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Journal of Nutritional Biochemistry 78 (2020) 108326

Journal of Nutritional Biochemistry

Carbohydrate-restricted diet alters the gut microbiota, promotes senescence and shortens the life span in senescence-accelerated prone mice☆

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Received 30 May 2019; received in revised form 8 October 2019; accepted 17 December 2019

Abstract

This study examined the effects of a carbohydrate-restricted diet on aging, brain function, intestinal bacteria and the life span to determine long-term carbohydrate-restriction effects on the aging process in senescence-accelerated prone mice (SAMP8). Three-week-old male SAMP8 were divided into three groups after a week of preliminary feeding. One group was given a controlled diet, while the others fed on high-fat and carbohydrate-restricted diets, respectively. The mice in each group were further divided into two subgroups, of which one was the longevity measurement group. The other groups fed *ad libitum* until the mice were 50 weeks old. Before the test period termination, passive avoidance test evaluated the learning and memory abilities. Following the test period, serum and various mice organs were obtained and submitted for analysis. The carbohydrate-restricted diet group exhibited significant decrease in the survival rate as compared to the other two diet groups. The passive avoidance test revealed a remarkable decrease in the learning and memory ability of carbohydrate-restricted diet group as compared to the control-diet group. Measurement of lipid peroxide level in tissues displayed a marked increase in the brain and spleen of carbohydrate-restricted diet group than the control-diet and high-fat diet groups. Furthermore, notable serum IL-6 and IL-1β level (inflammation indicators) elevations, decrease in *Enterobacteria* (with anti-inflammatory action), increase in inflammation-inducing *Enterobacteria* and lowering of short-chain fatty acids levels in cecum were observed in the carbohydrate-restricted diet group. Hence, carbohydrate-restricted diet was revealed to promote aging and shortening of life in SAMP8.

Keywords: Aging; Carbohydrate-restricted diet; Gut microbiota; Senescence-accelerated mice; Short-chain fatty acids

1. Introduction

Senescence, an age-dependent phenomenon, involves declining functions across various systems. To attend the call for delayed senescence and quality of life for the elderly, extensive research based on food(s) and diet(s) is under way. Among these is the Japanese diet, which is considered to be one of the factors responsible for longevity in Japanese people. We have conducted various studies to prove Japanese diet's health benefits, including an experiment on rats, which revealed that the Japanese diet was highly beneficial as compared to the American diet [1].

However, over the past 50 years, Japanese diet has become more westernized and has changed greatly [2]; also, the incidence of lifestyle-related diseases has increased [3]. For specifically determining the beneficial effects of Japanese diet, we recreated the diets of the years 1960, 1975, 1990 and 2005 under the guidance of a head dietitian. The foods were freeze-dried and ground into a powder to prepare the experimental feed for mice. The results demonstrated a large decrease in the visceral fat in the group that fed on the 1975 Japanese diet, wherein carbohydrate and lipid metabolism activation was the underlying mechanism [4]. When these diets were administered to senescence-accelerated prone mice (SAMP8), the 1975 Japanese diet prolonged the life span and delayed the onset of aging-related diseases [5, 6].

Through these experiments, we had demonstrated Japanese diet of the year 1975 to be highly health beneficial. We conducted a study to further determine the beneficial effects of this diet in humans. The results revealed that Japanese diet (1975) influenced nutriment and led to decreased visceral fat amount(s) in healthy or mildly obese subjects as compared to the contemporary diet [7–9], thus validating our hypothesis. The 1975 diet possesses five main characteristics [7, 8, 10]: (1) variety: the diet included various ingredients; (2) cooking methods: simmering (niru) played a large part in the 1975 diet; (3) ingredients: soy products, fish, shellfish, vegetables, fruits, green tea,

☆ This research was supported by the project grant of the NARO Bio-oriented Technology Research Advancement Institution (Advanced integration research for agriculture and interdisciplinary fields).

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https://doi.org/10.1016/j.jnutbio.2019.108326
0955-2863/

seaweed and mushrooms were commonly used; (4) seasoning(s): *dashi* stock and fermented seasoning [soy sauce, miso (fermented bean paste), vinegar, mirin (sweet rice wine), and *sake* (rice wine)] were often used; and (5) format: the *rice-and-soup set* was predominant in the 1975 diet. This indicated rice (staple food) as the centerpiece of the meal, accompanied by a soup and small portions of side dishes (okazu) of various ingredients.

Meanwhile, another type of diet that limits carbohydrates and replaces them with protein (without changing the overall portion size) is known. Such low-carbohydrate, high-protein diet, known as the carbohydrate-restricted diet, is in contrast to the carbohydrate-centric meals (typical of Japanese diet). Carbohydrate-restricted diets have been reported to reduce visceral fat; due to this, they have attracted attention and have been widely implemented [11]. However, long-term carbohydrate restriction in mice was reported to pose a negative effect on their cardiovascular system and induce aging disease [12, 13]. Recently, we have demonstrated that long-term ingestion of a carbohydrate-restricted diet promotes skin senescence [14]. Therefore, the safety of long-term adherence to carbohydrate-restricted diets appears doubtful.

Of late, as observed in humans and mice, gut microbiota alteration (aging-induced change) has drawn attention. In a recent study, the gut microbiota of healthy Japanese people (0–104 years) was analyzed by using a next-generation sequencer [15]. The results revealed a difference in the gut microbiota of young and elderly people. In particular, bifidobacteria decreased, whereas the disease-related microbes increased, in elderly than in young people. In addition, the alteration of gut microbiota correlated with certain biochemical parameters [16, 17]. The results suggested a close relation between the attenuation of vital function(s) and gut microbiota alteration(s) with aging. It is known that diet can greatly affect gut microbiota. For example, ingesting various carbohydrates increased *Lactobacillus* and bifidobacteria (beneficial for hosts) and decreased the disease-related microbes [18]. Moreover, the gut microbiota differs among elderly people due to their diet differences; in particular, those consuming more carbohydrates have well-balanced gut microbiota [19]. This suggested that the gut microbiota undergoes a change (apart from age dependency) due to the difference in diet(s). The carbohydrates used in these trials include dietary fiber. Since much research has been performed on dietary fiber, we were interested in the effect carbohydrates without dietary fiber. Therefore, we conducted a study using SAMP8 to examine carbohydrate (without dietary fiber)-restricted diet's effect(s) on the life span, gut microbiota and brain function to determine the outcome of long-term carbohydrate restriction on the aging process. SAM were developed by Kyoto University, Japan (1981) [20], and different strains indicating various signs of aging (SAMP1, P2, P3, P6, P7, P8, P9 and P10) exist [21]. After a normal growth process, SAMP8 underwent rapid senescence past 24 weeks of age. In addition, they exhibited pathologies such as learning disabilities and memory disorders [22, 23]. Their life span is of 48 weeks; therefore, they are widely used in studies based on the relationship between senescence and dietary composition [24–27]. In this study, we measured the life span and the learning and memory ability (passive avoidance test) to evaluate the progression of brain aging. Therefore, the aging markers for each tissue (lipid peroxides), serum and liver biochemical parameter concentrations, gut microbiota changes and the amount of short-chain fatty acids (SCFAs) in the cecum were measured.

2. Methods

2.1. Animals and diets

All animal procedures were performed in accordance with the Animal Experiment Guidelines of Tohoku University, Japan; and the animal protocol was approved by the Animal Use Committee at Tohoku University, Japan (2017AgA-015) [28, 29]. SAMP8 (3 weeks old, male; Japan SLC, Inc., Japan) were fed the standard laboratory chow [CE-2 (powdered); CLEA Japan Inc., Tokyo, Japan] for a week before the study. Further, they were randomly divided into 3 groups of 40 mice each such that the average weights of mice in the 3 groups were nearly equal. The mice had the access to their respective diet(s) and distilled water *ad libitum* in a temperature- and humidity-controlled room with a 12/12-h light/dark cycle. The three groups were group CO, fed on normal diet (AIN-93G); group HF, high-fat diet fed (wherein cornstarch of AIN-93G was replaced with lard and cholesterol); and group CHR, fed on carbohydrate-restricted diet (low-carbohydrate, high-protein diet wherein milk casein replaced corn starch of the high-fat diet) (Table 1). Each group was further divided into 2 subgroups with 20 mice each, out of which 1 was assessed for life span measurement. The others were evaluated for learning and memory ability at 49 weeks of age. At 50 weeks, after a 12-h fast, the mice were weighed and the blood samples were collected by decapitation. To obtain serum, blood was centrifuged (900×*g*, 5°C, 15 min). Several organs (brain, heart, lung, liver, pancreas, spleen, kidney, white adipose and cecum tissues) were removed. The serum and organs were stored at −80°C until further use.

2.2. Passive avoidance test

Passive avoidance test is a useful behavioral study for measuring learning and memory. The apparatus (Muromachi Kikai Co., Tokyo, Japan) used for the test consisted of a light and a dark compartment separated by a guillotine with an electrifiable grid floor. On the first day of testing, a training trial was performed. A mouse was placed in the light compartment, and after 40 s, the guillotine's door was opened. When the mouse moved into the dark compartment, the door automatically closed, and an electric foot-shock (0.1 mA/10 g body weight) was delivered through the grid floor for 3 s. The time taken to enter the dark compartment was recorded for each trial. The mouse that stayed up to 180 s in the light compartment was excluded from the experiment. Twenty hours after the training trial, a test trial was performed, and the latency for entering the dark compartment was recorded.

2.3. Peroxidized lipid in serum and various tissues

Peroxidized lipid [thiobarbituric acid-reactive substances (TBARS)] was measured as an indicator of oxidative stress and senescence, as

Table 1
Diet composition (g/100 g diet)

		Normal diet (AIN-93G)	High-fat diet	Carbohydrate-restricted diet
Protein	Milk casein	20	20	39.6
Fat	Soybean oil	7	7	7
	Lard	-	20	20
Carbohydrate	Corn starch	39.75	19.6	-
	α-Corn starch	13.2	13.2	13.2
	Sucrose	10	10	10
Other	Cellulose	5	5	5
	Mineral mix (AIN-93G-MX)	3.5	3.5	3.5
	Vitamin mix (AIN-93VX)	1	1	1
	L-cystine	0.3	0.3	0.3
	Choline bitartrate	0.25	0.25	0.25
	t - Butylhydroquinone	0.0014	0.0014	0.0014
	Cholesterol	-	0.15	0.15
Energy (kcal/100 g)		394.8	494.2	494.2

described previously [27, 30]. A microplate reader (Infinite F200; Tecan Japan Co., Ltd., Kanagawa, Japan) was used for absorbance measurements.

2.4. Serum and liver biochemical analyses

Biochemical analyses of serum and liver samples were performed as described previously [18, 31]. Serum and liver triacylglycerol (TG), total cholesterol (TC), serum phospholipid (PL) and glucose were measured using commercial enzyme kits (Wako Pure Chemical Industries Ltd., Osaka, Japan). Serum insulin was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). Liver PL was measured according to the procedure of Rouser et al. [32]. Serum IL-1β and IL-6 were determined by an ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA). A microplate reader (Infinite F200, Tecan Japan Co., Ltd., Kanagawa, Japan) was used for absorbance measurements.

2.5. Gut microbiota analysis

Intestinal bacterial DNA was extracted from mouse stool samples, and the gene encoding 16S rRNA was amplified by the polymerase chain reaction (PCR). The composition of intestinal bacteria in feces was examined by meta-analysis (16S) as described previously [9, 33]. Mouse feces were collected daily during the last week of the study period, and DNA was extracted from the recovered feces, which was pooled for each group. DNA extraction from the feces was performed using a QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany). PCR-based 16S rRNA gene amplicon sequencing was performed with 10 ng DNA, 10 μM of each barcoded forward and reverse primer, 2× Gflex PCR buffer (Mg^{2+}, dNTP plus) (Takara Bio Inc., Japan) and Tks Gflex DNA Polymerase (Takara Bio Inc., Japan) in 25-μl volume. To target 16S rRNA variable regions 3 and 4 (V3 and V4), forward primer 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAA-GAGACAGCCTACGGGNGGCWGCAG-3′) and reverse primer 806R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAC-TACHVGGGTWTCTAAT-3′) were used at 94°C (1 min), followed by 28 cycles of 98°C (10 s), 50°C (15 s) and 68°C (15 s). PCR products were tagged using a Nextera XT Index Kit (Illumina, San Diego, CA, USA) to distinguish the sample IDs using DNA (10 ng), Nextera XT Index Primer 1, Nextera XT Index Primer 2, 2× Gflex PCR buffer (Mg^{2+}, dNTP plus) and Tks Gflex DNA Polymerase (Takara Bio) in a volume of 25 μl at 94°C for 1 min. This was followed by 8 cycles of 98°C (10 s), 60°C (15 s) and 68°C (15 s). Final PCR products were mixed to provide 2 ng of DNA for each sample. This was sent to Takara Bio, Japan, for metagenomic analysis.

2.6. SCFA analyses

SCFAs were derivatized and analyzed by liquid chromatography–tandem mass spectrometry by following a previously described method [18, 34]. Acetic, propionic and butyric acids were quantified, and the results were expressed in μM/g of wet weight cecum contents.

2.7. Statistical analysis

Survival curves were estimated by the Kaplan–Meier test, and the curves were compared using the log-rank test [6, 25]. Results were expressed as mean±S.E.M. Data were analyzed using one-way analysis of variance followed by the Tukey–Kramer test for multiple comparisons among the three groups (CO, HF and CHR). A difference was considered significant at $P<.05$.

3. Results

3.1. Survival rate

Survival curves at the end of the life measurement test are shown in Fig. 1. When compared to the other groups, CHR group experienced a significant decrease in the survival rate ($P<.05$) from 46 weeks of age. The average life span for CO group was noted as 61.7±2.2 weeks, whereas it was 57.1±1.4 and 50.2±1.4 weeks for HF and CHR group, respectively. The average life span was significantly lower in CHR group than the CO and HF groups ($P<.001$ and $P<.05$, respectively). These results indicated that carbohydrate-restricted diet reduced the survival rate of SAMP8.

3.2. The learning and memory ability

During the 49-week study period, 2 mice from CO and HF group, respectively, and 8 mice from the CHR group died, leaving behind 18, 18 and 12 mice in CO, HF and CHR group, respectively, for euthanization and tissue collection.

The effect of a carbohydrate-restricted diet on learning and memory ability was examined by the passive avoidance test (Fig. 2). Latency time significantly decreased in the CHR group as compared to the CO group ($P<.05$). These results exhibited that the carbohydrate-restricted diet reduced the learning and memory ability of SAMP8 mice.

3.3. Growth parameters

During the 50-week study period, 2 mice from the CO group and 4 and 9 mice from HF and CHR group, respectively, died, leaving behind 18, 16 and 11 mice (in CO, HF and CHR groups, respectively) for euthanization and tissue collection. The effects of a carbohydrate-restricted diet on body weight, food intake, energy intake and tissue weights were examined (Table 2). No difference(s) in the body weight (s) was noted among all the groups. A significant decrease in food intake was observed in HF and CHR groups as compared to the CO group ($P<.05$, and $P<.05$, respectively); however, no difference existed in the energy intake among all the groups. Brain and lung weights notably increased in the CHR group as compared to HF group ($P<.05$ and .05, respectively). A marked decrease in the heart, kidney and liver weights was noticed in HD group as compared to the CO and CHR groups ($P<.05$, .05 and .05, respectively). No weight difference existed before the body weight correction of these tissues. Spleen weight significantly increased in the CHR group than in CO and HF groups ($P<.05$). However, there were no significant differences in the pancreas or adipose tissue(s) weight among the groups. The results revealed carbohydrate-restricted diet to have a strong impact on the spleen of SAMP8 mice.

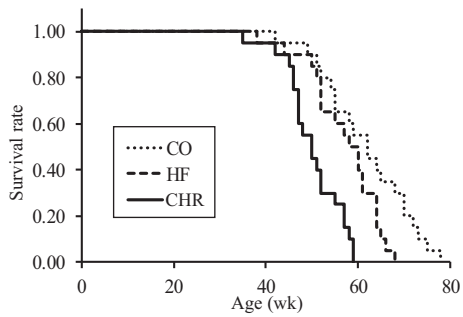

Fig. 1. The effect(s) of carbohydrate-restricted diet on the survival curves in SAMP8.

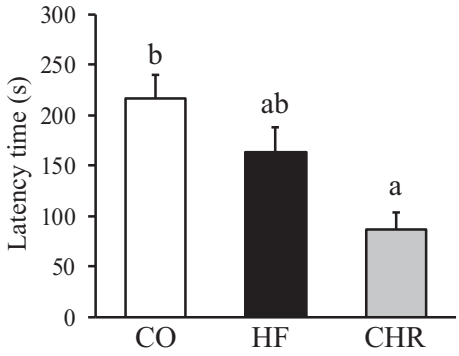

Fig. 2. The effect(s) of carbohydrate-restricted diet on the learning and memory ability in SAMP8.The values are mean±S.E.M., n=12–18. Superscript letters indicate significant differences ($^{a,b}P$<.05).

Table 3
Lipid peroxide levels of serum and each tissue

	CO	HF	CHR
	(μmol/L)		
Serum	10.8±1.1 ^{a}	12.6±0.8 ^{ab}	15.4±0.8 ^{b}
	(nmol/g tissue)		
Brain	130±3^{a}	154±8^{b}	186±7^{c}
Heart	66.1±4.2	67.4±5.6	72.3±5.7
Kidney	63.9±2.1	65.4±3.6	69.5±2.6
Liver	48.2±2.2^{a}	70.5±3.8^{b}	52.8±2.5^{a}
Lung	49.1±2.2	49.7±3.3	54.5±3.3
Pancreas	78.0±4.0^{a}	99.3±5.6^{b}	89.1±6.5^{ab}
Spleen	82.9±6.2^{a}	85.0±7.8^{a}	290±68^{b}
Epididymal adipose tissue	2.72±0.3^{a}	5.60±0.9^{b}	1.97±0.31^{a}
Mesenteric adipose tissue	5.36±0.36^{a}	13.5±4.65^{b}	11.3±0.98^{ab}
Perinephric adipose tissue	3.77±0.26^{a}	7.08±0.62^{b}	3.24±0.2^{a}

Values are mean±S.E., n=11–18. Different superscript letters indicate significantly different means at P<.05.

3.4. Peroxidized lipid and inflammation indicator level

Since reduction in learning and memory ability is one of the phenomena of senescence, senescence and oxidative stress indicator (TBARS) and factors inducing them (IL-1β and IL-6) were measured (Tables 3 and 4). TBARS levels of serum, brain and spleen increased significantly in the CHR group as compared to CO group (P<.05, .05 and .05, respectively), whereas TBARS levels of liver, pancreas and adipose tissues increased remarkably in the HF group than in the CO group (P<.05, .05 and .05, respectively). Since chronic inflammation promotes senescence, serum IL-1β and IL-6 levels, which are inflammation indicators, were measured. Serum IL-1β and IL-6 levels in the CHR group significantly increased as compared to the CO group (P<.05 and P<.05, respectively). The results suggested that a carbohydrate-restricted diet promoted inflammation and oxidative stress of SAMP8 mice.

3.5. Biochemical parameters in serum and liver

Biochemical parameters in the serum and liver are outlined in Table 4. Serum TC, liver TG and liver TC levels increased significantly in the HF group as compared to CO and CHR groups (P<.05, .05 and .05, respectively). Serum PL level notably increased in the HF group than in CHR group (P<.05). Serum glucose and insulin levels showed a marked increase in the HF group as compared to CO group (P<.05 and .05, respectively). Liver PL level was significantly reduced in the HF group as compared to CO and CHR groups (P<.05). The results indicated that a carbohydrate-restricted diet did not promote lipid accumulation in SAMP8.

Table 2
Growth parameters

	CO	HF	CHR
Initial body weight (g)	20.1±1.3	20.1±1.2	20.1±1.2
Final body weight (g)	37.0±2.1	41.8±1.8	35.8±2.2
Food intake (g/day)	3.98±0.08^{b}	3.23±0.13^{a}	3.13±0.05^{a}
Energy intake (kcal/day)	15.7±0.3	15.9±0.6	15.4±0.3
Tissue weight (g/100 g body weight)			
Brain	1.19±0.05^{ab}	1.06±0.05^{a}	1.30±0.07^{b}
Heart	0.52±0.02^{b}	0.44±0.02^{a}	0.54±0.02^{b}
Kidney	1.42±0.04^{b}	1.15±0.04^{a}	1.64±0.08^{c}
Liver	4.71±0.26^{a}	7.36±0.86^{b}	5.05±0.11^{a}
Lung	0.80±0.05^{ab}	0.73±0.03^{a}	0.97±0.11^{b}
Pancreas	0.56±0.03	0.52±0.03	0.55±0.05
Spleen	0.43±0.03^{a}	0.43±0.04^{a}	1.21±0.29^{b}
White adipose tissue			
Epididymal	1.07±0.14	1.27±0.14	0.66±0.11
Mesenteric	1.84±0.23	2.57±0.30	1.81±0.26
Perinephric	2.81±0.22	3.21±0.43	2.53±0.30

The values are means±S.E.M., n=11–18. Different superscript letters indicate significant differences ($^{a,b}P$<.05).

3.6. Gut microbiota

To understand the factors involved in the effect of carbohydrate restriction, changes in the gut microbiota of the CHR group were examined. Homology searches and lineage classification analyses were performed using the 16S rRNA database with obtained sequences, and the details of microorganisms in the samples were determined. Detailed analysis identified eight taxonomic units for which the proportion was at least twofold higher or lower in the CHR group when compared to CO and HF groups (Table 5 and Table S1). *Clostridiales;f__[Mogibacteriaceae];g__*, *Clostridium*, and *Sutterella* were twofold more abundant in the CHR group, whereas *Allobaculum*, *Lachnospiraceae;Other*, *Clostridiaceae;Other*, *Bifidobacterium* and *Turicibacter* were at least twofold less frequent in the CHR group. However, determining the genus of *Clostridiales;f__[Mogibacteriaceae];g__* was not possible. The differences in gut microbiota composition resulting from the carbohydrate-restricted diet were speculated to serve as the underlying factor(s) causing different effects of diet(s) on physiological functions.

3.7. SCFAs levels in cecum content

Given that gut microbiota substantially changed with respect to carbohydrate-restricted diet, the SCFA levels (whose production is promoted by the intestinal bacteria) were measured (Table 6). Acetic,

Table 4
Biochemical parameter of serum and liver

	CO	HF	CHR
Serum			
TG (mmol/L)	2.27±0.57	1.30±0.18	1.57±0.28
TC (mmol/L)	3.20±0.2^{a}	5.18±0.55^{b}	2.83±0.11^{a}
PL (mmol/L)	2.84±0.16^{ab}	3.23±0.18^{b}	2.45±0.10^{a}
Glucose (mmol/L)	6.94±0.65^{a}	10.3±1.00^{b}	7.54±0.55^{ab}
Insulin (ng/ml)	0.28±0.04^{a}	0.72±0.13^{b}	0.39±0.14^{ab}
IL-1β (pg/ml)	1.18±0.25^{a}	1.41±0.28^{ab}	1.77±0.34^{b}
IL-6 (pg/ml)	50.6±4.7^{a}	62.1±6.3^{ab}	79.8±9.0^{b}
Liver			
TG (μmol/g tissue)	7.67±0.79^{a}	11.5±0.5^{b}	7.7±0.53^{a}
TC (μmol/g tissue)	4.54±0.51^{a}	7.92±0.33^{b}	4.1±0.38^{a}
PL (μmol/g tissue)	28.6±0.8^{b}	25.1±0.8^{a}	29.2±1.3^{b}

Values are mean±S.E., n=11–18. Different superscript letters indicate significantly different means at P<.05.

Table 5
Gut microbiota (genus level) that increased by more than two times or decreased to one half or less in the CHR group compared to the CO and HF groups

Genus	CO	HF	CHR	CHR/CO	CHR/HF
	(Relative abundance)				
Clostridiales;f__[Mogibacteriaceae];g__	0.000	0.002	0.003	7.55	2.03
Clostridium	0.001	0.001	0.003	4.51	3.04
Sutterella	0.001	0.000	0.004	4.07	9.70
Allobaculum	0.012	0.009	0.004	0.36	0.44
Lachnospiraceae;Other	0.052	0.050	0.012	0.23	0.24
Clostridiaceae;Other	0.050	0.068	0.003	0.06	0.04
Bifidobacterium	0.031	0.031	0.000	0.00	0.00
Turicibacter	0.002	0.003	0.000	0.00	0.00

propionic and butyric acid levels in the cecum content were notably reduced in the CHR group when compared to CO group ($P<.05$, .05 and .05, respectively). In addition, propionic and butyric acid levels were lowered in the CHR group than in HF group ($P<.05$ and .05, respectively). The results highlighted a reduction in SCFA production by the carbohydrate-restricted diet.

4. Discussion

In this study, SAMP8 mice were used to examine the effects of a carbohydrate-restricted diet on the process of aging, particularly life span, brain function and the intestinal flora. An effort was made to determine that how a long-term carbohydrate restriction affects the aging process. It was observed that by consuming a carbohydrate-restricted diet, the survival rate, learning and memory capacity, and the beneficial enteric bacteria was reduced, and in turn, it increased the harmful enteric bacteria. Life span shortening and decreased learning and memory abilities are typical phenotypes of aging. Therefore, we (with respect to this study) suggest that carbohydrate-restricted diets accelerate senescence. Several indicators of senescence, such as the IL-6, IL-1β and TBARS levels (all of which are known to increase with aging) [6, 25, 35], were measured. These parameters exhibited high values under carbohydrate restriction, demonstrating that a carbohydrate-restricted diet promotes senescence. In addition, carbohydrate restriction identified a significant accumulation of lipid peroxide in the brain and spleen, suggesting that carbohydrate restriction is likely to affect brain function and the immune system.

The effect of carbohydrate restriction on gut microbiota was examined to elucidate a part of the mechanism underlying the induction of oxidative stress and chronic inflammation. Carbohydrate restriction resulted in a substantial rise in the following taxonomic groups: *Clostridiales;f__[Mogibacteriaceae];g__*, *Clostridium* and *Sutterella*. *Clostridium* includes harmful bacteria that cause food poisoning and inflammation in the host [36]. Meanwhile, *Sutterella* increases when diets depressing the intestinal function are consumed and due to an increase in the lipopolysaccharide concentration and intestinal inflammation [37]. In this study, it was speculated that the number of harmful bacteria increased due to carbohydrate restriction and induced oxidative stress and chronic inflammation. The function(s) of *Clostridiales;f__[Mogibacteriaceae];g__* remains undetermined. The genera that significantly decreased with carbohydrate-restricted diet consumption included *Allobaculum*, *Lachnospiraceae;Other*, *Clostridiaceae;Other*, *Bifidobacterium* and *Turicibacter*. *Allobaculum* is reported to increase with respect to the intestinal health-preserving dietary components [38, 39]. *Lachnospiraceae;Other* produces short-chain fatty acids, and their number decreases in the intestines of elderly people and patients with various diseases [40]. *Bifidobacterium* metabolizes sugars in the intestine to produce acetic acid and lactic acid [41]. SCFAs exert an anti-inflammatory effect by strengthening the intestinal barrier function [42]. In this study, the carbohydrate-restricted diet intake reduced SCFA concentrations in the cecum content. Consequently, this suggests that the effect of carbohydrate restriction might be due to the SCFAs. SCFAs inhibit the growth of *Clostridium* spp. by lowering the pH in the intestine [43, 44]. Therefore, it is possible that *Clostridium* increased due to the decrease in SCFAs caused by carbohydrate restriction. *Clostridiaceae;Other* and *Turicibacter* are involved in obesity formation [36, 45]. In the present study, the results of adipose tissue weight, liver and serum biochemical parameters exhibit that carbohydrate-restricted diet inhibited lipid accumulation as compared to high-fat diets. Therefore, it suggests that the reduction of *Clostridiaceae;Other* and *Turicibacter* was sighted for these reasons. It has been reported that blood lipid parameters do not affect the subsequent life expectancy in the elderly [46, 47]. This suggests that lipid metabolism parameters did not significantly affect aging in this study.

Table 6
SCFA levels in cecum content

	CO	HF	CHR
	(μmol/g)		
Acetic acid	34.5 ± 3.8^{b}	28.4 ± 2.6^{ab}	23.9 ± 2.2^{a}
Propionic acid	16.0 ± 0.4^{b}	15.0 ± 0.7^{b}	12.3 ± 0.5^{a}
Butyric acid	9.88 ± 0.73^{b}	7.69 ± 0.98^{b}	3.13 ± 0.65^{a}

Values are mean±S.E., $n=11$–18. Different superscript letters indicate significantly different means at $P<.05$.

Carbohydrate-restricted diets are completely in contrast to the carbohydrate-centric meals (typical of Japanese diet). Carbohydrate-restricted diets have been reported to reduce visceral fat, and because of that, they have attracted attention and have been implemented at various places [11]. However, long-term carbohydrate restriction in mice has been shown to have various adverse effects [12–14]. As a result, the safety of long-term adherence to carbohydrate-restricted diets is still doubtful. In addition, carbohydrate restriction did not have any beneficial effects on longevity in our study. One of the characteristics of Japanese diet is that it effectively promotes longevity as it uses a variety of ingredients without leaning heavily towards specific ingredients only [7, 8]. However, carbohydrate-restricted diets are extremely rich in proteins and fat (constitutes an imbalanced way of eating) and differs from a Japanese diet. Previous studies have reported an increase in mechanistic target of rapamycin (mTOR) activation with increasing protein-to-carbohydrate ratios [13]. Thus, as chronic mTOR activation promotes senescence [48, 49], carbohydrate restriction in a low-carbohydrate, high-protein diet was found to be ineffective for longevity. Long-term protein overdose has been reported to increase the incidence of cancer, diabetes and cardiovascular disease [50–52]. Therefore, long-term protein overdose may have promoted aging. It was not clear in this study whether accelerated aging was caused by carbohydrate restriction or protein overdose. In the future, we plan to determine which of these two factors is important for the promotion of aging.

Recently, a large-scale epidemiological study has reported that high carbohydrate intake leads to high mortality rates and increased risk of cardiovascular diseases [53]. However, the study did not observe a definite connection between low intake of carbohydrates (≤50% calories) and health. The study did not promote diets that are extremely low in carbohydrates and concluded that moderate intake of carbohydrates is optimal. Statistically significant difference in the carbohydrate intake and mortality rate in Asia (wherein carbohydrate intake is high) is absent. The mortality rate is extremely low (forms a U-shaped curve) when carbohydrates are composed of approximately 60% calories. In Asia, negative effects could be observed when carbohydrate intake is <50% for >70% calories. Previously, we have demonstrated that the life span of mice was shorter when fed on 1960

Japanese diet, wherein the carbohydrate content was 70% calories [6]. Thus, we conclude that carbohydrate intake of ≥70% calories is ineffective for longevity. In this study, the carbohydrate energy ratio of the carbohydrate-restricted diet was about 20%. This ratio assumes the lack of any snacks and drinking except during three staple meals per day in humans. While this diet is not impossible, it is very severe carbohydrate restriction. Therefore, it is possible that gradual carbohydrate restriction might not cause aging promotion. We plan to clarify this in the future. In addition, while there is a large regional disparity in the effects of carbohydrate intake, it is important to demonstrate the proportion of calories that is safe in carbohydrate intake over a long-term period. We suggest that more research data should be accumulated (with respect to humans) wherein carbohydrate intake is ≤50% calories.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2019.108326.

Author contributions

All authors contributed to the development, analysis and drafting of this article.

Acknowledgments

This research was supported by grants from the Project of the NARO Bio-oriented Technology Research Advancement Institution (Advanced integration research for agriculture and interdisciplinary fields).

Conflict of interest

There are no conflicts of interest.

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