Moderate-dose Regular Lifelong Alcohol Intake Changes the Intestinal Flora, Protects against Aging, and Keeps Spatial Memory in the Senescence-accelerated Mouse Prone 8 (SAMP8) Model

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ABSTRACT - Purpose: Heavy and long-term alcohol consumption increase the risk of alcohol-related diseases. Epidemiological studies show moderate drinking reduces the risk of mortality, cardiovascular diseases, and brain infarction in the J-shaped or U-shaped curve effect. However, why moderate drinkers may be healthy and non-drinkers may be ill in diverse populations remains controversial. Herein, we examined the relationship between moderate/lifelong alcohol intake and aging, especially aging-related cognitive functions in senescence-accelerated mouse prone 8 (SAMP8) model. **Methods:** SAMP8 model (5-week-old, male, n = 36), a model of age-related cognitive deficit, were group-housed (n = 6/cage) and provided free access to water (water group, n = 18) or 1% ethanol (EtOH group, n = 18, intake started when mice were 9 weeks old). The object recognition test (ORT) and object location test (OLT) were used to evaluate cognitive functions. The intestinal flora at the age of 87 weeks was analyzed by terminal restriction fragment length polymorphism (T-RFLP). **Results:** The lifespan of the EtOH-group mice was about 4 weeks longer than that of the water-group mice. In the EtOH group, spatial recognition impairment, assessed by OLT, was observed later (age, 73 weeks) than that in the water group (age, 52 weeks). The spinal curvature and skin conditions progressed significantly slower in the EtOH group than in the water group. Moreover, diarrhea symptoms only appeared in the water group, at the age of 82 weeks. The T-RFLP analysis of the intestinal flora indicated higher Lactobacillales order and lower *Clostridium* cluster XI in the EtOH group than in the water group, although those were extremely high in some mice close to death in both groups. Water-group mice with diarrhea presented significantly higher *Clostridium* cluster XI than did those without diarrhea (P = 0.017). **Conclusion:** Moderate alcohol intake changes intestinal flora and positively affects aging of SAMP8 model.

INTRODUCTION

Heavy and long-term alcohol consumption is associated with increased risk of alcohol-related diseases such as liver cirrhosis, cancer, pancreatitis, depression, and dementia (1, 2). Alcohol abuse is not only associated with serious physical and psychiatric illnesses, but also with familial dysfunction, violence, and economic losses, all of which place a substantial burden on society.

However, Marmot et al. reported that the mortality rate was lower in moderate-alcohol drinkers than in either non-drinkers or heavier drinkers (3). The relationship between the mortality and alcohol consumption shows a U-shaped curve. Many epidemiological studies also suggested that moderate alcohol consumption reduces the risk of mortality and some diseases such as cardiovascular diseases in the J-shaped curve or U-shaped curve (4, 5). Ruitenberg et al. reported that light-to-moderate drinking was significantly associated with a lower risk of dementia, including vascular dementia (the Rotterdam Study) than that in the non-drinkers (6). Other epidemiological studies

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have also indicated a reduction in the risk of dementia by moderate alcohol intake (7-11). Neafsey et al. reviewed 143 papers and concluded that light to moderate drinking did not impair cognition in younger subjects. In fact, it reduced the risk of dementia and cognitive decline in older subjects (9). However, epidemiological studies using populations of diverse backgrounds often present controversial results indicating that moderate drinkers might be healthy, but ex-drinkers and nondrinkers might be ill (12, 13). Bhala et al. reported ethnic variations in liver- and alcohol-related disease hospitalisations and mortality (14). Some studies (10, 11) supported the idea that moderate alcohol intake reduces the risk of dementia, but they pointed out the heterogeneity of methodology and backgrounds. Therefore, further studies are needed to clarify the relationship between moderate alcohol intake and long-term healthy-aging in congenic rodents.

Although numerous alcohol studies in rodents mainly focused on the responses to high alcohol doses, several studies have been conducted on moderate alcohol intake and healthy effects (15-22). However, the relationship between moderate/sustained alcohol intake and health during a lifespan has been poorly studied. Most of the alcohol studies in rodents are conducted for a certain period, not until the death of the animals. Therefore, the present study aimed to examine the relationship among moderate/lifelong alcohol intake, lifespan, and aging, focusing on aging-related cognitive functions during life using non-invasive tests.

We used senescence-accelerated mouse prone 8 (SAMP8) model in this study. The SAM models were established by Takeda et al. (23). The SAMP lines (from SAMP1 to SAMP11) show some age-associated disorders similar to the humans, including accelerated accumulation of senile features, early onset and fast progress of age-associated pathological phenotypes, impaired immune response, senile osteoporosis, and deficits in learning and memory (24). The SAMP substrains SAMP8 and SAMP10 show early deficits in learning and memory with changes in neuronal or glial components (25-28). In the previous studies, the cognitive functions of SAM mice were mainly evaluated by the passive avoidance test using electric shock (29, 30) or by forced swimming in Morris water maze (31, 32). Both of these tests are invasive tests for mice. However, the novel, object recognition test (ORT) and object location test (OLT) for spatial cognition (33-36) are non-invasive and are conducted under conditions that are close to the human cognitive assessment. Moreover, the lifelong temporal changes in both ORT and OLT are unknown in SAMP8 model, because the recognition functions have been usually evaluated only at one or some aging points.

Therefore, we investigated the changes in cognitive functions with age by ORT and OLT across the life span, with measurements about every seven weeks, and examined the age-appropriate object selection. In addition to evaluating the recognition functions, the physical activity, and skin and spinal curvatures were also scored after the age of 70 weeks.

Mitsuoka et al. found that the human intestinal flora begins to change during the transition from middle age to old age using culture-based methods (37, 38). With aging, *Bifidobacterium* decreases, but *Clostridium perfringens, Lactobacillus*, *Enterobacteriaceae*, and *Enterococcus* increase. Recently, Odamaki et al. reported the sequential changes in gut microflora composition in newborn to centenarian Japanese subjects (39). O'Toole et al. reported a framework for analyzing microflora-health associations, distinguishing correlation from causation, identifying microflora interaction with physiological aging processes, and developing microflora-based health surveillance for older adults (40). The relationship between each bacterial strain and clinical phenotypes of aging is complicated and still not well known. The gut-brain axis is the biochemical signaling pathway between the central and the enteric nervous systems and affects the health and disease (41). The intestinal flora plays a crucial role in the brain functions like dementia (42, 43). However, only a few studies have reported the relationship between moderate drinking and the intestinal flora (44, 45). Therefore, we conducted the intestinal flora analysis to examine the effects of moderate drinking on the intestinal flora.

The moderate human dose/intake cannot be applied in rodent models because the rate of alcohol metabolism is different between them (46). Holman et al. reported the relative risk of mortality in male drinkers compared with abstainers was lowest at 1.0-1.9 standard drinks per day on J-curve by meta-

analysis (47). Osaki et al. reported that 1% ethanol intake revealed a J-curve effect and significantly reduced the serum levels of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and ammonia as compared to the no-ethanol intake (18). In this study, the animals were provided free access to 1% ethanol through a drinking bottle as the moderate-alcohol dose based on the J-curve studies in rodents (18, 22) and other studies (48, 49).

This study is, to our knowledge, the first report of a relationship between moderate drinking and health during lifespan in mice.

MATERIALS AND METHODS

Subjects

Thirty-six SAMP8 male mice (5-weeks-old) (Japan SLC, Inc., Hamamatsu, Japan) were acclimated to the animal facility for 2 weeks. The floor of the cage was covered with pulp bedding (Palmas μ, Material Research Center, Tokyo, Japan), which was changed every week. The animals were group-housed (6 mice per cage) with free access to water (water group, n = 18) or 1% (v/v) EtOH (EtOH group; water until 8 weeks of age, 1% (v/v) EtOH (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) started from 9 weeks of age, n = 18), and standard chow (CRF-1, Charles River Laboratories, Yokohama, Japan). The animal facility was maintained at 23 ± 1°C with 55% humidity and a 12-h/12-h light/dark cycle.

The ORT and OLT experiments were conducted from 09:00 to 17:00. All mice were enrolled in the experiment around the same time to allow adaptation to the circadian rhythms. More than 1 h before the beginning of the experiments in the morning or afternoon, the cages were moved from the breeding rack to a different rack to calm the mice. After the experiments ended, the cages were moved back to the breeding racks.

All experiments were approved by the Institutional Animal Care and Use Committee of SAPPORO BREWERIES LTD. (permit numbers 2013-008 and 2014-006) and followed the Guidelines for Proper Conduct of Animal Experiments on Science Council of Japan.

Liquid Consumption, Food Consumption, and Body Weight

The body weight of each mouse was recorded every week. The food and liquid consumption per cage were recorded every week and 3 times every week, respectively. They were expressed as one mouse consumption per day calculated by dividing the total consumption per cage by the number of mice in a cage.

Grading Aging Scores

We examined the grading aging scores such as behaviors, skin and hair conditions (glossiness, coarseness, and hair loss), ulcer, eye (cataract, periophthalmic lesion, capacity of cornea, and ulcer of cornea), and skeleton (spinal curvature) every 4 weeks starting at 70 weeks of age as previously reported (50).

Blood Sugar

The blood sugar levels were measured at the age of 71–75 weeks. The mice were fasted for 6 h before blood sampling. The blood sample was taken from tail vein with a razor blade and blood sugar levels were measured immediately using a blood glucometer (PRECISION XCEED with G3b smart blue electrodes, ABOTT JAPAN, Tokyo, Japan).

Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a technique for assessment of complex microbial communities based on 16S ribosomal RNA gene analysis.

We conducted the intestinal flora analysis by using T-RFLP at the age of 87 weeks. The feces of SAMP8 mouse were frozen at -30°C. T-RFLP analysis (Nagashima method) (51, 52) to analyze the intestinal flora was conducted by TechnoSuruga Laboratory Co. Ltd. (Shizuoka, Japan). The constitutive bacterial ratios (*Bifidobacterium*, Lactobacillales order, *Bacteroides*, *Prevotella*, *Clostridium* cluster IV, *Clostridium* subcluster XIVa, *Clostridium* cluster XI, *Clostridium* cluster XVIII, others) were expressed as the relative peak area/total peak area (%).

ORT and OLT Apparatus

We used the same boxes (ORT boxes: D: 300 mm × W: 300 mm × H: 350 mm; Brain Science Idea Inc., Osaka, Japan) to evaluate both ORT and OLT (36). The floor was covered with sliced paper and pulp bedding (Palmas μ, Material Research Center, Tokyo, Japan) similar to the breeding cage. The

ORT box was placed in light- and sound-attenuating chamber equipped with a fan. Video cameras were placed on the ceiling of each chamber to record the behavior. The video was recorded and locomotor activity for 10 min (staying time and locomotor distance in each area of the ORT box) was quantified using the ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA). The illuminant conditions were 25 lux.

Objects for ORT and OLT Test

The shape and size of objects are important for ORT and OLT tests. Age-appropriate object sizes were used for ORT and OLT (Table 1). Small objects were used for young SAMP8 because the young mice bite the bulged or sharp figures and climbed figures, a behavior that leads to errors in the ORT preference time. During the aging progression, mice did not discriminate between the familiar and novel objects. Therefore, we replaced small objects by large objects and repeated the same large-objects every seven weeks to precisely evaluate the age-related changes in ORT and OLT.

For small ORT objects, many objects were screened and 2 sets of small objects with similar size and preference were selected: 1) pale green/plastic mice (25 mm tall) as the training object *vs.* pink triplet/plastic figure (23 mm tall) as the novel object, 2) dark blue/glass ball (15 mm diameter) as the training object *vs.* white ceramic cylinder with brown stripes put horizontally (23 mm tall × 12 mm diameter) as the novel object. For large ORT objects, a white golf ball (43 mm diameter) was selected as the training object *vs*. white film case (29 mm diameter × 50 mm tall) as the novel object.

We selected fluorescent pink super balls (19 mm diameter) as small OLT objects (short-term memory) and wood apple blocks without coloring (31 mm width and 50 mm height of main body and 15 mm height of stem end) as large OLT objects (short-term memory). Green cylindrical wood blocks (44 mm diameter × 44 mm tall put horizontally) were used as large OLT objects (long-term memory). After each test, the objects were cleaned with distilled water.

Table 1. Age of mice at the time of the experiments and the type of objects used for object recognition and object location tests

Object size	Training object	Test object	Age (weeks)	Object size	Training and test object	Age (weeks)
Small	glass marble (15 mm diameter)	ceramic cylinder (23 mm tall × 12 mm diameter)	8, 11	Small	super ball (pink) (19 mm diameter)	8-9
	green mice doll (25 mm tall)	pink doll (23 mm tall)	16			
	glass marble (15 mm diameter)	ceramic cylinder (23 mm tall × 12 mm diameter)	23			24
Large	golf ball (43 mm diameter)	film case (29 mm diameter × 50 mm tall)	30	Large	wooden apple block (31 mm width and 50 mm height of main body and 15 mm height of stem end)	31
						37-38
			51		wooden cylinder block (green) (44 mm diameter × 44 mm tall put horizontally)	52
			58			59
			65			66
			72			73
			79			80
			86			88

The interval between training and test phases was 24 h for long-term memory (ORT and OLT) and 70 min for short-term memory assessment.

ORT and OLT Procedures

ORT (33-35) and OLT (36) consisted of the following 3 phases: habituation, training, and test. The training and test object position are described in Figure 1.

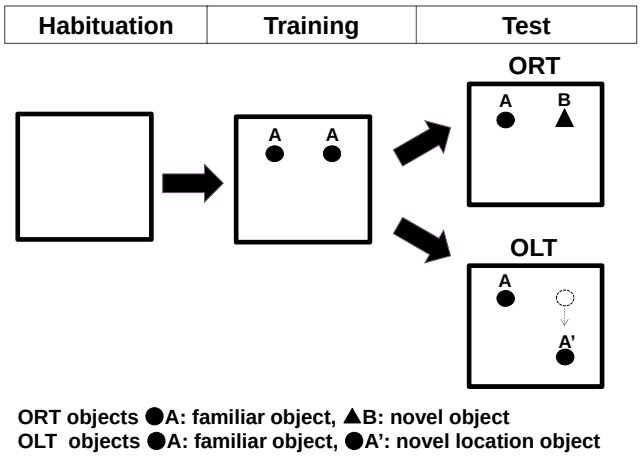

Figure 1. Schematic Representation of the Three Phases (Habituation, Training, and Test Phases) for the Object Recognition Test (ORT) and Object Location Test (OLT)

Habituation: Each mouse was free to explore the ORT box without objects for 10 min, once a day for 3 consecutive days during the ORT habituation phase and for 2 consecutive days during the OLT habituation phase. For the locomotor activities at 85 weeks of age, the locomotor activity at the first habituation day was measured. To properly assess the locomotor activities, a black patch was attached to the back of each mouse to enable correct tracking in the ORT chamber covered with white sliced paper.

Training: Two of the same objects (A) were fixed on the floor. In the ORT and OLT training phases, mice were placed in the box and allowed to freely access the two objects for 10 min.

Test: In the test phase, the right object was replaced by a novel object (B) for ORT or was placed at a different position (A') for OLT as shown in Figure 1. Each mouse was placed in the ORT box and allowed to explore the two objects freely for 10 min.

The interval between the training and test phase was 24 h for the assessment of long-term memory during the ORT and OLT or 70 min for short-term memory during the OLT.

The time of exploration of each object was measured with a CCD camera, played back, and was individually counted by two stopwatches. The exploration time was measured as the time when the mouse nose was at about 2 cm from each object, removing the time during which the mouse stepped on an object or dug the floor chips.

In the training phase, the recognition index of right and left objects during the training phase for each mouse was expressed as the ratio of the amount of time spent exploring object left A (Time left A×100)/(Time left A + Time right A) and the amount of time spent exploring object right A (Time right A ×100)/(Time left A + Time right A) for both ORT and OLT. During the test phase, the recognition index for each mouse was expressed as the ratio of the amount of time spent exploring familiar object A (Time A×100)/(Time A + Time B) and the amount of time spent exploring novel object B (Time B×100)/(Time A + Time B) for ORT or familiar object A (Time A×100)/(Time A + Time A') and the amount of time spent exploring novel location object A' (Time A'×100)/(Time A + Time A') for OLT.

Differences between recognition indexes of left and right (or novel location) objects were assessed by using the unpaired *t*-test for ORT (OLT) in each phase.

Data Analysis

SPSS software 10.0.7J for Windows (IBM Co., Armonk, NY, USA) was used for Fisher's exact test (between-group comparison of diarrhea symptoms and Lactobacillales order in T-RFLP analysis). Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used for unpaired *t*-tests (between-group comparisons for grading aging scores, T-RFLP analysis, blood sugar, locomotor activity, ORT, and OLT). Data in the text and figures are presented as mean ± standard error of the mean (SEM). In all analyses, a P-value <0.05 was considered statistically significant.

RESULTS

Liquid Consumption, Food Consumption, Blood Sugar, and Body Weight

Because EtOH has calories, we investigated

whether moderate EtOH intake changes food consumption, blood sugar level, and body weight.

Food and liquid consumption were expressed as the consumption of one mouse per day calculated by dividing the total consumption by the number of mice per cage. Liquid consumption gradually decreased with age. The average liquid consumption showed wide fluctuations in volume, especially at the age of about 100 weeks because the number of live animals was small. Low EtOH concentration, 1% EtOH, did not result in an escalation of the drinking volume.

Until about 50 weeks of age, the water consumption was higher than that of EtOH and there was no significant difference afterward (Figure 2A). Food consumption decreased with age and there was no difference between the water and EtOH groups (Figure 2B). The blood sugar levels were measured at the age of 71–75 weeks. The average blood sugar levels were 114 ± 19 mg/dL (water group, n = 9) and 105 ± 35 mg/dL (EtOH group, n = 10), which were not significantly different from each other (P = 0.520). Body weight increased until 30 weeks of age; however, it gradually decreased from 52 weeks (1 year) of age. The changes in body weight were similar between the water and EtOH groups (Figure 2C).

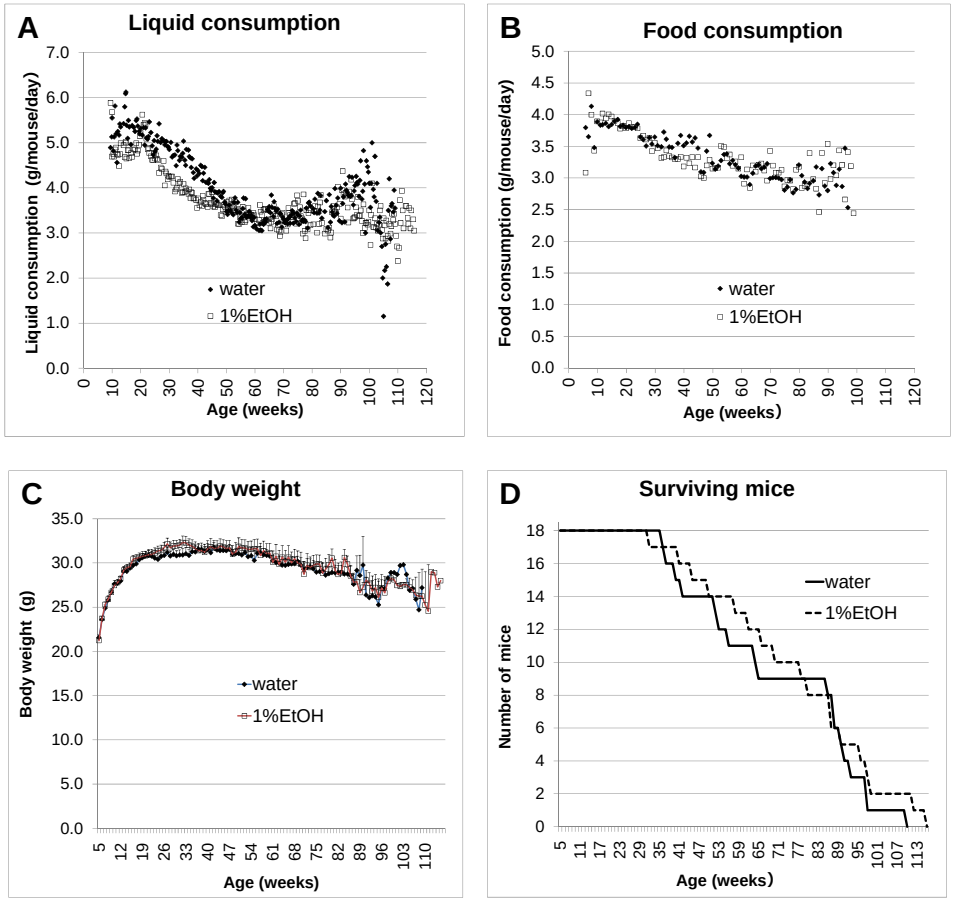

Figure 2. Liquid and Food Consumption, Body Weight, and Survival Analysis of the Mice Drinking Water or EtOH
A. Liquid consumption (g/mouse/day) changes during the lifespan of the mice.
B. Food consumption (g/mouse/day) changes during the lifespan of the mice.
C. Body weight (g/mouse/day) changes during the lifespan of the mice.
D. Survival analysis.
Line: water group; broken line: EtOH group
◆: water group, □: EtOH group

Aging Grading Scores, Locomotor Activity, and Lifespan

Next, we investigated whether moderate/lifelong EtOH intake protects aging and prolongs lifespan.

Aging Grading Scores: Physical activity, skin conditions, eye inflammation, and spinal curvature of SAMP8 model were examined starting from 70 weeks of age. The degrees of spinal curvature at the age of 70–71, 73-74, 77-78, and 82 weeks are described in Figure 3A. The spinal curvature in the EtOH group was significantly lower than that in the water group at each measurement (70–71 weeks, P = 0.017; 73-74 weeks of age, P = 0.010; 77-78 week of age, P = 0.027; and 82 weeks of age, P = 0.016). As shown in Figure 3B, the sum of three skin scores (glossiness, coarseness, and hair loss) of the water group at the age of 73-74 weeks was significantly higher than that of the EtOH group (P = 0.016), although those at the age of 70-71 weeks, 77-78 weeks, and 82 weeks were not significant (P = 0.074, P = 0.080, and P = 0.061, respectively). There was no significant difference in terms of physical activities and eye inflammation between the groups (data not shown, P > 0.05).

Locomotor Activity: Locomotive syndrome is one of the aging parameters. We investigated whether moderate/lifelong EtOH intake protects dysfunction of motor activity in mice. The locomotor activities for 10 min (ORT habituation day 1) at 85 weeks of age were 16.4 ± 1.8 m in the water group (n = 8) and 16.5 ± 2.0 m in the EtOH group. There was no significant difference between groups (data not shown: P > 0.05).

***Lifespan*:** The number of mice who survived at the end of experiments is shown in Figure 2D. Both lines crossed-over and there was no significant difference in term of lifespan between the water and EtOH groups. However, the average length of life was 71.0 ± 5.8 weeks (n = 18) in the water group and 75.4 ± 5.8 weeks (n = 18) in the EtOH group. The maximum ages were 109 weeks in the water group and 116 weeks in the EtOH group.

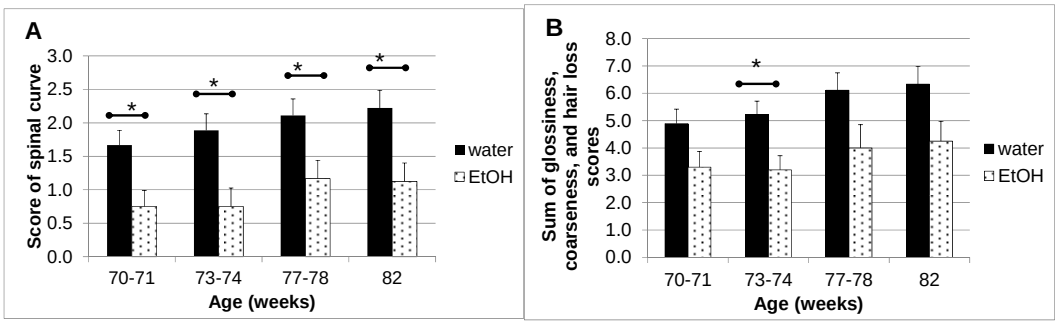

Figure 3. Comparison of Grading Aging Scores between the Water and EtOH Groups.
A. Spinal curvature changes in the water and EtOH groups from the age of 70–71 to 82 weeks.
B. Skin conditions (sum of glossiness, coarseness, and hair loss scores) between the water and EtOH groups from the age of 70–71 to 82 weeks.
*P < 0.05, unpaired *t*-test

ORT-Long-term Memory

Next, we investigated whether moderate/lifelong EtOH intake protects against the age-related impairment of recognition in ORT and OLT.

The recognition indexes of the familiar object (left) and novel object (right) during the test phase are illustrated in Figure 4A (water group) and Figure 4B (EtOH group). During the training phase, the recognition indexes were almost 50% and no significant difference was observed between the recognition of left and right objects (data not shown) because both objects were identical. In Figure 4A and Figure 4B, the circle surrounding the symbols of familiar and novel recognition indexes indicates no significant difference during the training phase, but a significant difference in the test phase. During the test phase at 8–11 weeks of age, the recognition index of the novel object

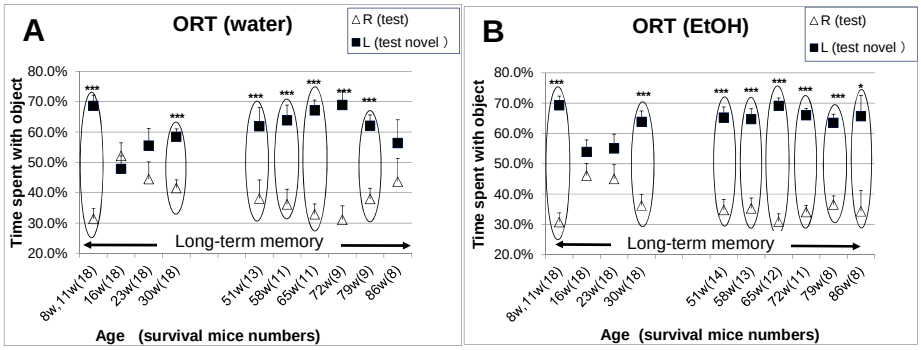

Figure 4. Comparison of the Age-Related Changes in Object Recognition between the Water and EtOH Groups.
A. Recognition indexes of the mice in the water group during the test phase of ORT.
B. Recognition indexes of the mice in the EtOH group during the test phase of ORT.

The circle surrounding the symbols of familiar and novel recognition indexes indicates no significant difference during the training phase, but a significant difference during the test phase. The numbers shown in parentheses correspond to the number of mice alive at the different ages.
△: right object (familiar object), ■: left object (novel object)
***P < 0.001, *P < 0.05; unpaired *t*-test

(ceramic cylinder) was significantly higher than that of the familiar object (glass marble) in both groups (P < 0.001).

The objects were replaced by the set of large objects, familiar object (white golf ball) or novel object (white film case), to perform long-term ORT starting at the age of 30 weeks. In both water and EtOH group mice, the recognition index of the novel object (film case) was significantly higher than that of the familiar object (golf ball) at the age of 51 weeks (both groups; P < 0.001), 58 weeks (water group, P = 0.002; EtOH group; P < 0.001), 65 weeks (both groups; P < 0.001), and 79 weeks (both groups; P < 0.001). However, there was also a significant difference between the right and left objects (same objects) in the training and test phases at the age of 72 weeks in the water group. Therefore, it was not evaluated in the test phase. In the last ORT at the age of 86 weeks, the long-term ORT was only significant in the EtOH group, but not in the water group (water group, P = 0.299; EtOH group, P = 0.014).

OLT–Short-term and Long-term Spatial Memory

The recognition indexes of the familiar object (left) and novel position during the test phase are shown in Figure 5A (water group) and Figure 5B (EtOH group). In short-term OLT, mice significantly recognized the novel position compared to the familiar position at about 70:30 in both groups (P < 0.001) at the age of 8–9, 24, and 31 weeks (both groups; P < 0.001) and 37–38 weeks (water group; P = 0.021, EtOH group; P < 0.001). We then assessed the long-term spatial recognition starting at the age of 52 weeks. At the age of 52 weeks, mice from both groups displayed long-term spatial recognition (both groups; P < 0.001). However, at the age of 59, 66, and 73 weeks, the recognition index of the novel position object was significantly higher than that of the familiar position object only in the EtOH group (59 weeks, P = 0.016; 66 weeks, P = 0.004; and 73 weeks, P = 0.017), but not in the water group. Furthermore, at the age of 73 weeks, there was a significant difference between the right and left objects (same objects) in the training phase in the water-group mice. Sometimes, it became difficult for the aging mice to approach the two same objects equally as compared to the young mice in the training phase.

Diarrhea Symptom and T-RFLP Flora Analysis

Probiotics such as lactobacilli and bifidobacteria prevent diarrhea by improving the intestinal bacterial balance (53). The intestinal flora changes with aging (37, 38). Caracciolo et al. reviewed the relationship among cognitive decline, dietary factors, and gut-brain interaction (43). Therefore, we investigated whether moderate/lifelong EtOH intake affects diarrhea and the intestinal flora.

***Diarrhea Symptom*:** Diarrhea appeared at the age of 82 weeks. At the age of 87 weeks, 8 mice in the water group and 8 mice in EtOH group were alive. The ratio of mice presenting diarrhea to healthy mice was 5/8 mice in the water group and 0/8 mice in the EtOH group. The number of mice with diarrhea was significantly higher in the water group than that in the EtOH group (Fisher's exact test, P = 0.026).

***T-RFLP Flora Analysis*:** The results of T-RFLP flora analysis of the mice feces at the age of 87 weeks is shown in Figure 6A. The relative bacterial ratios varied for each mouse. *Bifidobacterium* was not detected in any mouse. When comparing the water (n = 8) and EtOH groups (n = 8), there was no significant difference in *Bacteroides*, *Prevotella*, *Clostridium* cluster XVIII, and *Clostridium* cluster IV by (unpaired *t*-test, P > 0.05). Only 1 of 8 mice presented a ratio of less than 20% Lactobacillales order in the in EtOH group, whereas 6 of 8 mice (Figure 6B) showed this ratio. The EtOH-group mice presented a significantly higher ratio of Lactobacillales order (P = 0.041; Fisher's exact test). Four mice (two mice from the water group and two mice from the EtOH group) presenting an extremely high ratio of Lactobacillales order (more than 35%) died within three weeks. The ratio of *Clostridium* cluster XI was higher in the water group than that in the EtOH group (P = 0.052). Furthermore, the mice with diarrhea (n = 5) showed significantly higher *Clostridium* cluster XI ratio than that in the mice without diarrhea (n = 11) (P = 0.014, unpaired *t*-test) (Figure 6C). As shown in Figure 6D, the ratio of *Clostridium* subcluster XIVa was slightly higher in the EtOH group than that in the water group (P = 0.152, unpaired *t*-test). Moreover, the ratio of *Clostridium* subcluster XIVa in mice with diarrhea (n = 5) was lower than that in mice without diarrhea (n = 11) (P = 0.108, unpaired *t*-test). The other orders (including multiple candidates) were also significantly higher in the water group than in the EtOH group (Figure 6A, P = 0.005, unpaired *t*-test)

DISCUSSION

Liquid Consumption, Food Consumption, and Body Weight

In both (water and EtOH) groups, liquid consumption gradually decreased with age (Figure 2A). The intake of a low dose of EtOH, 1% EtOH, by SAMP8 model, did not increase the drinking volume as observed for addiction. A previous study reported that C57BL/6 mice showed a high alcohol preference, escalating their drinking volume, and adaptation to the high concentration of EtOH (54). However, why the liquid consumption was lesser in the EtOH group than in the water group until about 50 weeks of age in this study remains unknown. The EtOH group mice consumed water until the age of 8 weeks and were switched to 1% EtOH at the age of 9 weeks. The taste alteration in the EtOH group may affect the drinking volume. However, the decrease in the consumption with age was more significant than the difference in consumption between both groups. In mice, the EtOH metabolism is fast and the mice in EtOH group did not seem drunk after consuming 1% EtOH. We previously reported the relationship between moderate EtOH intake and reward assessed by using conditioned place preference (CPP) test and locomotor sensitization in DBA/2 CrSlc mice. We found that moderate, but long-term, EtOH dose may confer a lower risk for reward progression than the high dose for shorter periods (17). The results of this study supported the low risk of reward by low alcohol, although the oral intake in this experiment was different from i.p. administration in the previous report (17).

The consumption of 1% EtOH in place of water did not affect blood sugar levels at the age of 71–75 weeks or lifelong food consumption (Figure 2B). There are 7 kcal/g of EtOH and the consumption of 5 ml of 1% (v/v) EtOH represents about 0.28 kcal, while consumption of 4 g of solid food, CRF-1, represents 14.3 kcal. The EtOH-calories corresponded to only 2% of the total calories in the EtOH-group mice and barely contributed to the increase in blood sugar and body weight (Figure 2C). The blood sugar was measured at the age of 71–75 weeks. Cuesta et al. reported that the glucose metabolism of SAMP8 model changed with aging and growth hormone could improve insulin resistance (55). The diabetes increases a risk for cognitive impairment (56). However, there was no significant difference in the blood sugar levels between water and EtOH groups.

Aging Manifestation

There was no significant difference in term of locomotor activity and eye inflammation between

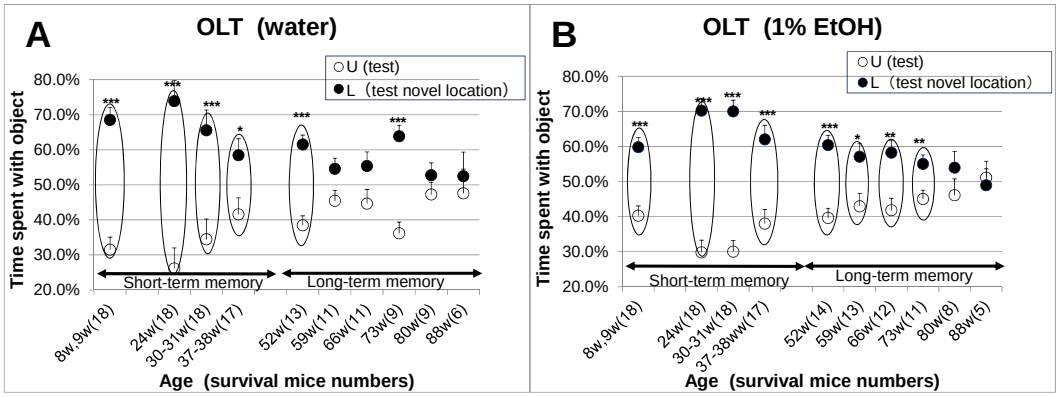

Figure 5. Comparison of the Age-Related Changes in Spatial Recognition between the Water and EtOH Groups.
A. Recognition indexes of the mice in the water group during the test phase of the OLT.
B. Recognition indexes of the mice in the EtOH group during the test phase of the OLT.
The circle surrounding the symbols of familiar and novel location recognition indexes indicates no significant difference during the training phase, but a significant difference during the test phase. The numbers in parenthesis correspond to the number of mice alive at the different ages.
○: upper location (familiar location) object, ●: lower location (novel location) object
***P < 0.001, *P < 0.05; unpaired *t*-test

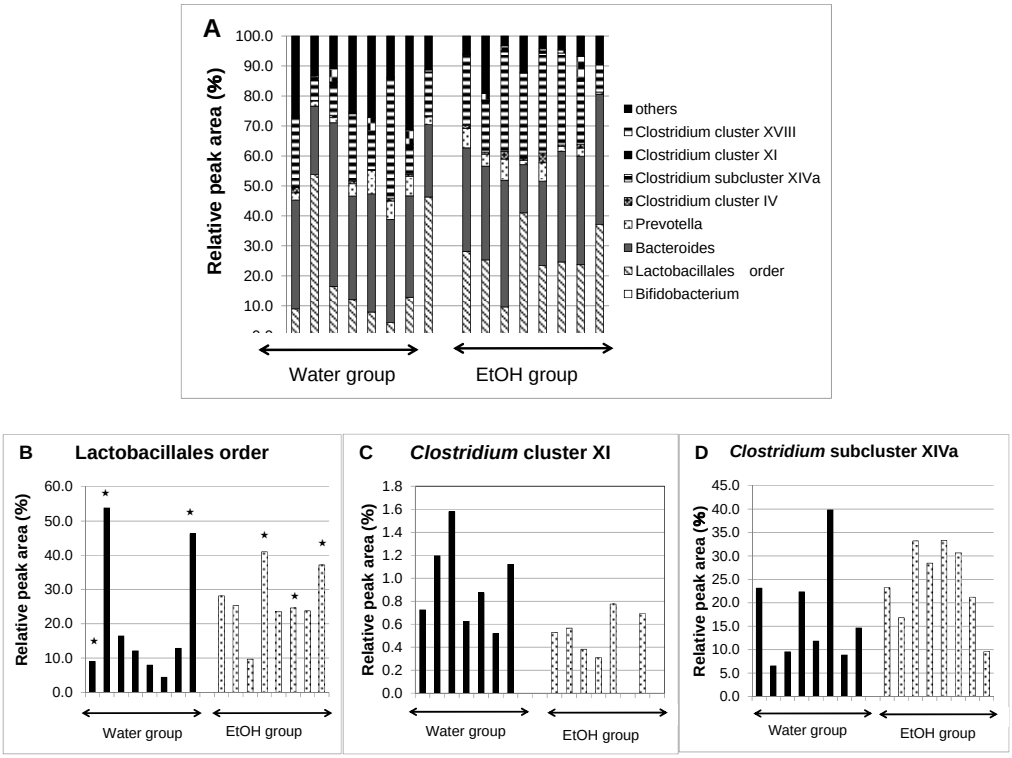

Figure 6. Comparison of the Intestinal Flora by T-RFLP Analyses of Mice Feces between the Water and EtOH Groups at the Age of 87 Weeks.
A. Bacterial classification of the intestinal flora of each mouse. (Water group, n = 8; EtOH group, n = 8)
B. Ratio of Lactobacillus order in the intestinal flora of each mouse. *: dead mice within 3 weeks from feces sampling.
C. Ratio of *Clostridium* Cluster XI in the intestinal flora of each mouse.
D. Ratio of *Clostridium* Cluster XIVa in the intestinal flora of each mouse.

the groups (data not shown), probably because the individual differences were large. In contrast, drinking 1% EtOH significantly protected against the aging of hair condition (glossiness, coarseness, and hair loss) and spinal curvature. Moderate drinking showed anti-aging effects (Figures 3A and 3B). Osaki et al. reported that the aging scores in SAMP1 mice drinking 1% EtOH were lower than those drinking water or 2% EtOH (22). The intake of 1% EtOH had anti-aging effects in SAMP lines. The SAMP lines are often used to evaluate the anti-aging effects of anti-oxidant foods such as reduced coenzyme Q10 (57) and teas (58, 59). Ethanol is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase, microsomal ethanol oxidation system (MEOS), and catalase. Ethanol metabolism causes oxidative stress and alcohol-related diseases (60, 61). Chronic and high-dose alcohol consumption has been shown to induce the MEOS and catalase that leads to the production of free radicals and oxidative stress (62). In contrast, the low dose alcohol intake showed anti-aging effects, like anti-oxidative food intake, in this study. Therefore, it can be suggested that the low-dose alcohol intake might induce anti-aging effects.

Lifespan

The average lifespan of mice in the EtOH group was about one month longer than mice in the water group (Figure 2D). We speculated that the cause of death for each mouse in both groups was different. Some mice died suddenly due to cancer (e.g., ascites with the increased body weight) or from aging, although they ate food the day before their death.

Moderate EtOH intake increases the levels of high-density lipoprotein cholesterol (63), decreasing the risk of coronary heart disease (CHD). However, the SAMP8 model is not a CHD model like the Apo-E knockout mice. Additionally, in this study, the SAMP8 model were fed on normal food (CRF-1), but not on high fat or high cholesterol food. Therefore, we did not measure the serum cholesterol levels. Knoops et al. reported that the physical activity and non-smoking were associated with a lower risk of all-cause mortality (64). In this study, there was no difference in term of food consumption and body weight between the groups, indicating similar feeding efficiency. Therefore, we speculated that there was no difference in term of physical activities between the groups.

Thus, the lifelong low dose EtOH intake did not negatively affect the survival of SAMP8 model fed on the normal diet.

ORT and OLT

Areas in the brain such as the insular cortex (65), perirhinal cortex (66), and the ventromedial prefrontal cortex (67) are associated with the object recognition in ORT, while the hippocampus, especially the CA1 region, is associated with the spatial recognition in OLT (68, 69).

The young SAMP8 model recognized small and novel objects in ORT (Figure 4A and Figure 4B); however, at 23 weeks of age, they did not. Once the larger objects replaced the small objects, the animals recognized the objects at the age of 30 weeks. The object selection was important for ORT. The recognition indexes of both the water and EtOH groups for the novel object (right) were significantly higher than those for the familiar object (left) until the age of 79 weeks, respectively. However, a significant difference was only observed for the EtOH group at the age of 86 weeks (Figures 4A and 4B). Mice in both groups presented a better long-term memory than we expected.

Murai et al. reported that there were significant differences in location of the familiar location and the novel location objects for only short-time memory (the interval between training and test within 2 h) in ICR mice (36). We also found that DBA/2 mice were unsuitable for OLT for the long-term memory (24 h) (data not shown). As shown in Figures 5A and 5B, the young SAMP8 model recognized the novel location, even, small objects (70 min, short-term memory). After changing the small OLT objects with larger objects, mice in both groups recognized the novel location (long-term memory) at the age of 52 weeks as well as at the age of 37–38 weeks (by using large apple object of woody color, short-term memory). The loss of spatial recognition in OLT (Figures 5A and 5B) progressed faster than that of object recognition in ORT (Figures 4A and 4B). The SAMP8 model possessed a long-term memory of spatial recognition under the conditions for OLT used in this study. However, the impairment of spatial recognition was observed in the water-group mice at the age of 59 weeks. The recognition index of the object at the novel location was significantly higher only in the EtOH group at the age of 59 weeks and

remained higher until the age of 73 weeks. Thus, the intake of a low dose of EtOH for long period protected against the loss of spatial memory by aging.

Matsui et al. reported that SAMP8 model show impairment of learning and memory at the age of 4 and 6 months in ORT (70). Similarly, Yokozawa et al. reported that SAMP8 model presented a deficit in object recognition and spatial recognition at 41-42 weeks of age (71). In these reports, the mice were younger than in our study when the memory deficit was detected. Furthermore, the experimental conditions were not the same, e.g., the type of objects and the experimental procedures.

Stragier et al. reported that chronic and moderate EtOH intake produces marked epigenetic changes that result in the overexpression of brain-derived neurotrophic factor (BDNF) and downstream hippocampal neurogenesis in C57BL/6J mice (72). In contrast, Ebada et al. reported that C57BL/6J mice suffered from memory deficit due to a chronic intermittent intake of a low dose (1% EtOH) of ethanol (73). They used the two-bottle choice (all procedures took about 2 months) and showed a reduction in the hippocampal BDNF levels, although 1% alcohol with a low concentration of corticosterone improved the memory deficit in both the ORT and OLT (73).

Yuan et al. reported that an enriched environment improves cognitive performance in SAMP8 model (74). The BDNF plays an intrinsic role in improving the synaptic plasticity and cognition by environmental enrichment (75, 76).

In this study, we could not evaluate if the continuous measurement of ORT and OLT every 7 weeks stressed or enriched the life of SAMP8 model. However, not only low-dose EtOH but also low stress or enrichment-like physical activities by repeated ORT and OLT unlike mice housed in the standard environment may contribute to improving the memory deficit with aging.

Diarrhea Symptoms

Diarrhea appeared only in the water group at about 82 weeks of age. At the age of 87 weeks, the number of mice presenting diarrhea in the water group was significantly higher than that in the EtOH group. The diarrhea symptoms in mice continued until death. Eighteen mice of the water group were divided into 3 cages at the beginning of this study (at the age of 5 weeks). Five diarrhea mice were detected in all 3 cages at the similar ages. Therefore, it was speculated that the increase in the number of mice with diarrhea was spontaneously caused in each mouse, but not caused by mouse-to-mouse infection within a cage. We speculated that the intestinal flora was affected by the low-dose alcohol intake and prevented diarrhea in the EtOH-group mice.

T-RFLP Flora Analysis

The results of T-RFLP flora analysis of the feces at the age of 87 weeks varied for each mouse (Figure 6A). Between-group differences were observed in Lactobacillales order, *Clostridium* cluster XI, and other orders (including multiple candidates). Mitsuoka et al. and other researchers have reported that the intestinal flora changes with aging and the *Lactobacillus* strains are increased with aging (37, 38), although some *Lactobacillus* strains are used as probiotic strains (53, 77). In this study, the average ratio of Lactobacillales order in the EtOH group was higher than that in the water group. This result seemed to be opposite to the anti-aging phenomenon. Surprisingly, some mice nearing death (within 3 weeks) in both groups presented an extremely high ratio of Lactobacillales order (Figure 6B). These results suggest the complexity of *Lactobacillus* contribution to the functions of the intestinal flora. The role of Lactobacillales order intestinal flora might result from a balance between the probiotic-like positive effects and dominant negative effects close to the death.

Clostridium difficile causes diarrhea (78) and is a member of *Clostridium* cluster XI. In this study, the mice presenting diarrhea (n = 5) showed significantly higher *Clostridium* cluster XI than that in mice with no symptoms of diarrhea (n = 11) (Figure 6C).

It was recently demonstrated that not all *Clostridium* species in the intestinal flora are bad for health (79). Furusawa et al. reported that commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells that play a central role in the cell-mediated immunity (79). *Clostridium* subcluster XIVa, which is mainly composed of butyrate-producing strains, was slightly higher in the EtOH group than in the water group (Figure 6D). Finally, we speculated that the difference in the spinal curvature was associated with the difference in calcium absorption by the

flora in the intestine.

Yang et al. reported that dietary lactoferrin up-regulates the intestinal gene expression of BDNF, ubiquitin thiolesterase (UCHL1), and alkaline phosphatase activity to alleviate early weaning diarrhea (80). The gut–brain axis is the biochemical signaling between the central and the enteric nervous system, linking emotional and cognitive centers of the brain with peripheral intestinal functions (81). Although the causal association between the intestinal flora and aging progression could not be demonstrated from these results, the change in the intestinal flora might contribute to the phenotype difference between the water and EtOH groups.

The lifelong clinical study is impractical in human under various life environments. Therefore, animal studies are important to understand EtOH intake in human as pre-clinical studies. We believe that some aspects of moderate alcohol on human health aging are extrapolatable from animal data. The relative density of microflora between human and mice are different (52, 82-85). For example, that of *Lactobacillus* in mice is higher than in human, while that of *Bifidobacterium* in human is higher than in mice. In addition, there are many examples of food ingredients on microflora changes in rodent. These changes are similar to those in human microflora (86-93).

Overall Effects

Our results confirmed the previous reports that moderate alcohol consumption is associated with improved cognitive functions (7-11, 94).

The effect of moderate alcohol intake, harm or benefit, depends on the individual constitution or living environment in the human.

CONCLUSIONS

The effects of low-dose alcohol intake on aging were investigated using SAMP8 model. Low-dose alcohol intake had no negative effect on the lifespan. The average lifespan in the EtOH group was about 4 weeks longer than that in the water group. The spatial memory deficit, skin aging, and the spinal curvature progressed more slowly in the low-dose EtOH group compared to the water group. Moreover, the diarrhea symptoms only appeared in the water group and the intestinal flora was different between the water and EtOH groups. Although further research is required to identify the mechanisms underlying the effects of chronic low-dose EtOH intake on the intestinal flora, the spatial memory, and aging and their relationship, our results suggested that the low-dose alcohol intake has positive effects on healthy aging in SAMP8 model.

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CONFLICT OF INTEREST

Toshitaka Nabeshima serves as a consultant to SAPPORO HOLDINGS LTD. Chikako Shimizu, Yutaka Mitani, and Youichi Tsuchiya are employees of SAPPORO HOLDINGS LTD. Yasuhiro Oki is an employee of SAPPORO BREWERIES LTD.

REFERENCES

1. Schmidt W, Popham RE. Heavy alcohol consumption and physical health problems: a review of the epidemiological evidence. Drug Alcohol Depend, 1975; 1:27-50. DOI: http://dx.doi.org/10.1016/0376-8716(75)90005-8.
2. Bagnardi V, Blangiardo M, La Vecchia C, Corrao G. Alcohol consumption and the risk of cancer. a meta-analysis. Alcohol Res. Health. 2001; 25: 263-270.
3. Marmot MG, Rose G, Shipley MJ, Thomas BJ. Alcohol and mortality: a U-shaped curve. Lancet, 1981; 14:580-3. DOI: http://dx.doi.org/10.1016/s0140-6736(81)92032-8.
4. Higuchi S, Matsushita S, Maesato H, Osaki Y. Japan: alcohol today. Addiction, 2007; 102:1849-62. DOI: http://dx.doi.org/10.1111/j.1360-0443.2007.01902.x.
5. Wannamethee SG, Shaper AG. Alcohol, coronary heart disease and stroke: an examination of the J-shaped curve. Neuroepidemiology, 1998; 17:288-95. DOI: http://dx.doi.org/10.1159/000026182.
6. Ruitenberg A, van Swieten JC, Witteman JC, Mehta KM, van Duijn CM, Hofman A, Breteler MM. Alcohol consumption and risk of dementia: the Rotterdam Study. Lancet, 2002; 359:281-6. DOI: http://dx.doi.org/10.1016/s0140-6736(02)07493-7.

7. Letenneur L. Risk of dementia and alcohol and wine consumption: a review of recent results. Biol Res, 2004; 37:189-93. DOI: http://dx.doi.org/10.4067/s0716-97602004000200003.
8. Anstey KJ, Mack HA, Cherbuin N. Alcohol consumption as a risk factor for dementia and cognitive decline: meta-analysis of prospective studies. Am J Geriatr Psychiatry, 2009; 17:542-55. DOI: http://dx.doi.org/10.1097/jgp.0b013e3181a2fd07.
9. Neafsey EJ, Collins MA. Moderate alcohol consumption and cognitive risk. Neuropsychiatr Dis Treat, 2011; 7:465-84. DOI: http://dx.doi.org/10.2147/ndt.s23159.
10. Kim JW, Lee DY, Lee BC, Jung MH, Kim H, Choi YS, Choi IG. Alcohol and cognition in the elderly: a review. Psychiatry Investig, 2012; 9:8-16. DOI: http://dx.doi.org/10.4306/pi.2012.9.1.8.
11. Peters R, Peters J, Warner J, et al. Alcohol, dementia and cognitive decline in the elderly: a systematic review, Age Ageing 2008; 37:505-512. DOI: http://dx.doi.org/10.1093/ageing/afn095.
12. Klatsky AL, Armstrong MA, Friedman GD. Risk of cardiovascular mortality in alcohol drinkers, ex-drinkers and nondrinkers. Am J Cardiol, 1990; 66:1237-42. DOI: http://dx.doi.org/10.1016/0002-9149(90)91107-h.
13. Fillmore KM, Stockwell T, Chikritzhs T, Bostrom A, Kerr W. Moderate alcohol use and reduced mortality risk: systematic error in prospective studies and new hypotheses. Ann Epidemiol, 2007; S16-23. DOI: http://dx.doi.org/10.1016/j.annepidem.2007.01.005.
14. Bhala N, Cézard G, Ward HJ, Bansal N, Bhopal R; Scottish Health and Ethnicity Linkage Study (SHELS) Collaboration. Ethnic variations in liver- and alcohol-related disease hospitalisations and mortality: The Scottish health and ethnicity linkage study. Alcohol Alcohol. 2016; 51:593-601. DOI: http://dx.doi.org/10.1093/alcalc/agw018.
15. Martin CG, Agapito VV, Obeso A, Prieto-Lloret J, Bustamante R, Castañeda J, Agapito T, Gonzalez C. Moderate ethanol ingestion, redox status, and cardiovascular system in the rat. Alcohol, 2011; 45: 381-91. DOI: http://dx.doi.org/10.1016/j.alcohol.2010.08.003.
16. Liu W, Redmond EM, Morrow D, Cullen JP. Differential effects of daily-moderate versus weekend-binge alcohol consumption on atherosclerotic plaque development in mice. Atherosclerosis, 2011; 219:448-54. DOI: http://dx.doi.org/10.1016/j.atherosclerosis.2011.08.034.
17. Shimizu C, Oki Y, Mitani Y, Nakamura T, Nabeshima T. Factors affecting ethanol-induced conditioned place preference and locomotor sensitization in mice. Biol Pharm Bull, 2015; 38:1935-45. DOI: http://dx.doi.org/10.1248/bpb.b15-00626.
18. Osaki A, Okazaki Y, Kimoto A, Izu H, Kato N. Beneficial effect of low dose of ethanol on liver function and serum urate in rats fed a high-fat diet. J Nutr Sci Vitaminol, 2014; 60: 408-412. DOI: http://dx.doi.org/10.3177/jnsv.60.408.
19. Nammi S, Roufogalis BD. Light-to-moderate ethanol feeding augments AMPK-α phosphorylation and attenuates SREBP-1 expression in the liver of rats. J Pharm Pharm Sci, 2013; 16:342-51. DOI: http://dx.doi.org/10.18433/j3788x.
20. Kalev-Zylinska ML, During MJ. Paradoxical facilitatory effect of low-dose alcohol consumption on memory mediated by NMDA receptors. J Neurosci, 2007; 27:10456-67. DOI: http://dx.doi.org/10.1523/jneurosci.2789-07.2007.
21. Bate C, Williams A. Ethanol protects cultured neurons against amyloid-β and α-synuclein-induced synapse damage. Neuropharmacology, 2011; 61:1406-12. DOI: http://dx.doi.org/10.1016/j.neuropharm.2011.08.030.
22. Osaki A, Kimoto A, Kato N, Izu H, Matsumaru K. Beneficial effect of low intake of alcohol on high-fat diet fed rats and senescence-accelerated mice (SAMP1). IUNS 20th International Congress of Nutrition Book of Abstracts, 2013; PO1873.
23. Takeda T, Hosokawa M., Takeshita S, Irino M, Higuchi K, Matushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M and Yamamuro T. A new murine model of accelerated senescence. Mech. Ageing Dev, 1981; 17:183-194. DOI: http://dx.doi.org/10.1016/0047-6374(81)90084-1.
24. Takahashi R. Anti-aging studies on the senescence accelerated mouse (SAM) strains. Yakugaku Zasshi. 2010; 130:11-8 (Japanese). DOI: http://dx.doi.org/10.1248/yakushi.130.11.
25. Kawamata T, Akiguchi I, Yagi H, Irino M, Sugiyama H, Akiyama H, Shimada A, Takemura M, Ueno M, Kitabayashi T, Ohnishi K, Seriu N, Higuchi K, Hosokawa M, Takeda T. Neuropathological studies on strains of senescence-accelerated mice (SAM) with age-related deficits in learning and memory. Exp Gerontol, 1997; 32:161-9. DOI: http://dx.doi.org/10.1016/s0531-5565(96)00063-0.
26. Takeda T. Senescence-accelerated mouse (SAM) with special references to neurodegeneration models, SAMP8 and SAMP10 mice. Neurochem Res, 2009; 34:639-59. DOI: http://dx.doi.org/10.1007/s11064-009-9922-y.
27. Butterfield DA, Poon HF. The senescence-accelerated prone mouse (SAMP8): a model of age-related cognitive decline with relevance to alterations of the gene expression and protein

abnormalities in Alzheimer's disease. Exp Gerontol, 2005; 40:774-83. DOI: http://dx.doi.org/10.1016/j.exger.2005.05.007.
28. Morley JE, Armbrecht HJ, Farr SA, Kumar VB. The senescence accelerated mouse (SAMP8) as a model for oxidative stress and Alzheimer's disease. Biochim Biophys Acta, 2012; 1822:650-6. DOI: http://dx.doi.org/10.1016/j.bbadis.2011.11.015.
29. Kawamata T, Akiguchi I, Maeda K, Tanaka C, Higuchi K, Hosokawa M, Takeda T. Age-related changes in the brains of senescence-accelerated mice (SAM): association with glial and endothelial reactions. Microsc Res Tech, 1998; 43:59-67. DOI: http://dx.doi.org/10.1002/(sici)1097-0029(19981001)43:1<59::aid-jemt9>3.0.co;2-x.
30. Komatsu T, Chiba T, Yamaza H, Yamashita K, Shimada A, Hoshiyama Y, Henmi T, Ohtani H, Higami Y, de Cabo R, Ingram DK, Shimokawa I. Manipulation of caloric content but not diet composition, attenuates the deficit in learning and memory of senescence-accelerated mouse strain P8. Exp Gerontol, 2008; 43:339-46. DOI: http://dx.doi.org/10.1016/j.exger.2008.01.008.
31. Wang F, Chen H, Sun X. Age-related spatial cognitive impairment is correlated with a decrease in ChAT in the cerebral cortex, hippocampus and forebrain of SAMP8 mice. Neurosci Lett, 2009; 454:212-7. DOI: http://dx.doi.org/10.1016/j.neulet.2009.03.030.
32. Wang HM, Wang LW, Liu XM, Li CL, Xu SP, Farooq AD. Neuroprotective effects of forsythiaside on learning and memory deficits in senescence-accelerated mouse prone (SAMP8) mice. Pharmacol Biochem Behav, 2013; 105:134-41. DOI: http://dx.doi.org/10.1016/j.pbb.2012.12.016.
33. Brooks SP, Pask T, Jones L, Dunnett SB. Behavioural profiles of inbred mouse strains used as transgenic backgrounds. II: cognitive tests. Genes Brain Behav, 2005;4:307-17. DOI: http://dx.doi.org/10.1111/j.1601-183x.2004.00109.x.
34. Kamei H, Nagai T, Nakano H, Togan Y, Takayanagi M, Takahashi K, Kobayashi K, Yoshida S, Maeda K, Takuma K, Nabeshima T, Yamada K. Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. Biol Psychiatry, 2006; 59:75-84. DOI: http://dx.doi.org/10.1016/j.biopsych.2005.06.006.
35. Wang D, Noda Y, Zhou Y, Mouri A, Mizoguchi H, Nitta A, Chen W, Nabeshima T. The allosteric potentiation of nicotinic acetylcholine receptors by galantamine ameliorates the cognitive dysfunction in beta amyloid25-35 i.c.v.-injected mice: involvement of dopaminergic systems. Neuropsychopharmacology; 2007; 32:1261-71. DOI: http://dx.doi.org/10.1038/sj.npp.1301256.
36. Murai T, Okuda S, Tanaka T, Ohta H. Characteristics of object location memory in mice: Behavioral and pharmacological studies. Physiology & Behavior, 2007; 90,116-124. DOI: http://dx.doi.org/10.1016/j.physbeh.2006.09.013.
37. Mitsuoka T, Hayakawa K. The fecal flora in man. I. Composition of the fecal flora of various age groups. Zentralbl Bakteriol Orig A, 1973; 223:333-42 (German).
38. Mitsuoka T. Intestinal flora and aging. Nutr Rev; 1992; 50:438-46. DOI: http://dx.doi.org/10.1111/j.1753-4887.1992.tb02499.x.
39. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, Abe F, Osawa R. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol. 2016; 16:90. DOI: http://dx.doi.org/10.1186/s12866-016-0708-5.
40. O'Toole PW, Jeffery IB. Gut microbiota and aging. Science, 2015; 350:1214-5. DOI: http://dx.doi.org/10.1126/science.aac8469.
41. Grenham S, Clarke G, Cryan JF, Dinan TG. Brain-gut-microbe communication in health and disease. Front Physiol, 2011; 2:94. DOI: http://dx.doi.org/10.3389/fphys.2011.00094.
42. Bercik P, Collins SM, Verdu EF. Microbes and the gut-brain axis. Neurogastroenterol Motil, 2012; 24:405-13. DOI: http://dx.doi.org/10.1111/j.1365-2982.2012.01906.x.
43. Caracciolo B, Xu W, Collins S, Fratiglioni L. Cognitive decline, dietary factors and gut-brain interactions. Mech Ageing Dev, 2014; 136-137:59-69. DOI: http://dx.doi.org/10.1016/j.mad.2013.11.011.
44. Gabbard SL, Lacy BE, Levine GM, Crowell MD. The impact of alcohol consumption and cholecystectomy on small intestinal bacterial overgrowth. Dig Dis Sci; 2014; 59:638-44. DOI: http://dx.doi.org/10.1007/s10620-013-2960-y.
45. Cuervo A, Reyes-Gavilán CG, Ruas-Madiedo P, Lopez P, Suarez A, Gueimonde M, González S. Red wine consumption is associated with fecal microbiota and malondialdehyde in a human population. J Am Coll Nutr, 2015; 34:135-41. DOI: http://dx.doi.org/10.1080/07315724.2014.904763.
46. Dole VP, Gentry RT. Toward an analogue of alcoholism in mice: scale factors in the model. Proc Natl Acad Sci U S A, 1984; 81:3543-6. DOI: http://dx.doi.org/10.1073/pnas.81.11.3543.
47. Holman CD, English DR, Milne E, Winter MG. Meta-analysis of alcohol and all-cause mortality: a validation of NHMRC recommendations. Med J Aust. 1996; 164:141-5. PMID: 8628131.
48. Martin CG, Agapito VV, Obeso A, Prieto-Lloret J, Bustamante R, Castañeda J, Agapito T, Gonzalez C. Moderate ethanol ingestion, redox status, and

cardiovascular system in the rat. Alcohol, 2011; 45:381-91. DOI: http://dx.doi.org/10.1016/j.alcohol.2010.08.003.
49. Sun H, Xiong W, Arrick DM, Mayhan WG. Low-dose alcohol consumption protects against transient focal cerebral ischemia in mice: possible role of PPARγ. PLoS One, 2012; 7:e41716. DOI: http://dx.doi.org/10.1371/journal.pone.0041716.
50. Hosokawa M, Kasai R, Higuchi K, Takeshita S, Shimizu K, Hamamoto H, Honma A, Irino M, Toda K, Matsumura A, Matsushita M, Takeda T. Grading score system: a method for evaluation of the degree of senescence in senescence accelerated mouse (SAM). Mech Ageing Dev, 1984; 26:91-102. DOI: http://dx.doi.org/10.1016/0047-6374(84)90168-4.
51. Nagashima K, Mochizuki J, Hisada T, Suzuki S, Shimomura K. Phylogenetic analysis of 16S ribosomal RNA Gene Sequences from human fecal microbiota and improved utility of terminal restriction fragment length polymorphism profiling. Biosci Microflora, 2006; 25:99–107. DOI: http://dx.doi.org/10.12938/bifidus.25.99.
52. Emoto T, Yamashita T, Sasaki N, Hirota Y, Hayashi T, So A, Kasahara K, Yodoi K, Matsumoto T, Mizoguchi T, Ogawa W, Hirata K. Analysis of gut microbiota in coronary artery disease patients: a possible link between gut microbiota and coronary artery disease. J Atheroscler Thromb. 2016; 23:908-21. DOI: http://dx.doi.org/10.5551/jat.32672.
53. Mitsuoka T. Development of functional foods. Biosci Microbiota Food Health, 2014; 33:117-28. DOI: http://dx.doi.org/10.12938/bmfh.33.117.
54. Hwa LS, Chu A, Levinson SA, Kayyali TM, DeBold JF, Miczek KA. Persistent escalation of alcohol drinking in C57BL/6J mice with intermittent access to 20% ethanol. Alcohol Clin Exp Res, 2011; 35:1938-47. DOI: http://dx.doi.org/10.1111/j.1530-0277.2011.01545.x.
55. Cuesta S, Kireev R, Forman K, García C, Acuña D, Vara E, Tresguerres JA. Growth hormone can improve insulin resistance and differentiation in pancreas of senescence accelerated prone male mice (SAMP8). Growth Horm IGF Res, 2011; 21:63-8. DOI: http://dx.doi.org/10.1016/j.ghir.2010.12.007.
56. Umegaki H. Type 2 diabetes as a risk factor for cognitive impairment: current insights. Clin Interv Aging. 2014; 9:1011-9. DOI: http://dx.doi.org/10.2147/CIA.S48926.
57. Yan J, Fujii K, Yao J, Kishida H, Hosoe K, Sawashita J, Takeda T, Mori M, Higuchi K. Reduced coenzyme Q10 supplementation decelerates senescence in SAMP1 mice. Exp Gerontol, 2006; 41:130-40. DOI: http://dx.doi.org/10.1016/j.exger.2005.11.007.
58. Chan YC, Hosoda K, Tsai CJ, Yamamoto S, Wang MF. Chan YC1, Hosoda K, Tsai CJ, Yamamoto S, Wang MF. Favorable effects of tea on reducing the cognitive deficits and brain morphological changes in senescence-accelerated mice. J Nutr Sci Vitaminol (Tokyo), 2006; 52:266-73. DOI: http://dx.doi.org/10.3177/jnsv.52.266.
59. Unno K, Takabayashi F, Yoshida H, Choba D, Fukutomi R, Kikunaga N, Kishido T, Oku N, Hoshino M. Daily consumption of green tea catechin delays memory regression in aged mice. Biogerontology, 2007; 8:89-95. DOI: http://dx.doi.org/10.1007/s10522-006-9036-8.
60. Mansouri A, Demeilliers C, Amsellem S, Pessayre D, Fromenty B. Acute ethanol administration oxidatively damages and depletes mitochondrial DNA in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. J Pharmacol Exp Ther, 2001; 298:737-43. PMID:11454938.
61. Zima T, Fialová L, Mestek O, Janebová M, Crkovská J, Malbohan I, Stípek S, Mikulíková L, Popov P. Oxidative stress, metabolism of ethanol and alcohol-related diseases. J Biomed Sci, 2001; 8:59-70. DOI: http://dx.doi.org/10.1007/bf02255972.
62. Ishii H, Horie Y, Tomita K, Yamagishi Y. Recent progress in alcoholic liver diseases. Nihon Shokakibyo Gakkai Zasshi, 2003; 100:1187-97. (Japanese)
63. Frohlich JJ. Effects of alcohol on plasma lipoprotein metabolism. Clin Chim Acta, 1996; 246:39-49. DOI: http://dx.doi.org/10.1016/0009-8981(96)06225-0.
64. Knoops KT, de Groot LC, Kromhout D, Perrin AE, Moreiras-Varela O, Menotti A, van Staveren WA. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. JAMA, 2004; 292:1433-9. DOI: http://dx.doi.org/10.1001/jama.292.12.1433.
65. Bermudez-Rattoni F, Okuda S, Roozendaal B, McGaugh JL. Insular cortex is involved in consolidation of object recognition memory. Learn. Mem, 2005; 12:447-449. DOI: http://dx.doi.org/10.1101/lm.97605.
66. Winters BD, Forwood SE, Cowell RA, Saksida LM, Bussey TJ. Double dissociation between the effects of peri-postrhinal cortex and hippocampal lesions on tests of object recognition and spatial memory: heterogeneity of function within the temporal lobe. J Neurosci, 2004; 24:5901-8. DOI: http://dx.doi.org/10.1523/jneurosci.1346-04.2004.
67. Akirav I, Maroun M. Ventromedial prefrontal cortex is obligatory for consolidation and reconsolidation of object recognition memory. Cereb Cortex, 2006; 216:1759-65. DOI: http://dx.doi.org/10.1093/cercor/bhj114.
68. Mumby DG, Gaskin S, Glenn MJ, Schramek TE, Lehmann H. Hippocampal damage and exploratory

preferences in rats: memory for objects, places, and contexts. Learn Mem, 2002; 9:49-57. DOI: http://dx.doi.org/10.1101/lm.41302.

69. Haettig J, Stefanko DP, Multani ML, Figueroa DX, McQuown SC, Wood MA. HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. Learn Mem, 2011; 18:71-9. DOI: http://dx.doi.org/10.1101/lm.1986911.
70. Matsui N, Takahashi K, Takeichi M, Kuroshita T, Noguchi K, Yamazaki K, Tagashira H, Tsutsui K, Okada H, Kido Y, Yasui Y, Fukuishi N, Fukuyama Y, Akagi M. Magnolol and honokiol prevent learning and memory impairment and cholinergic deficit in SAMP8 mice. Brain Res, 2009; 1305:108-17. DOI: http://dx.doi.org/10.1016/j.brainres.2009.09.107.
71. Yokozawa T, Lee YA, Cho EJ, Matsumoto K, Park CH, Shibahara N. Anti-aging effects of oligomeric proanthocyanidins isolated from persimmon fruits. Drug Discov Ther, 2011; 5:109-18. DOI: http://dx.doi.org/10.5582/ddt.2011.v5.3.109.
72. Stragier E, Massart R, Salery M, Hamon M, Geny D, Martin V, Boulle F, Lanfumey L. Ethanol-induced epigenetic regulations at the Bdnf gene in C57BL/6J mice. Mol Psychiatry, 2015; 20:405-12. DOI: http://dx.doi.org/10.1038/mp.2014.38.
73. Ebada ME, Latif LM, Kendall DA, Pardon MC. Corticosterone protects against memory impairments and reduced hippocampal BDNF levels induced by a chronic low dose of ethanol in C57BL/6J mice. Rom J Morphol Embryol, 2014; 55:1303-16. PMID:25611260.
74. Yuan Z, Wang M, Yan B, Gu P, Jiang X, Yang X, Cui D. An enriched environment improves cognitive performance in mice from the senescence-accelerated prone mouse 8 strain: Role of upregulated neurotrophic factor expression in the hippocampus. Neural Regen Res, 2012; 7:1797-804. DOI: http://dx.doi.org/10.3969/j.issn.1673-5374.2012.23.006.
75. Novkovic T, Mittmann T, Manahan-Vaughan D. BDNF contributes to the facilitation of hippocampal synaptic plasticity and learning enabled by environmental enrichment. Hippocampus, 2015; 25:1-15. DOI: http://dx.doi.org/10.1002/hipo.22342.
76. Griñan-Ferré C, Pérez-Cáceres D, Gutiérrez-Zetina SM, Camins A, Palomera-Avalos V, Ortuño-Sahagún D, Rodrigo MT, Pallàs M. Environmental enrichment improves behavior, cognition, and brain functional markers in young senescence-accelerated prone mice (SAMP8). Mol Neurobiol, 2016; 53:2435-50. DOI: http://dx.doi.org/10.1007/s12035-015-9210-6.
77. Hébuterne X. Gut changes attributed to ageing: effects on intestinal microflora. Curr Opin Clin Nutr Metab Care, 2003; 6:49-54. DOI: http://dx.doi.org/10.1097/00075197-200301000-00008.
78. Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A. Evidence-based review of probiotics for antibiotic-associated diarrhea and Clostridium difficile infections. Anaerobe, 2009; 15:274-80. DOI: http://dx.doi.org/10.1016/j.anaerobe.2009.09.002.
79. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature, 2013; 504:446-50. DOI: http://dx.doi.org/10.1038/nature12721.
80. Yang C, Zhu X, Liu N, Chen Y, Gan H, Troy FA 2nd, Wang B. Lactoferrin up-regulates intestinal gene expression of brain-derived neurotrophic factors BDNF, UCHL1 and alkaline phosphatase activity to alleviate early weaning diarrhea in postnatal piglets. J Nutr Biochem, 2014; 25:834-42. DOI: http://dx.doi.org/10.1016/j.jnutbio.2014.03.015.
81. Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann Gastroenterol, 2015; 28:203-209. PMCID: PMC4367209.
82. Ishikawa, H., Takaki, A., Tsuzaki, R., Yasunaka, T., Koike, K., Shimomura, Y., Seki, H., Matsushita, H., Miyake, Y. & Ikeda, F. L-carnitine prevents progression of non-alcoholic steatohepatitis in a mouse model with upregulation of mitochondrial pathway. PLoS One. 2014; 9:e100627. DOI: http://dx.doi.org/10.1371/journal.pone.0100627.
83. Jin, J. S., Touyama, M., Hisada, T. & Benno, Y. Effects of green tea consumption on human fecal microbiota with special reference to Bifidobacterium species. Microbiol Immunol. 2012; 56:729-39. DOI: http://dx.doi.org/10.1111/j.1348-0421.2012.00502.x.
84. Yoda K, He F, Miyazawa K, Kawase M, Kubota A, Hiramatsu M. Orally administered heat-killed Lactobacillus gasseri TMC0356 alters respiratory immune responses and intestinal microbiota of diet-induced obese mice. J Appl Microbiol. 2012; 113:155-62. DOI: http://dx.doi.org/10.1111/j.1365-2672.2012.05316.x.
85. Jin JS, Touyama M, Kibe R, Tanaka Y, Benno Y, Kobayashi T, Shimakawa M, Maruo T, Toda T, Matsuda I, Tagami H, Matsumoto M, Seo G, Chonan O, Benno Y. Analysis of the human intestinal microbiota from 92 volunteers after ingestion of identical meals. Benef Microbes. 2013;

4:187-93. DOI: http://dx.doi.org/10.3920/BM2012.0045.
86. Sakai K, Aramaki K, Takasaki M, Inaba H, Tokunaga T, Ohta A. Effect of dietary short-chain fructooligosaccharides on the cecal microflora in gastrectomized rats. Biosci Biotechnol Biochem. 2001; 65:264-9. DOI: http://dx.doi.org/10.1271/bbb.65.264.
87. Nakamura S, Kondo N, Yamaguchi Y, Hashiguchi M, Tanabe K, Ushiroda C, Kawahashi-Tokuhisa M, Yui K, Miyakoda M, Oku T. Daily feeding of fructooligosaccharide or glucomannan delays onset of senescence in SAMP8 mice. Gastroenterol Res Pract. 2014; 2014: Article ID 303184. DOI: http://dx.doi.org/10.1155/2014/303184.
88. Tokunaga T, Nakada Y, Tashiro Y, Hirayama M, Hidaka H. Effects of Fructooligosaccharides Intake on the Intestinal Microflora and Defecation in Healthy Volunteers. Bifidus. 1993; 6:143-150. (Japanese) DOI: https://www.jstage.jst.go.jp/article/jim1987/6/2/6_2_143/_pdf.
89. Paineau D, Respondek F, Menet V, Sauvage R, Bornet F, Wagner A. Effects of short-chain fructooligosaccharides on faecal bifidobacteria and specific immune response in formula-fed term infants: a randomized, double-blind, placebo-controlled trial. J Nutr Sci Vitaminol (Tokyo). 2014; 60:167-75. DOI: http://doi.org/10.3177/jnsv.60.167.
90. Kaur S, Vaishnavi C, Prasad KK, Ray P, Kochhar R. Effect of Lactobacillus acidophilus & epidermal growth factor on experimentally induced Clostridium difficile infection. Indian J Med Res. 2011; 133:434-41. PMCID: PMC3103179.
91. Lau CS, Chamberlain RS. Probiotics are effective at preventing Clostridium difficile-associated diarrhea: a systematic review and meta-analysis. Int J Gen Med. 2016; 9:27-37. DOI: http://dx.doi.org/10.2147/IJGM.S98280.
92. Kanauchi O, Iwanaga T, Mitsuyama K, Saiki T, Tsuruta O, Noguchi K, Toyonaga A. Butyrate from bacterial fermentation of germinated barley foodstuff preserves intestinal barrier function in experimental colitis in the rat model. J Gastroenterol Hepatol. 1999; 14:880-8. DOI: http://dx.doi.org/10.1046/j.1440-1746.1999.01971.x.
93. Kanauchi O, Matsumoto Y, Matsumura M, Fukuoka M, Bamba T. The beneficial effects of microflora, especially obligate anaerobes, and their products on the colonic environment in inflammatory bowel disease. Curr Pharm Des. 2005; 11:1047-53. PMID: 15777254.
94. Reas ET, Laughlin GA, Kritz-Silverstein D, Barrett-Connor E, McEvoy LK. Moderate, regular alcohol consumption is associated with higher cognitive function in older community-dwelling adults. J Prev Alzheimers Dis, 2016; 3:105-113. DOI: http://dx.doi.org/10.14283/jpad.2016.89.