

Short Communication

Influence of long-term consumption of a *Lactococcus lactis* strain on the intestinal immunity and intestinal flora of the senescence-accelerated mouse

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The senescence-accelerated mouse develops normally until 5–6 months of age and then displays rapid and irreversible advancement of senescence manifesting as clinical signs and gross lesions. To clarify the effect of lactic acid bacteria on the physiological changes with increasing age, heat-killed *Lactococcus lactis* G50 was administered to 1-month-old senescence-accelerated-prone mouse (SAMP)6 mice for 11 months, a senescence-accelerated mouse strain that develops senile osteoporosis. Mice fed G50 gained more weight than the control mice (not fed G50) during the feeding experiment. Faecal IgA levels in the mice fed G50 at 3 months were higher than those of the control mice but decreased to control levels with increasing age. The numbers of viable cells of *Bacteroides* sp., *Lactobacillus* sp., *Staphylococcus* sp., *Enterococcus/Streptococcus* sp. and *Enterobacteriaceae* sp. in faeces were similar for mice fed the G50 and control diets at any age, but strain G50 suppressed the intestinal growth of H₂S-producing bacteria. Bone density of the thigh bone did not differ between aged G50 and control mice. Strain G50 would be a beneficial bacterium for the enhancement of intestinal immunity during youth and to suppress the growth of harmful intestinal bacteria. The applicability of strain G50 for the food and animal industries has been proposed in the present study.

Lactic acid bacteria: *Lactococcus*: Ageing: Senescence-accelerated mouse

Many countries are faced with the challenge of maintaining good health and quality of life in an ageing population living longer than ever before. Physiological phenomena associated with ageing include impaired memory acquisition, decreased immune responses, increased peroxidation *in vivo* and the loss of bone density. Intestinal microbiota also displays changes with increasing age, for example, the presence of *Bifidobacterium* sp. in the elderly may decrease⁽¹⁾. In addition, ageing compromises the intestinal mucosal immune responses in animals and human subjects⁽²⁾. Consequences of this immune dysfunction include increases in the incidences of infectious diseases and in the associated rates of morbidity and mortality in the elderly⁽²⁾.

The probiotic properties of lactic acid bacteria have garnered particular interest. Probiotics have been defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit to the host' by the FAO/WHO. For example, some lactic acid bacteria and bifidobacteria are immunostimulatory in elderly humans and animals. It has been reported that the *Lactobacillus casei* strain Shirota

activates immune responses in aged mice and ameliorates influenza viral infections⁽³⁾. The term 'immunobiotics' has been proposed to identify bacteria that promote health by driving mucosal immune mechanisms⁽⁴⁾. Many studies of the immunomodulatory activity of lactic acid bacteria include both viable and non-viable micro-organisms⁽⁵⁾. These and other studies have shown that the anti-inflammatory effects of probiotics are mediated by the bacterial DNA and that live micro-organisms are not required to attenuate murine experimental colitis⁽⁶⁾. Currently, little is known about the immunomodulatory activity of viable and non-viable lactic acid bacteria and their effect on the physiological changes associated with increasing age.

The senescence-accelerated mouse is a good model for studying the physiological phenomena associated with increasing age⁽⁷⁾. Senescence-accelerated mice develop normally, and then start senescence at approximately 6 months of age and irreversible advancement of senescence, manifested by signs such as loss of hair and increased lordokyphosis. Eight senescence-accelerated mouse strains are senescence

Abbreviation: SAMP, senescence-accelerated-prone mouse.

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prone, each of which develops specific age-related conditions. The senescence-accelerated-prone mouse (SAMP)6 strain provides a model of senile osteoporosis because after 4–5 months of age, these mice exhibit decreasing bone mass and reduced bone remodelling⁽⁸⁾. Few reports address the physiological effects of administering lactic acid bacteria to senescence-accelerated mouse strains⁽⁹⁾. Previously, we reported that oral administration of *Lactococcus lactis* H61 to aged SAMP6 mice was associated with reduced bone-density loss, improved gross lesion and enhanced immune responses, compared with those of controls (without receiving strain H61)⁽¹⁰⁾.

L. lactis G50 modulates immune responses in young Balb/c mice⁽¹¹⁾. We surmised that strain G50 could modulate immune responses in aged mice also, and lead to beneficial effects on the ageing process. The present study investigated the effects of orally administered strain G50 on phenotypic changes such as the immune responses and bone density of SAMP6 mice. Here, we focused on the aspects of intestinal immunity, such as IgA antibody production and numbers and types of intestinal bacteria, because oral agents directly affect intestinal function. For example, some lactic acid bacteria enhance faecal IgA levels⁽¹²⁾. We used heat-killed cells of strain G50 for oral administration to facilitate comparing the results with those of heat-killed cells of strain H61 in our previous study and because non-viable micro-organisms are thought to be as immunomodulatory as live organisms, as mentioned previously. Furthermore, we prolonged the G50 administration period in the present study, compared with that in our previous study using strain H61 in the hope of increasing any beneficial effects.

Materials and methods

Mice

Male, 1-month-old, SAMP6 mice (eighteen per experimental group) were purchased from SLC Japan (Shizuoka, Japan). Two to four mice were housed in each cage (27 × 15 × 10 mm) and maintained at 22 ± 2°C with a 24 h light/dark cycle. Mice were fed MM-3 diet (Funabashi Farm, Chiba, Japan), the composition of which has been described previously⁽¹⁰⁾. Food and water were provided *ad libitum*. Animal experiments followed the animal experimentation guidelines of our institute.

Preparation of bacterial cells

L. lactis ssp. *lactis* G50 was maintained by the International Patent Organism Depository (FERM P-18415, Tsukuba, Japan). The strain was cultured in M17 broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.5 % glucose by subculturing 1 % inocula for 18 h at 30°C. Heat-killed G50 cells were prepared as described previously⁽¹⁰⁾ and were added to MM-3 to a final concentration of 0.5 g/kg (w/w)⁽¹⁰⁾. The control mice received MM-3 without G50.

Quantification of IgA in faeces

Faeces were taken from 2, 3, 4, 7, 9 and 11-month-old mice. Faecal samples from two SAMP6 mice were pooled to obtain sufficient material for the analysis of the IgA levels.

Pooled faeces were suspended in PBS (1.9 mM-NaH₂PO₄, 8.1 mM-Na₂HPO₄, 154 mM-NaCl (pH 7.2)) supplemented with 1 % fetal calf serum (Sigma Chemical Co., St Louis, MO, USA) to a final concentration of 0.1 g/ml (w/v) and vortexed until suspended. Supernatants were obtained by centrifugation at 10 000 g for 10 min. Total IgA levels in the supernatants were determined by ELISA using biotinylated rat anti-mouse IgA antibody (PharMingen, San Diego, CA, USA) and a streptavidin–horseradish peroxidase conjugate (PharMingen).

Detection of enteric bacteria in faeces

Faeces were obtained from 2-, 7- and 10-month-old control and G50-fed mice. Faeces from three or four mice of the same mice were pooled, mixed with sterile 0.85 % NaCl solution to a concentration of 0.1 g/ml (w/v) and vortexed until suspended. Serial dilutions of stool samples were streaked on to selective agar for *Lactobacillus* sp., *Bacteroides* sp., *Bifidobacterium* sp., *Staphylococcus* sp., *Enterobacteriaceae* sp., *Enterococcus/Streptococcus* sp. and H₂S-producing bacteria⁽¹³⁾. Plates for *Bacteroides* sp., *Bifidobacterium* sp. and *Lactobacillus* sp. were incubated at 37°C anaerobically; those for *Staphylococcus* sp., *Enterococcus/Streptococcus* sp., *Enterobacteriaceae* sp. and H₂S-producing bacteria were incubated at 37°C aerobically. Colonies were counted after 24–48 h incubation.

Bone-density analysis

The right femurs of the 12-month-old G50-fed and control mice (eight per group) were removed and immersed in 70 % ethanol for 1 week. Bone density was determined by single-energy X-ray absorption (DCS-600R; Aloka, Tokyo, Japan), which measures bone mineral and bone area.

Statistical analysis

Statistical analyses were done using Statistical Analysis System software version 9.1 (SAS Institute, Cary, NC, USA). Bone mineral densities were analysed according to the general linear model procedure with one-way allocation. Changes in the body weight, faecal IgA level and viable count of intestinal bacteria with increasing age were analysed by the general linear model procedure, in which the diet was designated as the main plot, and age and diet × age interaction as the sub-plot; the error term for the main plot was animal or cage within the diet, whereas the error term for the sub-plot was the residual error. Comparisons of the least-squares means were analysed using the PDIF option of the general linear model procedure. Data are expressed as means with their standard errors. *P* < 0.05 was considered statistically significant.

Results and discussion

Body weight

Before the start of the experiment, body weight did not differ between groups (the mean body weight of the mice ranged from 20.2 to 21.4 g). However, thereafter body weight (mean

(SE)) began to differ between the G50 and control animals. At 2 months of age, the mice fed G50 weighed 29.7 (SE 0.59) g (n 18) and the control mice weighed 27.5 (SE 0.59) g (n 18; $P=0.0087$). Body weight at 7 months was 39.4 (SE 0.67) g for the G50 group (n 15) and 34.6 (SE 0.65) g for the control group (n 15; $P<0.0001$). Among the 10-month-old mice, the body weight of the G50 group was 39.6 (SE 0.80) g (n 10) and 35.7 (SE 0.67) g for the control group (n 14; $P=0.0002$). Body weight was 39.1 (SE 1.72) g for the 12-month-old mice fed G50 (n 8) and 34.7 (SE 1.35) g for their age-matched controls (n 13; $P=0.0634$). The number of mice was changed during the experiment because three mice from each group were killed at 6 months of age for bone-density analysis. In addition, several mice died due to increased age.

Immune response

Faecal IgA levels (Fig. 1) were significantly ($P=0.0252$) higher in the mice fed G50 than in the controls at 3 months of age (i.e. after the G50 animals had been eating the supplemented diet for 2 months), but thereafter no significant difference between the G50 and control mice was found. The mucosal immune responses to antigenic challenges reportedly decreases with increasing age⁽¹⁴⁾, as did IgA production in the present study. Therefore, the intestinal immunity of aged mice likely would be poorly susceptible to stimulation by oral administration of strain G50. A similar result occurred in the rats fed kefir milk fermented by using several bacteria and yeast. In that study, enhanced intestinal immunity occurred in young adult, but not old, rats⁽¹⁵⁾.

The immunomodulatory factors of lactic acid bacteria include the slime products produced by *L. lactis* ssp. *cremoris*, which are B-cell mitogens⁽¹⁶⁾. Although strain G50 does not produce slime products, cell wall fractions prepared from the strain in our preliminary experiments stimulated cytokine production (such as IL-6) from a macrophage cell line (data not shown). IL-6 can enhance intestinal immunity by

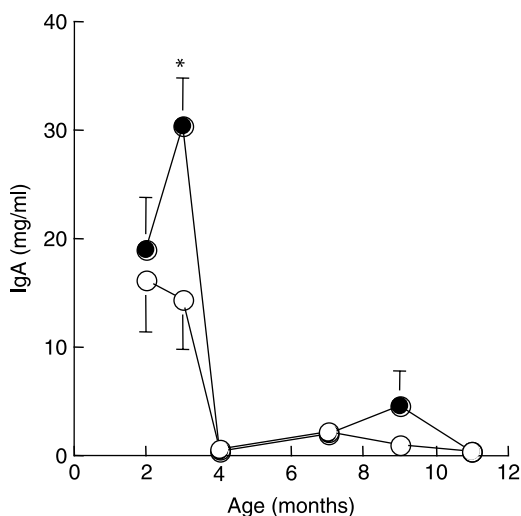


Fig. 1. Changes in total faecal IgA level with increasing age (control, ○; G50, ●). Values are the means of 5–9 independent trials, each with two mice, with their standard errors depicted by vertical bars. * Mean values were significantly different between the two groups on the corresponding age ($P=0.0252$).

stimulating IgA antibody production⁽¹⁷⁾. Various cell wall components, present in heat-killed cells, may be immunomodulatory factors of strain G50.

Effect on growth of enteric bacteria

In humans, the viable counts of *Clostridium* sp., *Enterococcus* sp., *Lactobacillus* sp. and *Enterobacteriaceae* sp. increase with age, and those of *Bifidobacterium* sp. decrease⁽¹⁾. Table 1 shows that the viable counts of *Lactobacillus* sp., *Bacteroides* sp., *Enterococcus/Streptococcus* sp. and *Enterobacteriaceae* sp. did not differ with increasing age in either control or G50-fed mice; *Bifidobacterium* sp. was not ever detected in either group. In terms of the effect of diet, there was no difference in the viable counts of those bacteria between the control and G50-fed mice at any age. This result might reflect the use of heat-killed cells that are unable to multiply and cannot produce antimicrobial substances such as lactic acid or bacteriocin. However, viable H₂S-producing bacteria were significantly ($P=0.0289$) fewer in the G50 than control mice. The H₂S-producing intestinal bacteria include *Salmonella* sp., *Shigella* sp. and *Citrobacter* sp., all of which are associated with various chronic diseases including inflammatory bowel disease and colorectal cancer⁽¹⁸⁾. Furthermore, heat-killed *Lactobacillus acidophilus* strain LB inhibits the adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells⁽¹⁹⁾. Adhesion ability and hydrophobicity are correlated in some lactobacilli⁽²⁰⁾, and hydrophobicity increased when the bacterial cells were heat inactivated⁽²¹⁾. In addition, some lactic acid bacteria co-aggregate with pathogen⁽²¹⁾. Elucidating whether strain G50 adheres to the intestinal cells or whether it co-aggregates with pathogen *in vitro* will help clarify the mechanism through which the heat-killed cells of strain G50 inhibit the H₂S-producing intestinal bacteria *in vivo*.

Bone-density analysis

The heat-killed G50 cells were administered to 1-month-old SAMP6 mice for 11 months, after which their bone density was measured. Body density (mean (SE)) was 39.7 (SE 0.88) mg/cm² for the 12-month-old mice fed G50, and 38.7 (SE 0.88) mg/cm² for their controls; these values do not differ significantly. Similarly, in our preliminary experiment, bone density did not differ between the 6-month-old control mice (n 3, 40.6 (SE 0.91) mg/cm²) and their age-matched G50-fed cohort (n 3, 42.8 (SE 0.91) mg/cm²), which had received the supplemented diet for 5 months.

In our previous study, oral administration of *L. lactis* ssp. *cremoris* H61 suppressed bone-density loss with increasing age⁽¹⁰⁾. However, in that study, compared with that of the controls, the greater mean body weight of the H61 group might account for their higher bone density, because bone density usually increases with increasing body weight⁽²²⁾. In the present study, to exclude a perceived effect of strain G50 on bone density because of the organism's effect on weight gain, we only compared mice whose body weights were similar to the mean value for their group. Alternatively, the differing effects of strains G50 and H61 on bone density may reflect species (ssp. *lactis* or ssp. *cremoris*) or strain specificity. Furthermore, because osteoporosis is associated

Table 1. Effect of heat-killed strain G50 on mean viable count of intestinal bacteria of senescence-accelerated-prone mouse (SAMP)6 mice (Means with their standard errors for two independent trials, each with three or four mice)

Effect	log colony-forming units/ml											
	<i>Bacteroides</i> sp.		<i>Lactobacillus</i> sp.		<i>Enterococcus</i> / <i>Streptococcus</i> sp.		<i>Staphylococcus</i> sp.		<i>Enterobacteriaceae</i> sp.		H ₂ S-producing bacteria	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Diet	NS		NS		NS		NS		NS		*	
Control	9.80	0.12	9.26	0.16	6.28	0.08	5.80	0.17	6.65	0.23	5.93	0.06
Strain G50	9.93	0.12	8.90	0.16	6.15	0.08	5.83	0.16	6.21	0.23	5.42	0.06
Age (months)	NS		NS		NS		NS		NS		NS	
2	9.65	0.15	8.85	0.23	5.78	0.27	6.08	0.27	6.00	0.27	5.20	0.24
7	9.62	0.15	9.22	0.23	6.55	0.27	5.50	0.27	6.38	0.27	5.78	0.24
10	10.3	0.15	9.17	0.23	6.33	0.27	5.88	0.27	6.93	0.27	6.0	0.24
Diet × age	NS		NS		NS		NS		NS		NS	

Difference between values in the same diet or age column is NS.

* $P=0.0289$, significance of effect.

with immune dysfunction⁽²³⁾, the effects of strains G50 and H61 on the immune system should be studied.

In our previous study, oral administration of the heat-killed strain H61 reduced grading scores following senescence-associated changes such as hair loss, skin ulceration and altered immune responses such as enhancement of Th1-type immune response⁽¹⁰⁾. In the present study, the 12-month-old G50-fed mice showed some of the same manifestations of increasing age as did the control mice. We hypothesised that prolonged supplementation with strain G50 would have a stronger anti-ageing effect than that of strain H61, because strain G50 had higher immunomodulatory activity *in vitro* than did strain H61⁽¹¹⁾. However, although administered to young (1 month old) through to aged (12 months old) mice in the present study, strain G50 lacked the anti-ageing effect of strain H61, which was administered to 5-month-old to aged (9 months old) mice. During the analysis of the mechanism of the anti-ageing effect of strain H61, G50 may be an effective negative control for that process.

Strain G50 enhanced the intestinal immunity of young mice and suppressed the growth of enteric H₂S-producing bacteria. Strain G50 may be useful in ameliorating the disease caused by the intestinal bacterial pathogens. In addition, supplementation with strain G50 increased the body weight of SAMP6 mice. Similarly, oral administration of probiotic bacteria including lactic acid bacteria has a positive but poorly understood effect on body weight in the animal industry context⁽²⁴⁾. Perhaps, strain G50 can help maintain the health in or suppress some diseases of young livestock, which often have decreased resistance to pathogens. The present study used the heat-killed cells of strain G50; as such, these organisms do not meet the accepted definition of probiotics. However, non-viable microorganisms with beneficial effects on animal health, like strain G50, might also be useful as food supplements for humans or livestock.

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