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Effects of semi-purified diet on depressive behaviors in aged mice

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ABSTRACT

Diet is a key modifiable factor influencing the composition of gut microbiota. There are two types of commercially available diets for experimental animals: non-purified and semi-purified diets. Non-purified diets are composed of complex ingredients from multiple sources, while semi-purified diets are formulated with refined ingredients. Accumulating evidence has demonstrated a link between the gut microbiota and depression, and feed ingredients may influence depressive physiology and behaviors. To test this hypothesis, we examined how chronic non-purified (CRF-1) and semi-purified (AIN-93G) diets affected phenotypes, including depressive behaviors, plasma corticosterone levels, and small-intestine microbiota in young (2 months old) and aged (22 months old) inbred C57BL/JJcl mice. In young mice, similar phenotypes were associated with non-purified and semi-purified diets. However, in aged mice, semi-purified diets increased depressive behaviors in the tail suspension ($P < 0.05$) and forced swimming tests ($P < 0.01$). The corticosterone levels were similar between the two diets under normal rearing conditions. However, immediately after exposure to the stressful conditions of the forced swimming test, the corticosterone levels in the aged mice fed the semi-purified diet were higher than those of mice fed the non-purified diet ($P < 0.05$). There were fewer *Lactobacillales* in the small intestines of aged mice fed the semi-purified diet compared to those fed the non-purified diet ($P < 0.01$). Further, α-diversity was lower in aged mice fed the semi-purified versus non-purified diet ($P < 0.01$). Our results indicate that host physiology and gut microbiota differed according to whether the aged mice were fed a non-purified or semi-purified diet. Specifically, those fed the semi-purified diet were more vulnerable to stress than age-matched mice fed the non-purified diet. Our findings indicate that researchers should consider the effects of feed ingredients on depressive physiology and behaviors, and select diets that are appropriate for their particular research design. Further, identification of the ingredients in non-purified diets could facilitate examination of the mechanisms by which gut microbiota composition might increase resistance to stress and depression.

1. Introduction

Elderly people, especially those over 80 years of age, are the fastest growing segment of the world's population. The physical and mental consequences of depression complicate other health conditions [1], which has important implications for clinical management as well as basic research. Aging is a complicated phenomenon [2], strongly affected by genetics and husbandry factors such as diet, water, housing, enrichment, cage density, room size, and noise level [3]. However, few studies have systematically investigated the effects of these husbandry factors on depressive phenotypes in aging mice.

Experimental animals are fed various commercially available feeds. Non-purified diets have complex ingredients including phytoestrogens, such as isoflavones, which are structurally and functionally similar to mammalian estrogens [4]. Among groups of 2-month-old female rats, those fed non-purified diets (TD8728C; Harlan Teklad, WI, USA) exhibited altered estrous cycles, decreased body weight (BW) gain, and higher basal urinary corticosterone (CORT) levels compared to those fed semi-purified (AIN-93G; Harlan Teklad) diets [5]. Ovarian hormonal fluctuations have been connected with the incidence of depression [6,7], and higher CORT levels have been associated with the development of depression [8]. These results suggest that diet type is an important factor in depression research. Further, the reliability of behavioral data obtained using model animals has been questioned in recent years [9]. Although diet type influences the reproducibility of research using animal models [9], little information is available regarding the most suitable diets for depression research.

Feed ingredients can affect animal health [10]. Fiber, which is critical to the gut microbiota [11], has two forms: soluble and insoluble. Non-purified diets typically contain both soluble and insoluble fiber,

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from multiple sources. In contrast, semi-purified diets contain soluble fibers. Gut bacteria ferment soluble fiber, whereas insoluble fiber is largely not fermentable, and release short-chain fatty acids (SCFAs) [12]. SCFAs help reduce the amount of host-harmful intestinal bacteria in the gut by changing the gut pH [13].

In the last decade, a strong association between microbiota changes (dysbiosis) and host diseases has been uncovered [14]. Disruption of the composition of intestinal microbiota can cause depression [15–17]. A diet with high microbiota content, such as probiotics or fermented foods, has been shown to assist recovering of the balance of intestinal microbiota [18]. Diets containing probiotics [18–23], soluble fiber [18], and fermented protein [18,24] play a role in reducing depressive behavior. Bidirectional communication exists between the gut and brain (gut--brain axis) [17], and gut microbiota regulate depressive behaviors through the glucocorticoid receptor pathway [25]. These results indicate that alterations in gut microbiota-derived metabolites can lead to depressive-like behavior through changes in activity of the gut-brain axis. Physiological elements of depression are regulated not only by the gut-brain axis, but also by the hypothalamic-pituitary-adrenocortical (HPA) axis [26]. Recent studies have shown a relationship between dysregulation of the stress-induced activation of the HPA axis and alterations in gut microbiota composition in patients with mood disorders and psychosis [27], as well as depressive mice [28]. Dysregulation of the HPA axis can lead to neuroendocrine responses, such as CORT secretion [29]. CORT, as a major product of the HPA axis, stimulates adaptation and recovery from stress, and restores homeostasis following exposure to stressors [30]. However, long-term stimulation of the HPA axis and corresponding increases in CORT levels can impact psychological function, alter the composition of microbiota [27], and lead to adverse health consequences [31]. It is not yet clear whether the main cause of depression is altered gut microbiota or dysregulated gut-brain axis, or dysregulated HPA axis.

Diet is a key modifiable factor influencing the composition of gut microbiota. The above-mentioned studies suggest that feed ingredients may be linked to depressive physiology and behaviors. As a first step in examining this hypothesis, we evaluated the effects of non- and semi-purified diets on BW gain, depression, plasma CORT levels, and small-intestine microbiota in young and aged C57BL/JJcl (B6) male mice. We found that diet type significantly affected stress sensitivity and gut microbiota composition in older mice.

2. Materials and methods

2.1. Animals

Three-week-old male non-sibling C57BL/JJcl (B6) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). We used males because a previous study reported that a non-purified diet influenced CORT levels in female mice without stress exposure [5]. Immediately after arrival at the experimental facility, forty mice were randomly divided into the following four groups (n = 10 per group): young mice fed CRF-1 (Y-CRF); young mice fed AIN-93G (Y-AIN); aged mice fed CRF-1 (A-CRF); and aged mice fed AIN-93G (A-AIN). They were given either irradiated non-purified pellet food (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) or irradiated semi-purified pellet food (AIN-93G; Oriental Yeast Co. Ltd.) (Supplemental Table 1) and kept in group-housed cages (MBS7115RHMV, 19.1 × 29.2 × 12.7 cm; Allentown Caging, Allentown, NJ, USA) with paper chips for bedding (Paper Clean; Japan SLC, Inc., Shizuoka, Japan). The mice had unlimited access to water and food, and were housed in a room maintained at a temperature of 23 ± 2 °C under a 12/12-h light/dark cycle (lights on at 08:00 until 20:00). Young (2 months old) and aged (22 months old) mice were used in this study. We conducted the animal experiments at HAMRI Co. Ltd. (Ibaraki, Japan) after obtaining approval (Approval number: 14-H116) from the Institutional Animal Care and Use Committee of HAMRI Co. Ltd. We analyzed the data at Kyushu University.

2.2. Behavioral study

All behavioral analyses were performed by a well-trained, blinded researcher between 10:00 and 14:00. The mice were moved into the behavioral testing room at least 1 h before testing. Fig. 1 shows the experimental design. After each test, the apparatuses were cleaned with water and hypochlorous acid to eliminate any olfactory cues. The behavioral study was conducted after the young mice had been fed the non-purified or semi-purified food for 5 weeks; aged mice had received the non-purified or semi-purified food for 85 weeks.

2.2.1. Body weight measurements

Body weight (BW) gain was calculated by subtracting the weight on the day of arrival at the experimental facility from the weight 1 day before the experiment (Day 0).

2.2.2. Locomotor activity test

The mice were subjected to a locomotor activity test (LAT) on Days 1 and 6. Locomotor activity was monitored for 1 h using the LOCOMO LS-5 photobeam interruption sensor (Melquest, Toyama, Japan) while the mouse was housed in a transparent cage (CL-0104-1, 22.5 × 33.8 × 14.0 cm; CLEA Japan, Inc.) [32]. A light intensity of 150 lx was maintained in the experimental room. The number of beam breaks was counted in accordance with the manufacturer's instructions for evaluating locomotor activity.

2.2.3. Tail suspension test

The mice were subjected to a tail suspension test (TST) on Day 3. Each mouse was suspended from a 4-cm hook, 21 cm from the floor of an opaque compartment (15.0 × 16.0 × 25.0 cm), using adhesive tape placed 2 cm from the tail end. A light intensity of 150 lx was maintained. Seven-minute videos were recorded and the total amount of time (s) spent immobile was measured between minutes 2 and 7 [33].

2.2.4. Forced swimming test

The mice were subjected to a forced swimming test (FST) on Day 5. Each mouse was placed in a 19-cm glass cylinder (diameter = 11.0 cm) filled with water up to 13 cm (temperature = 24 ± 1 °C). A mouse was deemed immobile if it floated via small movements of the forelimbs but kept the hindlimbs immobile. A light intensity of 150 lx was maintained.

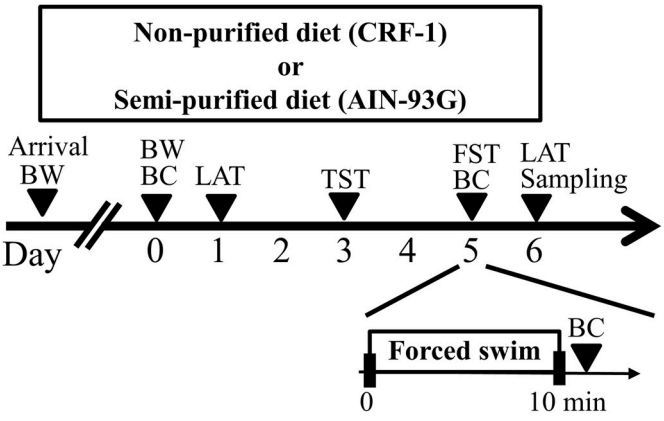

Fig. 1. Schematic diagram of the experimental procedures. Mice (n = 10 per group) were subjected to body weight (BW) measurement on the day of arrival at the experimental facility and on Day 0, blood collection (BC) on Day 0, and behavioral tests including the locomotor activity test (LAT) on Days 1 and 6, the tail suspension test (TST) on Day 3, and the forced swimming test (FST) on Day 5. Blood was also collected on Day 5, immediately after the FST. Small-intestine samples were collected after euthanasia on Day 6.

Seven-minute videos were recorded and the total amount of time (s) spent immobile was measured between minutes 2 and 7 [33].

2.3. Corticosterone assay

A day before the experiment (Day 0), approximately 0.1 mL blood was collected from the facial or submandibular vein into tubes containing 10 units of sodium heparin (Wako Pure Chemical Industries Ltd., Osaka, Japan) using Goldenrod Animal Lancets (Medipoint, Inc., Mineola, NY, USA). To investigate the effects of stress on plasma CORT levels, blood samples were obtained immediately after FST on Day 5. Blood was collected between 14:00 and 15:00 at the same time on Days 0 and 5 to avoid effects of circadian changes on CORT [34]. Plasma CORT levels were measured using the Corticosterone EIA Kit (Enzo Life Sciences, Inc., PA, USA) in accordance with the manufacturer's instructions.

2.4. Bacteriological analysis

Bacterial analysis of the small intestines was performed using the terminal restriction fragment length polymorphism (T-RFLP) method [35]. The small intestines were sampled between 13:00 and 15:00. The mice (Y-CRF, Y-AIN, A-CRF, A-AIN groups; n = 10 per group) were anesthetized with isoflurane (Pfizer Japan, Tokyo, Japan; 5% induction, 2% maintenance inhaled until euthanized). Immediately after euthanasia, the small intestines were quickly and aseptically removed (intact) and placed on a sterile Petri plate using sterile instruments. The contents of the small intestines were collected into DNase and RNase-free tubes by manually massaging the intestine. Immediately after collection, the tubes were flash-frozen in liquid nitrogen and stored at −80 °C for further analysis. Total DNA was isolated from the contents using a commercial kit (Fast DNA spin kit; MO Bio Laboratories, CA, USA). The amplified polymerase chain reaction products of the 16S rRNA gene were digested with the *Bsl*I (New England Bio Labs, Beverly, MA, USA) using primers of 516f (5′-TGCCAGCAGCCGCGGTA-3′; *Escherichia coli* positions, 516–532) and 1510r (5′-GGTTACCTTGTTACGACTT-3′; *E. coli* positions, 1510-1492). The digested fragments were profiled using the ABI PRISM 3130x1 DNA Sequencer and GeneMapper (Applied Biosystems LLC, Foster City, CA, USA), and divided into groups according to operational taxonomic units (OTUs). The groups were distinguished according to the percentages of individual OTUs per total OTU area, expressed as the peak percent area under the curve, and analyzed using GeneMaths (Applied Maths, Sint-Martens-Latem, Belgium). The Shannon diversity index was calculated as the microbial α-diversity index, according to the distribution of all obtained OTUs.

2.5. Data analysis

Data are presented as means ± standard errors. All statistical analyses were performed using GraphPad Prism software (version 9.1.2; GraphPad Software Inc., La Jolla, CA, USA). Data were analyzed using analysis of variance (ANOVA), followed by the Bonferroni correction post hoc test for multiple comparisons between groups. A p-value < 0.05 was considered significant.

3. Results

We used a set of depressive behavioral tests to investigate the effects of age on mice fed with different diets. We also investigated the effects of different diets on plasma CORT levels and small-intestine microbiota in young and aged mice. There was no hair loss, emaciation, or apparent abnormal behaviors (stereotyped behavior, aggressivity, immobility) during daily observations.

3.1. BW gain

The BW gain did not differ significantly among the Y-CRF (12.2 ± 0.83 g), Y-AIN (12.1 ± 0.52 g), A-CRF (22.0 ± 1.13 g), and A-ANI (21.7 ± 1.28 g) groups [diet × age interaction (F1,9 = 0.02, $P > 0.05$)].

3.2. Behavioral tests

In the LAT, no significant differences were found in the integrated counts among the Y-CRF group on Day 1 (136.1 ± 10.13 counts), Y-AIN on Day 1 (138.5 ± 10.70 counts), A-CRF on Day 1 (135.4 ± 14.28 counts), A-ANI on Day 1 (139.5 ± 11.06 counts), Y-CRF on Day 6 (129.1 ± 9.03 counts), Y-AIN on Day 6 (130.4 ± 10.32 counts), A-CRF on Day 6 (128.9 ± 13.87 counts), and A-ANI on Day 6 (131.3 ± 10.94 counts) [diet × age interaction (F(3, 27) = 0.006, $P > 0.05$].

In the TST (Fig. 2A), we observed significant differences in immobility time among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 10.57, $P < 0.01$]. The immobility time was significantly greater in the A-AIN compared to Y-AIN group ($P < 0.01$) and A-AIN compared to A-CRF group ($P < 0.05$), but there was no significant difference between the Y-CRF and Y-AIN groups.

In the FST (Fig. 2B), we observed significant differences in immobility time among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 21.09, $P < 0.01$]. The immobility time was similar between the Y-CRF and Y-AIN groups, but was significantly longer in the A-AIN compared to Y-AIN group ($P < 0.01$) and A-AIN compared to A-CRF group ($P < 0.01$).

3.3. CORT assay

The CORT assay, performed on Day 0, revealed no significant differences among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 0.09, $P > 0.05$] (Fig. 3A). To investigate the effects of stress on CORT levels, blood samples were collected immediately after the FST (Fig. 3B). The post-FST CORT assay showed significant differences among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 9.46, $P < 0.05$]. CORT levels were similar between the Y-CRF and Y-AIN groups. However, CORT levels were significantly higher in the A-AIN compared to Y-AIN group ($P < 0.05$) and A-AIN compared to A-CRF group ($P < 0.05$).

3.4. Small-intestine microbiota

The microbiota composition was characterized on the basis of fragment patterns analyzed by T-RFLP. The dominant microbiota in the small intestines were *Lactobacillales* (Table 1). There were significant differences in *Lactobacillales* quantities among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 16.21, $P < 0.01$]. The Y-CRF and Y-AIN groups had similar quantities of *Lactobacillales*, but the A-AIN group had a significantly lower quantity of *Lactobacillales* compared to the A-CRF group ($P < 0.01$) and Y-AIN group ($P < 0.01$). There were significant differences in α-diversity among the Y-CRF, Y-AIN, A-CRF, and A-ANI groups [diet × age interaction, F(1, 9) = 12.48, $P < 0.01$] (Fig. 4). The A-AIN group had significantly lower α-diversity compared to the A-CRF group ($P < 0.01$) and Y-AIN group ($P < 0.01$).

4. Discussion

Depression is a serious problem in the aging population worldwide [36]. Depression is a mood disorder characterized by persistent feelings of melancholy, apathy, and indifference. It may lead to suicide in severe cases. Currently, there is no reliable cure for this disorder, but there are a few strategies that can help to manage the symptoms. These strategies include pharmacological and non-pharmacological approaches. Diet is one of the non-pharmacological approaches for managing depression [37]. Imbalances in intestinal microbiota can lead to the development of

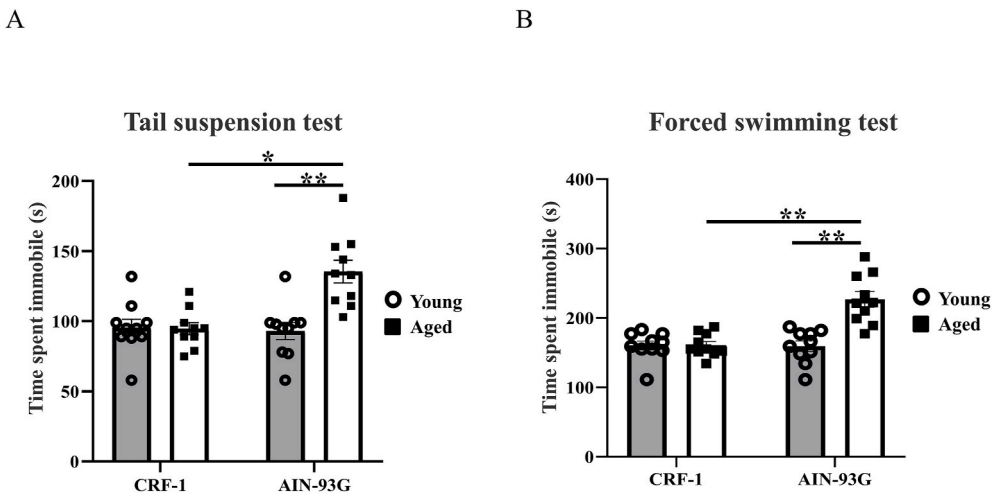

Fig. 2. Effects of the CRF-1 and AIN-93G diets on immobility times (s) in the TST (A) and FST (B). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01.

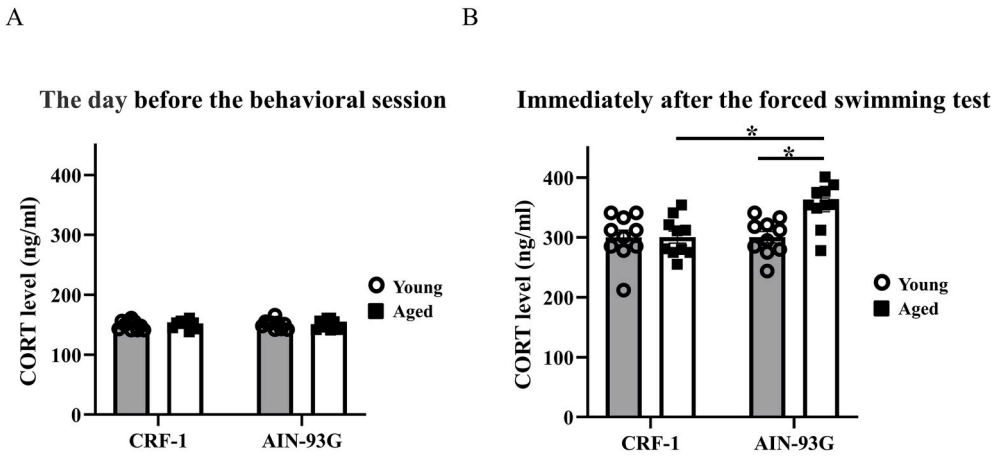

Fig. 3. Effects of the CRF-1 and AIN-93G diets on plasma CORT levels 1 day before behavioral testing (A) and immediately after the FST (B). Data are presented as mean ± SEM. *P < 0.05.

Table 1
Comparison of small-intestine microbiota using terminal restriction fragment length polymorphism (T-RFLP) analysis.

	Young mice		Aged mice	
	CRF-1 (Y-CRF)	AIN-93G (Y-AIN)	CRF-1 (A-CRF)	AIN-93G (A-AIN)
Bifidobacteriales	2.1 ± 0.23	2.0 ± 0.28	2.0 ± 0.25	2.2 ± 0.45
Lactobacillales	54.3 ± 1.13	55.6 ± 0.99	50.4 ± 2.35	40.3 ± 2.65 **,††
Bacteroides	4.7 ± 0.50	5.0 ± 0.17	4.8 ± 0.52	4.9 ± 0.45
Prevotella	4.8 ± 0.40	4.8 ± 0.32	4.8 ± 0.55	4.9 ± 0.56
Clostridium cluster IV	0.1 ± 0.12	0.1 ± 0.01	0.1 ± 0.10	0.1 ± 0.01
Clostridium subcluster XIVa	2.6 ± 0.63	2.9 ± 2.65	2.7 ± 0.67	3.1 ± 2.69
Clostridium cluster XI	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01
Clostridium cluster XVIII	2.3 ± 1.45	2.3 ± 1.32	2.7 ± 1.17	2.6 ± 1.13
Others	29.9 ± 8.59	27.2 ± 9.58	32.7 ± 9.88	42.7 ± 9.52

Data are presented as mean ± SEM, **P < 0.01 (A-AIN vs A-CRF), ††P < 0.01 (A-AIN vs Y-AIN).

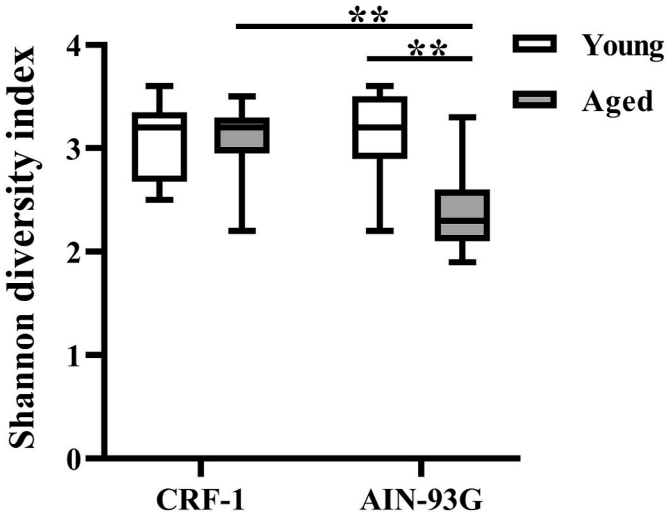

Fig. 4. Box plots showing Shannon diversity. Data are presented as mean ± SEM. **P < 0.01.

seemingly unrelated neurological disorders, such as abnormal CORT release and depression [38]. Different diets have been used in investigations of depression in model mice. However, few studies have

systematically investigated the effects of diet on the gut microbiota. In this study, we investigated the influence of diet on aging, depressive, model mice. We tested two widely used non-purified and semi-purified commercial diets in young and aged inbred mouse strains, to determine their effects on depressive behaviors, plasma CORT levels, and small-intestine microbiota. This study aimed to expand current knowledge of the effects of diet on depression in old age, and to develop reliable and reproducible animal experimental methods. Researchers have often used experimental diets based on personal judgment, because no standard diet has been developed for research on aging, depressive model animals.

The BW gain and locomotor activities were similar between the CRF and AIN groups. Although AIN (359 kcal/100 g) has a slightly higher caloric content than CRF (347 kcal/100 g) according to data from ORIENTAL YEAST Co., Ltd., our results suggest that basal energy metabolism and consequent BW gain was similar between both diets in B6 mice. The TST and FST have commonly been used in animal studies of depression. Immobility during the tests can be interpreted as loss of motivation or "behavioral despair", due to the inability of the animals to escape from the uncomfortable situation. There were no differences in immobility between young mice fed with CRF and AIN, on either the FST or TST. However, aged mice fed with AIN had significantly greater immobility compared to aged mice fed with CRF. There were 6 days on which the mice received the experimental diets between the LAT and FST. That there were no differences in locomotor activity between Days 1 and 6 in all groups indicates that the timing of the tests (2 days apart) did not affect the results, and that motor function was not a confounding factor in the TST or FST.

The CORT levels were similar between the CRF and AIN groups before the behavioral session, and during chronic feeding under normal rearing conditions. However, the CORT levels immediately after stress were influenced by the diets. These changes in CORT levels were reflected in the depressive behaviors of mice. Greater CORT elevation in response to stress was reported in germ-free mice compared to specific-pathogen-free mice [39], indicating an association between stress and the gut microbiota, mediated by dietary conditions.

Lactobacillales has been reported to constitute 30–60% of small-intestine bacteria in rodent models [40] in both CRF- and AIN-fed mice. In this study, *Lactobacillales* quantities were similar between the Y-CRF and Y-AIN groups, but levels were significantly lower in the A-AIN group compared to the A-CRF group. *Lactobacillales* has been shown to improve symptoms of depression in probiotic studies [41,42]. These results indicate that vulnerability to stress in aged mice fed with AIN may be related to decreased quantities of *Lactobacillales*. Lower α-diversity was reported in patients with depression compared to healthy controls [43]. Our results also revealed lower α-diversity in the A-AIN compared to A-CRF group. Thus, long-term dietary differences may influence metabolism via the alteration of microbiota composition. The gut-brain axis and HPA axis coordinate the adaptive responses of organisms to stressors [14,44,45] under various physiological and homeostatic conditions. Accumulated biological changes induced by gut microbiota may modulate the threshold of CORT release, as well as depressive behaviors via dysregulation of the gut-brain axis and HPA axis. Non-purified diets contain unknown ingredients that are available to the gut microbiota and change the gut environment with advancing age. Thus, aged mice fed non-purified diets might be more resistant to stress than age-matched mice fed a semi-purified diet.

Although our results showed that levels of Bacteroidetes were similarly abundant among groups, Bacteroidetes levels were significantly higher in 8-month-old C57BL/JJmsSLc mice following 6 months of a non-purified diet (MF; ORIENTAL YEAST Co., Ltd.) compared to a semi-purified diet (AIN-98G) [46], suggesting that dietary conditions alter gut microbiota even in C57BL substrains. Screening of natural ingredients in non-purified foods is important for the identification of novel compounds that alter gut microbiota. Further studies are needed to screen ingredients, and next-generation sequencing should be used to investigate differences in the gut microbiota.

In conclusion, the present study showed that long-term dietary differences influenced host gut microbiota. To ensure experimental reproducibility and minimize biases, researchers should be aware of the differences between diets, select appropriate diets for their particular research, and share data on the microbiota composition with the scientific community. In the future, journals may require a description of the composition of the microbiota of laboratory animals used in research.

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Role of the funding source

The funding source had no role in experimental design and execution, data analysis, and decision to submit results.

Authors' contributions

Eiki Takahashi: Conceptualization, Investigation, Methodology, Data curation, Visualization, Validation, Formal analysis, Funding acquisition, Resources, Project administration, Writing- Original draft preparation. Etsuro Ono: Supervision, Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101152.

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