RDSW-susceptible and -resilient (p=0.0176), *Bifidobacterium*: $F_{(4,72)}$=5.03, p=0.0067, significant differences were detected between water-control and -susceptible (p=0.0287), and water-susceptible and RDSW-control (p=0.0300), *Candidatus Saccharimonas*: $F_{(4,72)}$=4.18, p=0.0144, significant differences were detected between water-control and -susceptible (p=0.0274), water-susceptible and RDSW-control (p=0.0272), and water-susceptible and RDSW-susceptible (p=0.0409), and *Dubosiella*: $F_{(4,72)}$=9.29, p<0.001, significant differences were detected between water-control and RDSW-susceptible (p<0.001), RDSW-control and RDSW-susceptible (p<0.001), water-susceptible and RDSW-susceptible (p=0.0387), and RDSW-susceptible and -resilient (p=0.0118).

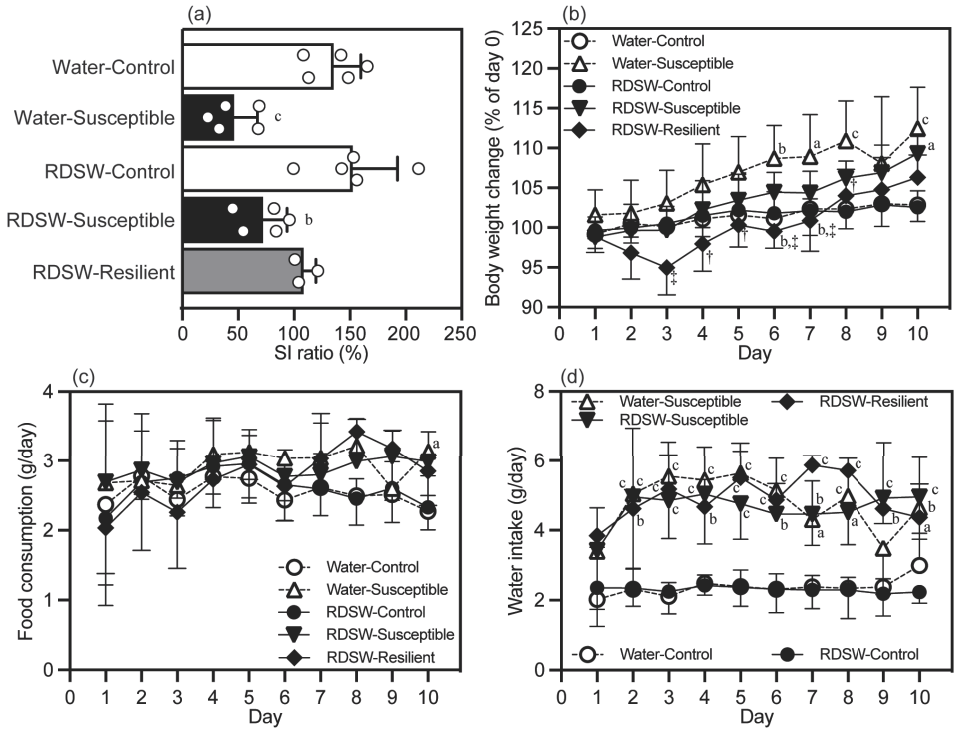

Figure 1. Alteration of SI ratio, body weight gain, food consumption and water intake in SDS-subjected mice.
(a) After 10-days SDS exposure, SI tests were performed to evaluate the sociability of mice. Body weight (b), food consumption (c), and water intake (d) of mice were measured from Day 0 to 10 daily just before SDS-exposure. Body weight changes of mice are shown as percent of Day 0. Each bar/point represents the mean ± SD (water-control and -susceptible, and RDSW-control and -susceptible: N=5, RDSW-resilient: N=3). [a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$, significantly different from the value in the respective control group (a) or in the respective control group on the corresponding day (b-d). † $p<0.05$ and ‡ $p<0.01$ significantly different between water susceptible and RDSW-resilient mice on the corresponding day (b). In panel a, the SI ratio was significantly lower in water- and RDSW-susceptible mice than in water- and RDSW-control ones ($p<0.001$ and $p=0.0012$, respectively). The results of one-way ANOVA for comparison of SI ratios were as follows: $F_{(4,72)}=13.7$ and $p<0.001$, significant differences were detected between water-control and -susceptible mice ($p<0.001$), water-control and RDSW-susceptible ones ($p=0.0106$), water-susceptible and RDSW-control ones ($p<0.001$), RDSW-control and -susceptible ones ($p=0.0012$), and water-susceptible and RDSW-resilient ones ($p=0.0330$). In panel b, the body weight change was significantly greater in water-susceptible mice than in water-control, and RDSW-control, -susceptible and -resilient ones ($p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively), significantly greater in RDSW-susceptible ones than in RDSW-control ones ($p=0.0222$), and water-control ones ($p=0.0136$), and significantly less in RDSW-resilient ones than in RDSW-susceptible ones ($p<0.001$) (a significant main effect of phenotypes ($F_{(4,72)}=27.1$, $p<0.001$), a significant main effect of day ($F_{(9,162)}=13.1$, $p<0.001$), and no significant interaction between phenotypes and day ($F_{(36,162)}=1.06$, $p=0.390$)). In panel c, the food consumption was significantly greater in water-susceptible mice than in water-control ones ($p=0.0024$) and greater in RDSW-susceptible ones than in water-control ones ($p=0.0087$), but there was no significant difference in food consumption between RDSW-control and -susceptible ones ($p=0.0609$), RDSW-susceptible and -resilient ones ($p=0.821$), and water- and RDSW-susceptible ones ($p=0.996$) (a significant main effect of phenotypes ($F_{(4,72)}=5,28$, $p<0.001$), a significant main effect of day ($F_{(9,162)}=2.47$, $p=0.0110$), and no significant interaction between phenotypes and day ($F_{(36,162)}=0.722$, $p=0.876$)). In panel d, the water intake was significantly greater in water-susceptible, RDSW-susceptible and RDSW-resilient mice than in the respective control ones ($p<0.001$, $p<0.001$, $p<0.001$ and $p<0.001$, respectively), significantly greater in water susceptible ones than in RDSW-control ones ($p<0.001$), and greater in RDSW-susceptible and -resilient ones than in water-control ones ($p<0.001$ and $p<0.001$, respectively), but there was no significant difference in water intake between water- and RDSW-susceptible ones ($p=0.961$) (a significant main effect of susceptibility ($F_{(4,72)}=97.1$, $p<0.001$), a significant main effect of day ($F_{(9,162)}=2.63$, $p=0.0070$), and no significant interaction between susceptibility and day ($F_{(36,162)}=0.992$, $p=0.490$)).

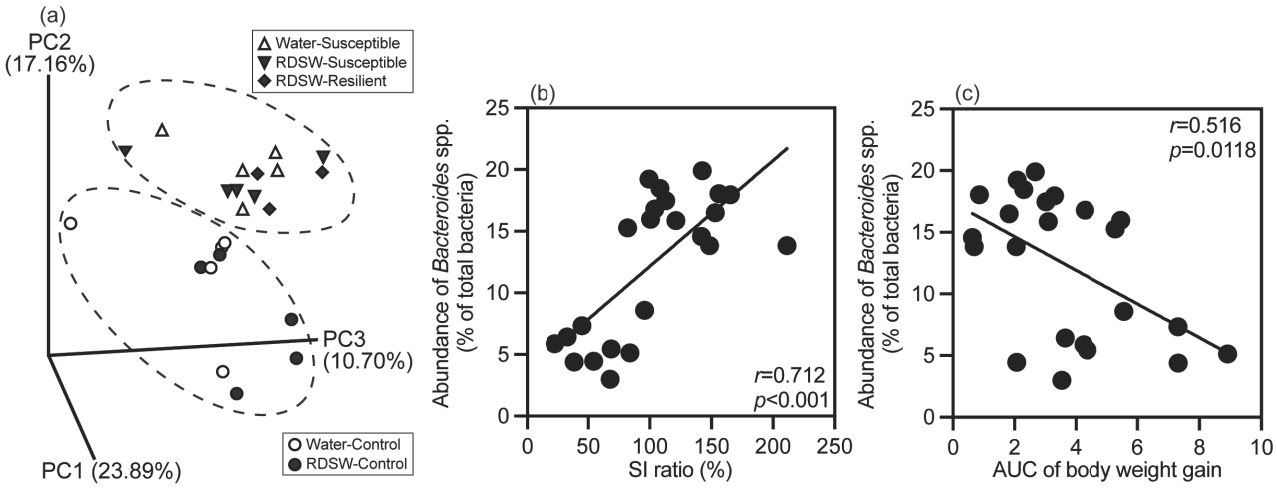

Figure 2. Effects of RDSW ingestion on feature of colonic microbiota in control and SDS-susceptible mice.
Using colonic feces of mice obtained on Day 10, the β-diversity of the colonic microbiota was examined by unweighted Unifrac distance principal coordinate analyses, based on the results of 16S rRNA metagenome analysis (a). The number of analyzed mice was 5 in water-control, water-susceptible, RDSW-control, RDSW-susceptible groups, and 3 in RDSW-resilient one. Panels B and C show the correlation of relative abundance of *Bacteroides* spp. in colonic microbiota with SI ratios (b) and AUC of body weight gain (c). The numbers analyzed were 23 mice from water-control, water-susceptible, RDSW-control, RDSW-susceptible and RDSW-resilient groups, *r* and *p* values being given in each panel.

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Data availability
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contribution
Conceptualization: Y.U., and K.N.; methodology: T.Y., and K.N.; investigation: W.Y., Y.T., H.M., and T.Y.; validation: W.Y., Y.T., K.M., K.T., T.Y., and K.N.; formal analysis: W.Y., Y.T., H.M., and T.Y.; writing-original draft: K.N.; review and editing: K.M., K.T., T.Y., Y.U., and K.N.; funding acquisition: K.M., K.T., Y.U., and K.N.; supervision: Y.U., and K.N.

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