

# Dietary heat-killed *Lactobacillus brevis* SBC8803 (SBL88™) improves hippocampus-dependent memory performance and adult hippocampal neurogenesis

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## Abstract

**Aims:** *Lactobacillus* species are used widely as various food and supplements to improve health. Previous studies have shown that heat-killed *Lactobacillus brevis* SBC8803 induces serotonin release from intestinal cells and affects sleep rhythm and the autonomic nervous system. However, the effect of SBC8803 on cognitive function remains unknown. Here, we examined the effects of dietary heat-killed SBC8803 on hippocampus-dependent memory and adult hippocampal neurogenesis.

**Methods:** Hippocampus-dependent memory performance was assessed in mice fed heat-killed SBC8803 using social recognition and contextual fear conditioning tasks. Adult hippocampal neurogenesis was evaluated before, during, and after feeding heat-killed SBC8803 by measuring the number of 5-bromo-2-deoxyuridine (BrdU)-positive cells following systemic injections of BrdU using immunohistochemistry.

**Results:** Mice fed a heat-killed SBC8803 diet showed an improvement of hippocampus-dependent social recognition and contextual fear memories and enhanced adult hippocampal neurogenesis by increasing the survival, but not proliferation, of newborn neurons.

**Conclusion:** Dietary heat-killed SBC8803 functions as memory and neurogenesis enhancers.

## KEYWORDS

adult hippocampal neurogenesis, contextual fear conditioning, hippocampus-dependent memory, *Lactobacillus brevis*, social recognition

RI and HF contributed equally to this work.

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## 1 | INTRODUCTION

There is increasing evidence that intestinal microbiota improves human health including brain function.<sup>1</sup> *Lactobacillus* species are used widely as various food and supplements to improve health through modulation of the immune system, enhance intestinal functions, lower blood lipid levels and reduce diet-induced obesity.<sup>2-5</sup> Interestingly, heat-killed *Lactobacillus brevis* SBC8803 induces serotonin release from intestinal cells<sup>6</sup> and affects sleep rhythm and the autonomic nervous system in animal experiments.<sup>7,8</sup> Importantly, human trials with heat-killed SBC8803 raise the possibility of a beneficial effect on sleep.<sup>9,10</sup> Thus, SBC8803 may have impacts on brain function. However, the effect of SBC8803 on cognitive function, especially learning and memory, still remains unknown. In this study, we examined the effects of dietary SBC8803 on memory performance and adult hippocampal neurogenesis.

## 2 | METHODS

### 2.1 | Animals

All experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals of the Japan Neuroscience Society and the*

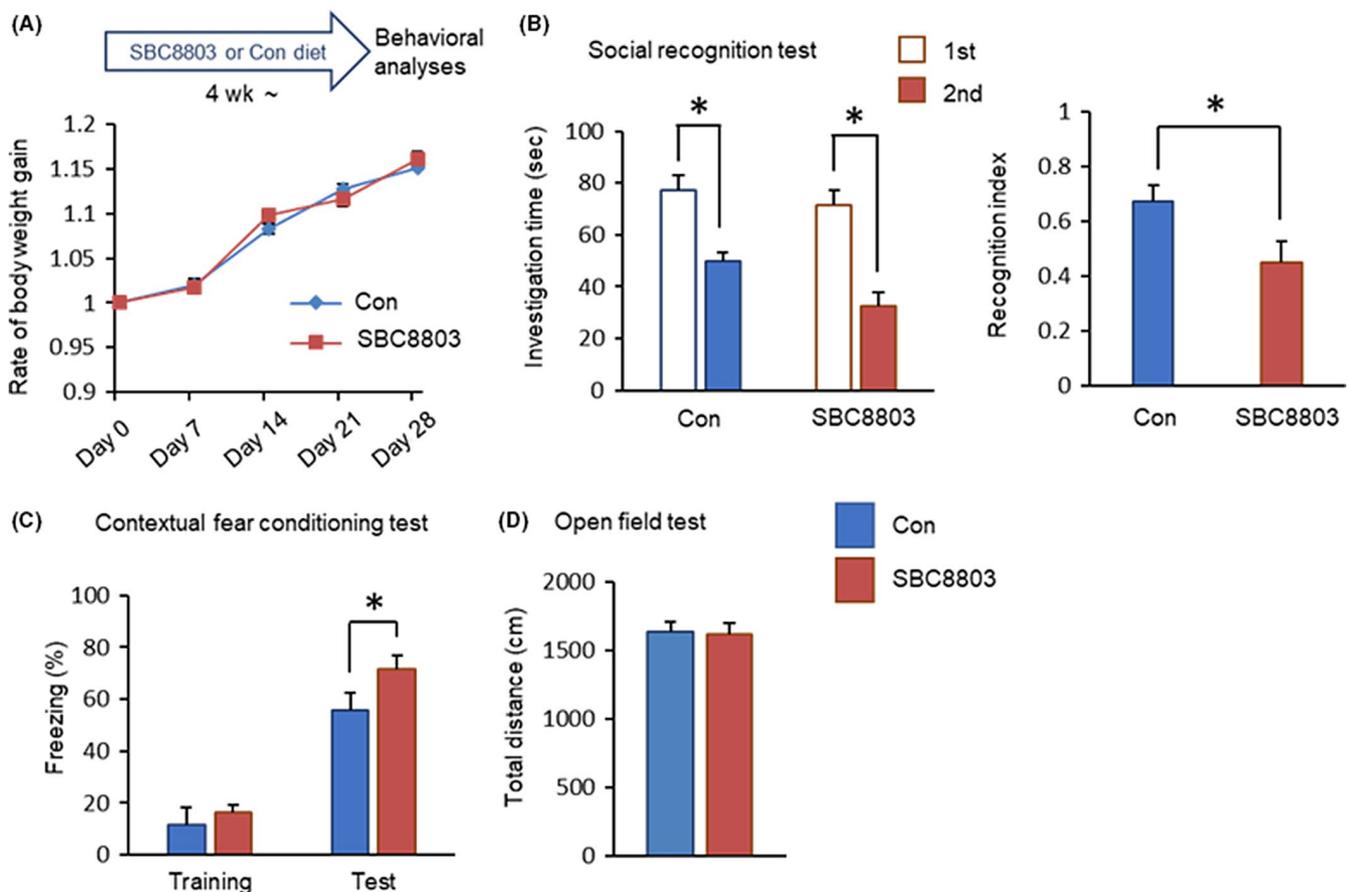
*Guide for the Tokyo University of Agriculture*. All animal experiments were approved by the Animal Care and Use Committee of Tokyo University of Agriculture. Male C57BL/6N mice were obtained from Charles River (Yokohama, Japan). The mice were housed in cages of 4, maintained on a 12 hour light/dark cycle, and allowed ad libitum access to pellet food and water. The mice were at least 8 weeks of age at the start of the experiments, and all behavioral procedures were conducted during the light phase of the cycle. All experiments were conducted blind to the treatment condition. Animal behavior was recorded using a video camera (Figure 1).

### 2.2 | Preparation of the SBC8803 diet

*Lactobacillus brevis* SBC8803 was propagated for 24 hours at 33°C in broth. Bacterial cells were collected by membrane concentration and washed with deionized water. Concentrated bacterial cells were heat-killed at 115°C for 15 minutes and lyophilized. The bacterial powder was mixed thoroughly with CRF-1 (mouse chow; ORIENTAL YEAST CO., LTD., Kyoto, Japan) to a final ratio of 0.5% and pelletized.

### 2.3 | Social recognition task

Adult mice were placed into individual plastic cages, identical to those in which they were normally housed (30 × 17 × 12 cm), in



**FIGURE 1** Effects of dietary SBC8803 on memory performance. A, Experimental procedure (top). Rate of body weight gain during SBC8803 feeding (bottom, control [Con],  $n = 24$ ; SBC8803,  $n = 24$ ). B, Social recognition task (Con,  $n = 8$ ; SBC8803,  $n = 9$ ). Investigation time (left,  $*P < 0.05$ , compared with the first exposure) and recognition index (right,  $*P < 0.05$ , compared with the Con group). C, Contextual fear conditioning task (Con,  $n = 12$ ; SBC8803,  $n = 12$ ).  $*P < 0.05$ . D, Open field test (Con,  $n = 24$ ; SBC8803,  $n = 24$ ). Error bars, SEM

the experimental room. After a period of 60 minutes, a juvenile mouse was placed into a cage with an adult mouse for 3 minutes (first exposure).<sup>11,12</sup> The duration of the adult's social investigation behavior was quantified using a stopwatch. Social investigation was defined as described previously.<sup>13</sup> Memory was assessed 24 hours later by recording the length of social investigation time exhibited by the subject to the same juvenile (second exposure) for 3 minutes. A recognition index was calculated as the ratio of the social investigation times during the second and first exposures.

## 2.4 | Contextual fear conditioning task

The mice were trained and tested in conditioning chambers (17.5 × 17.5 × 15 cm) with a stainless-steel grid floor through which a footshock could be delivered.<sup>10,14,15</sup> Training consisted of placing the mice in the chamber and delivering an unsignaled footshock (2 seconds duration, 0.4 mA) after 148 seconds, and the mice were returned to their home cage at 30 seconds after the footshock (training). Memory was assessed at 24 hours later by calculating the percentage of time spent freezing during 5 minutes when replaced in the training context (test). Freezing behavior (defined as a complete lack of movement, except for respiration) was measured automatically as described previously (O'Hara & Co., Ltd., Tokyo, Japan).<sup>16</sup>

## 2.5 | Open field test

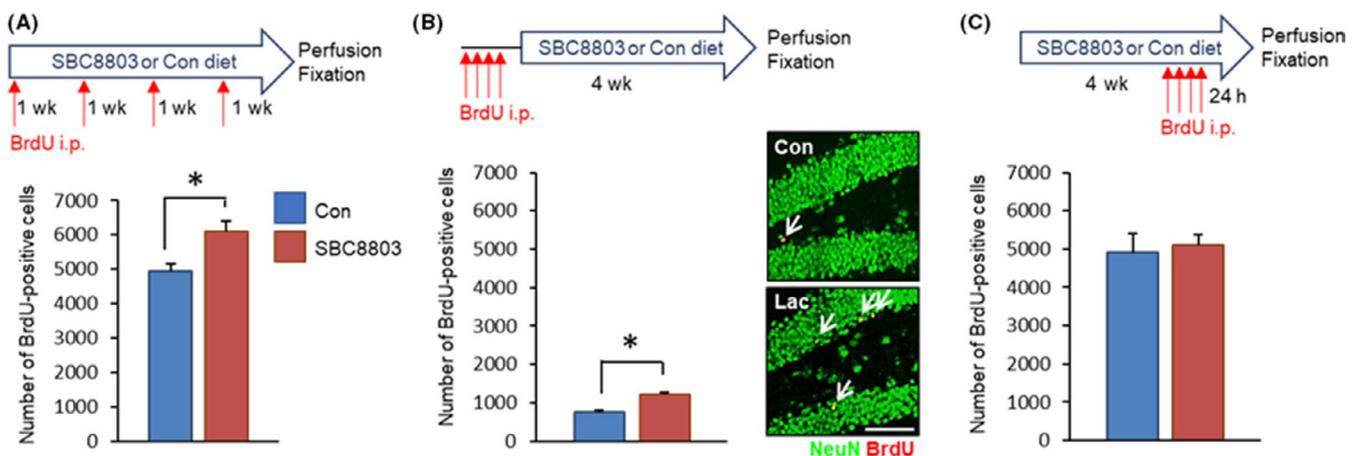
The mice were placed into the center of a square open field chamber (50 × 50 × 40 cm) that was surrounded by white walls. The total length of the path traveled (total distance) was measured using an automatic monitoring system (O'Hara & Co., Ltd.).<sup>12</sup>

## 2.6 | Immunohistochemistry

Immunohistochemistry was performed as described previously.<sup>11,14</sup> After anesthetization, all mice were perfused with 4% paraformaldehyde containing 0.5% picric acid. The brains were removed, fixed overnight, transferred to 30% sucrose, and stored at 4°C. Coronal sections (14 μm) were generated using a cryostat. Consecutive sections were boiled in citrate buffer solution for 5 minutes and incubated with 2N HCl at 37°C for 30 minutes, followed by incubation in a blocking solution. The sections were incubated overnight with a monoclonal rat anti-5-bromo-2-deoxyuridine (BrdU) primary antibody (1:5000; Novus Biologicals, Littleton, CO) and a monoclonal mouse anti-NeuN primary antibody (1:500; Millipore, Hayward, CA) in the blocking solution. Subsequently, the sections were incubated for 2 hours with Alexa Fluor 594-conjugated goat antirat IgG (1:500; Invitrogen, Grand Island, NY) and Alexa Fluor 488-conjugated goat antimouse IgG (1:500; Invitrogen).<sup>11,14</sup>

## 2.7 | Quantification

All fluorescence images were acquired using a confocal microscope (TCS SP8; Leica, Wetzlar, Germany). Equal cutoff thresholds were applied to all slices using LAS X software (Leica). BrdU-positive cells throughout the rostro-caudal extent of the dentate gyrus (DG) were counted in every eighth section, and the total number of BrdU-positive cells was calculated by multiplying the count in each section by 8 and then totaling the values.<sup>11,14</sup> BrdU-positive cells were colocalized with NeuN, a marker of mature neurons, and the number of these cells was measured in Figure 2A,B, whereas all BrdU-positive cells were measured in Figure 2C.



**FIGURE 2** Effects of SBC8803 on adult hippocampal neurogenesis. A, Experimental procedure (top). Quantification of 5-bromo-2-deoxyuridine (BrdU)-positive cells (bottom, control [Con],  $n = 4$ ; SBC8803,  $n = 4$ ). \* $P < 0.05$ . B, Experimental procedure to assess the survival of newborn neurons (top). Quantification of BrdU-positive cells (bottom left; Con,  $n = 4$ ; SBC8803,  $n = 4$ ). \* $P < 0.05$ . Representative immunofluorescent staining of BrdU-positive cells (red) and NeuN-positive cells (green; bottom right). Scale bar, 100 μm. C, Experimental procedure to assess the proliferation of newborn neurons (top). Quantification of BrdU-positive cells (bottom; Con,  $n = 4$ ; SBC8803,  $n = 4$ ). Error bars, SEM



## 2.8 | Data analysis

Two-way repeated analysis of variance (ANOVA) was used to analyze the effects of SBC8803 and time. Student's *t* test was used to analyze differences in social investigation time, recognition index, freezing levels, locomotor activity, and the number of BrdU-positive cells within each group.

## 3 | RESULTS

In this study, the mice were fed CRF-1 mixed with or without heat-killed SBC8803 for at least 4 weeks (SBC group and control group, respectively, Figure 1A). Consistent with previous observation,<sup>7</sup> the SBC8803 and control groups showed comparable body weight gain (two-way repeated ANOVA; diet,  $F_{1,46} = 0.06$ ,  $P > 0.05$ ; time,  $F_{4,184} = 204$ ,  $P < 0.05$ ; interaction,  $F_{4,184} = 0.3$ ,  $P > 0.05$ ; Figure 1A).

To examine the effect of dietary heat-killed SBC8803 on memory performance, we first performed a social recognition task. In this task, the mice form a hippocampus-dependent nonaversive social memory.<sup>11,12,17</sup> Mice were exposed to a juvenile male mouse twice for 3 minutes at an interval of 24 hours (first and second exposures). Both groups showed significant decreases in social investigation time at the second exposure compared with the first exposure ( $P_s < 0.05$ ; Figure 1B), suggesting that they formed a social recognition memory. Importantly, the SBC8803 group showed a significantly lower recognition index compared with control group, confirming that SBC8803 group shows an improved memory (Figure 1B). This result indicated that dietary SBC8803 improved social recognition memory.

We next performed a contextual fear conditioning task to examine the ability to form a hippocampus-dependent aversive memory.<sup>18</sup> The mice were trained with a single footshock (0.4 mA, training) and 24 hours later, behavioral freezing was assessed (test). SBC8803 group showed significantly more freezing compared with control group during test ( $P < 0.05$ ; Figure 1C). Consistently with the results shown in Figure 1B, this result suggests that SBC8803 mice showed improved contextual fear memory.

Importantly, the SBC8803 group showed normal locomotor activity in an open field test compared with the control group ( $P > 0.05$ ; Figure 1D), suggesting that the memory enhancement observed in the SBC8803 group was not attributable to abnormal locomotor activity.

Previous studies have suggested that hippocampus-dependent memory performance is improved by increased adult hippocampal neurogenesis.<sup>11,19,20</sup> Therefore, the effect of SBC8803 on adult hippocampal neurogenesis was examined. Mice with or without heat-killed SBC8803 received systemic injections of BrdU (50 mg/kg bw) to label proliferating cells once a week for 4 weeks after the onset of SBC8803 supplementation. Then, the number of BrdU-positive cells in DG was quantified using immunohistochemistry (Figure 2A). Interestingly, the SBC8803 group showed significantly more BrdU-positive cells than the control group ( $P < 0.05$ ; Figure 2A), suggesting that SBC8803 promoted adult hippocampal neurogenesis.

We next examined whether SBC8803 enhanced the survival or proliferation of newborn neurons in the DG. To examine the effect of SBC8803 on the cell survival, newborn neurons were labeled by systemic injections of BrdU four times at intervals of 2 hours. At 24 hours after the last injection, the mice were started to feed SBC8803 for 4 weeks. The SBC8803 group showed significantly more BrdU-positive cells than the control group (Figure 2B), suggesting that SBC8803 promoted the survival of newborn neurons in the DG.

We finally examined the proliferation of newborn neurons in the DG of SBC8803 mice. Newborn neurons were labeled/measured using BrdU at 4 weeks after the onset of supplementation with SBC8803 using the same procedure of Figure 2B. In contrast to the data shown in Figure 2B, comparable numbers of BrdU-positive cells were observed in the control and SBC8803 groups when the cells were measured 24 hours after BrdU injections (Figure 2C), suggesting that SBC8803 did not affect the proliferation of newborn cells in the DG. Collectively, these observations suggest that dietary SBC8803 enhanced adult hippocampal neurogenesis by promoting the survival, but not proliferation, of newborn neurons.

## 4 | DISCUSSION

In this study, dietary heat-killed SBC8803 improved memory performance in two different hippocampus-dependent memory tasks and enhanced adult hippocampal neurogenesis through increases in survival, but not proliferation, of newborn neurons. Thus, our results suggest that dietary SBC8803 functions as memory and neurogenesis enhancers<sup>14</sup> and that SBC8803 may be used to improve cognitive impairments such as deficits in learning and memory.

Young neurons (3–8 weeks old) generated through adult hippocampal neurogenesis are incorporated more frequently into the memory trace and are more plastic compared with the other generations of neurons, thereby improving memory performance.<sup>11,21,22</sup> Therefore, it is possible that the young neurons generated by feeding the SBC8803 diet contribute greatly to improved memory performance. Further studies are required to investigate the relationship between memory performance and increased neurogenesis following SBC8803-feeding.

SBC8803 improved memory and adult hippocampal neurogenesis. SBC8803 may increase adult hippocampal neurogenesis through the gut-brain axis or the autonomic nervous system, thereby improving memory performance, although further investigations are required to examine these possibilities. Moreover, it is important to examine the requirement of the gut-brain axis and/or autonomic nervous system for the enhancement of memory performances in SBC8803 mice by inhibiting/disrupting these systems.

Previous findings showed that SBC8803 displays positive impacts on biological rhythms and the quality of sleep,<sup>7</sup> and that SBC8803 induces serotonin release from intestinal cells and affects the autonomic nervous system.<sup>6,8</sup> Therefore, in addition to the possibility discussed above, the improved quality of sleep may



contribute to enhanced memory formation since sleep plays critical roles in memory performance.<sup>23,24</sup> It is important to examine these possibilities to understand the mechanisms underlying the improvement of memory performance by dietary SBC8803.

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## CONFLICT OF INTEREST

Some parts of this study were funded by SAPPORO HOLDINGS Ltd.

## DATA REPOSITORY

All relevant data are included in Supporting Information.

## ANIMAL STUDIES

All animal experiments were approved by the Animal Care and Use Committee of Tokyo University of Agriculture.

## AUTHOR CONTRIBUTION

SK is responsible for the hypothesis development and overall design of the research and experiment and supervised the experimental analyses. RI and SK cowrote the paper. RI and HF performed behavioral experiments. RI performed immunohistochemical experiments. RI and HF analyzed data. YN and HK contribute to the hypothesis development and overall design of the research and experiment and prepared the control and SBC8803 diets. All authors read and approved this paper.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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