

Heat-Killed *Lactobacillus brevis* SBC8803 Induces Serotonin Release from Intestinal Cells

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ABSTRACT

Previously, we reported that changes induced in autonomic neurotransmission in rats by *Lactobacillus brevis* SBC8803 may be mediated by serotonin 3 (5-HT₃) receptors. In this study, we evaluated the effects of heat-killed *L. brevis* SBC8803 on serotonin (5-HT) releasing from intestinal cells. In the *in vitro* study, *L. brevis* SBC8803 stimulated 5-HT release from cultured rat endocrine RIN-14B cells (SBC8803 vs. sterile water; $P < 0.01$). For *in vivo* study, 2 mg of heat-killed *L. brevis* SBC8803 was administered using a stomach sonde (feeding needle) to C57BL/6J mice. Analysis of plasma by ELISA showed gradually increase in 5-HT concentrations (0 min vs. 60 min; $P < 0.05$). ELISA of *ex vivo* cultured intestinal loops composed of duodenum and part of the jejunum, from C3H/HeN and C57BL/6J male mice indicated that *L. brevis* SBC8803 effectively induced 5-HT release (SBC8803 vs. sterile water; $P < 0.01$). These experimental results suggest that heat-killed *L. brevis* SBC8803 may stimulate 5-HT release from mouse intestinal cells such as enterochromaffin cells.

Keywords: Serotonin; Lactobacillus Brevis; Mice; Intestinal Cells; 5-HT₃ Receptors

1. Introduction

Neurogastroenterology is a research area of gastroenterology that investigates interactions between the central nervous system (CNS; brain) and the gut [1-3]. The enteric nervous system (ENS) communicates with the CNS through the parasympathetic and sympathetic nervous systems, which comprise nerves and hormones such as enteric hormones.

Lactic acid bacteria (LAB) are one of the most important types of bacteria found in the human and animal gastrointestinal tract. Tanida *et al.* reported that *Lactobacillus* strain affects autonomic neurotransmissions and alter physiological phenomena [4,5]. These reports also described that *Lactobacillus paracasei* ST11 may induce changes in autonomic neurotransmission through the central histaminergic nerves in brain [5], although the molecules that may be involved such as hormones, neuropeptides, and cytokines in the gut have not been identified.

We examined the effects of heat-killed *Lactobacillus brevis* SBC8803 [6-8] on autonomic neurotransmissions and found that changes induced in autonomic neuro-

transmissions by this heat-killed strain may be mediated by serotonin 3 (5-HT₃) receptors [9]. On the basis of these results, it was suggested that intestinally injected *L. brevis* SBC8803 stimulates afferent intestinal vagal nerve activity (IVNA) through 5-HT₃ receptors; this information is then transmitted to the CNS, which in turn increases efferent gastric vagal nerve activity (GVNA), and GVNA stimulation enhances appetite [9]. To the best of our knowledge, this is the first report that 5-HT₃ receptors may be involved in the interactions between LABs and autonomic nerves.

To clarify whether the heat-killed *L. brevis* SBC8803 stimulates the release of serotonin (5-HT) from intestinal cells, we designed experiments to examine 5-HT release from intestinal cells induced by *L. brevis* SBC8803 and performed an “*in vitro* cell-based study”, an “*in vivo* plasma study”, and an “*ex vivo* intestinal loop study” experiments.

2. Materials and Methods

2.1. Animals

Male C3H/HeN (Japan SLC Inc.) and C57BL/6J (Charles

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River Laboratories Japan) mice with a body weight of 20 - 23 g at the time of experiment were used in the *in vivo* and *ex vivo* studies. Mice were housed in a cage with free access to chow and water at $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 12 h light-dark cycle (Lights on: 08:00 - 20:00). The animals were allowed at least 1 week to acclimatize to their housing conditions before experiments. The Animal Care and Use Committee of Frontier Laboratories of Value Creation, Sapporo Breweries Ltd. approved all animal care and handling procedures.

2.2. Preparation of Heat-Killed SBC8803

L. brevis SBC8803 were cultured for 24 h at 30°C in broth containing 2% maltose, 1.4% yeast extract, 0.5% sodium acetate, and 0.005% $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$. Bacteria were collected by centrifugation at $8000 \times g$ for 10 min at 10°C (Himac CR21G; Hitachi Koki Co. Ltd.) and washed 3 times with deionized water. After washing, bacteria were heat-killed at 105°C for 10 min and lyophilized.

2.3. In Vitro Cell Based Study

The rat endocrine RIN-14B cell line (ATCC, CRL-2059) was suspended in RPMI1640 medium supplemented with 10% fetal calf serum (Gibco, Life Technologies) at 1×10^5 cells/0.5 mL, seeded in 24-well plates, and cultured for 3 d at 37°C in a 5% CO_2 incubator (MCO-20 AIC; SANYO Co.). The medium was removed, and the cells were washed with Hank's balanced salt solution (HBSS) containing Ca and Mg, (Wako Pure Chemical Industries, Ltd.). After washing, HBSS was removed and replaced with 0.5 mL HBSS containing samples (0 - 0.3 mg/mL), and the cells were further incubated for either 30 min or 60 min at 37°C . The assay solution was collected and centrifuged for 5 min to remove any detached cells. Serotonin concentrations in supernatants were measured using a serotonin-ELISA kit (Enzo Life Science). Results are represented as mean \pm SD ($n = 4 - 6$). Statistical evaluation of the results was performed using one-way analysis of variance (ANOVA).

2.4. In Vivo Plasma Study

The sample was suspended in sterile water and then orally administered using a stomach sonde (feeding needle) to male C57BL/6J mice (2 mg of sample/mouse). At 0, 15, 30, 60, and 90 min after administration, blood samples were collected from mice and treated with heparin, followed by centrifugation with 5000 rpm for 10 min at 4°C . Plasma 5-HT concentrations were measured using 5-HT ELISA kits. Results are represented as the mean \pm SD ($n = 5$). Statistical evaluation of the results was performed using one-way ANOVA.

2.5. Ex Vivo Intestinal Loop Study

Male C3H/HeN and C57BL/6J mice were killed. An intestinal loop (approximately 5 cm), composed of duodenum and part of the jejunum, was then removed from the small intestine. Each end of the intestinal loop was ligated with silk sutures and the loops filled with 0.1 ml sterilized water containing 2 mg/mL sample. To measure releasing effects, the loops were incubated for 60 min at 37°C in a 5% CO_2 incubator. The loops were placed into the center section of the organ culture dish (3.5 cm in diameter) in 3 mL of RPMI 1640. RPMI 1640 media in the culture dish were collected at 60 min to determine 5-HT release from the lumen to the medium bathing the loops. The 5-HT level in the medium was measured using the 5-HT-ELISA kit. Results are represented as mean \pm SD ($n = 5$). Statistical evaluation of the results was performed using one-way ANOVA.

3. Results

3.1. 5-HT Release from RIN-14B Cells (in Vitro Studies)

The *in vitro* experiments were performed to evaluate 5-HT release from the rat endocrine RIN-14B cells, which were used as a tentative model for the investigation of enterochromaffin (EC) cell function [10]. As shown in **Figure 1 (a)**, 5-HT release from RIN-14B cells increased depending on the concentration of SBC8803 (0 - 0.3 mg/mL) after incubation for 30 min (0 ppm vs. 30 ppm; $P < 0.05$, 0 ppm vs. 100 ppm and 300 ppm; $P < 0.01$). Moreover, maximum 5-HT release from RIN-14B cells was observed after incubation for 60 min (**Figure 1 (b)**). Subsequently, we performed an *in vitro* study using cellulose powder (100 ppm) as a physical stimulus in the 60 min reaction. The 5-HT concentration at 60 min after cellulose powder addition was equal to that obtained after addition of sterile water (**Figure 2**). Thus, cellulose powder had no effect on 5-HT release from these cells. On the other hand, 5-HT release was observed after addition of heat-killed *L. brevis* SBC8803 cells (SBC8803 vs. sterile water and cellulose powder; $P < 0.01$).

3.2. 5-HT Increase in Plasma of Mice (in Vivo Studies)

We determined 5-HT concentrations in blood after administration of heat-killed *L. brevis* SBC8803. Heat-killed *L. brevis* SBC8803 was suspended in sterile water and administered using a stomach sonde (feeding needle) to C57BL/6J mice (2 mg of SBC8803/mouse). This dose was equivalent to the daily intake of food containing 0.05% *L. brevis* SBC8803. At 0, 15, 30, and 60 min after administration, plasma 5-HT concentrations were determined using 5-HT ELISA kits. As shown in **Figure 3**,

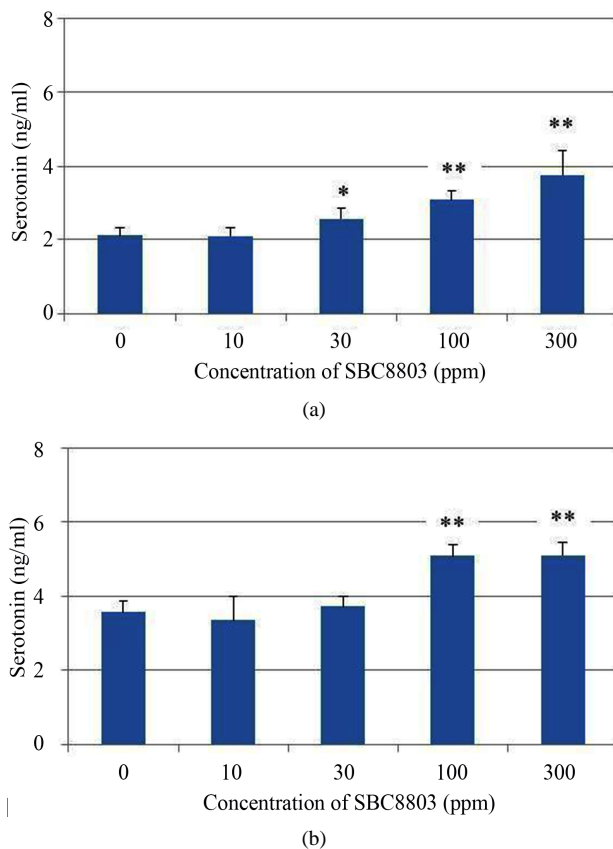


Figure 1. Influence of concentration of *Lactobacillus brevis* SBC8803 on 5-HT release from rat endocrine RIN-14B cells. The results obtained after incubation for 30 min are shown in (a). * $P < 0.05$ for 0 ppm vs. 30 ppm; ** $P < 0.01$ for 0 ppm vs. 100 ppm and 300 ppm. The results obtained after incubation for 60 min are shown in (b). ** $P < 0.01$ for 0 ppm vs. 100 ppm and 300 ppm. Results are represented as mean \pm SD ($n = 4$).

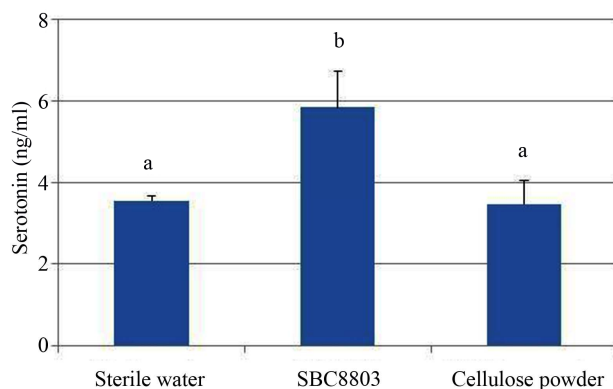


Figure 2. Comparison of the effects of *Lactobacillus brevis* SBC8803 and cellulose powder on 5-HT release from rat endocrine RIN-14B cells. The ability of heat-killed *L. brevis* SBC8803 and cellulose powder (both 100 ppm) to stimulate of 5-HT release from RIN-14B cells was evaluated after incubation for 60 min. Results are represented as mean \pm SD ($n = 6$). The different letters indicate statistical differences ($P < 0.01$).

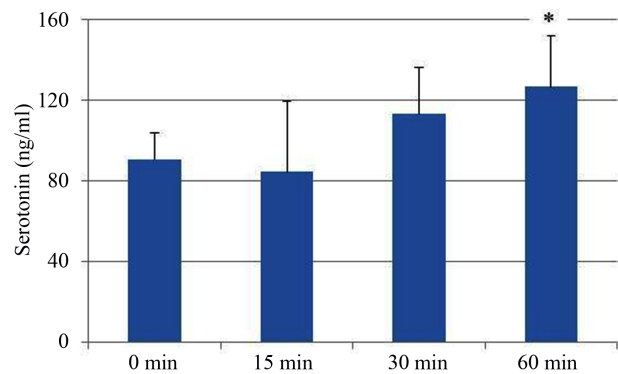


Figure 3. *In vivo* plasma study of the effects of *Lactobacillus brevis* SBC8803. Heat-killed *L. brevis* SBC8803 was administered using a stomach sonde to C57Bl/6J mice (2 mg/mouse). At 0, 15, 30, and 60 min after administration, the mice were killed humanely and blood samples were collected. Plasma 5-HT concentrations were evaluated. Results are represented as the mean \pm SD ($n = 5$). * $P < 0.05$ for 0 min vs. 60 min.

plasma 5-HT concentrations gradually increased after *L. brevis* SBC8803 administration (0 min vs. 60 min; $P < 0.05$). Subsequently, we performed an *in vivo* administration study using cellulose powder (2 mg/mouse) as a physical stimulus. The 5-HT concentration at 90 min after cellulose powder administration was equal to that observed after administration of sterile water (Figure 4), which was similar to that observed in the *in vitro* study using RIN-14B cells.

On the other hand, administration of heat-killed *L. brevis* SBC8803 led to higher plasma 5-HT concentrations than did sterile water or cellulose powder (SBC 8803 vs. sterile water; $P = 0.08$). These *in vivo* studies results (Figures 3 and 4) showed that oral administration of heat-killed *L. brevis* SBC8803 in mice increased 5-HT concentrations in the blood.

3.3. 5-HT Release from the Small Intestine of Mice (*ex Vivo* Studies)

Finally, we carried out *ex vivo* experiments using the intestinal loop, which is composed of the duodenum and part of the jejunum from male C3H/HeN and C57BL/6J mice. We injected 0.1 mL of sterile water containing heat-killed *L. brevis* SBC8803 (2 mg/mL) into the intestinal loop. We then evaluated the 5-HT-releasing ability of *L. brevis* SBC8803 by using the procedure described in the Material and Methods section. In C57BL/6J mice, injection of *L. brevis* SBC8803 led to 5-HT release from the intestinal cells into the RPMI1640 medium, whereas the amount of 5-HT released did not differ statistically from the amount release after injection of sterile water into intestinal loops, as shown in Figure 5. However, injection of heat-killed *L. brevis* SBC8803 into the intestinal loop of C3H/HeN mice led to release of significantly

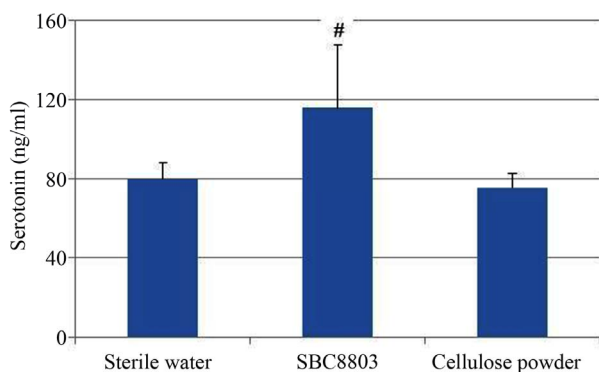


Figure 4. *In vivo* plasma study for comparing the effects of *Lactobacillus brevis* SBC8803 and cellulose powder. Samples were administrated using a stomach sonde to C57BL/6J mice (2 mg/mouse). At 90 min after administration, mice were killed humanely and blood samples were collected. Plasma 5-HT concentrations were determined. Results are represented as mean \pm SD (n = 5). [#]P < 0.1 for SBC8803 vs. sterile water and cellulose powder.

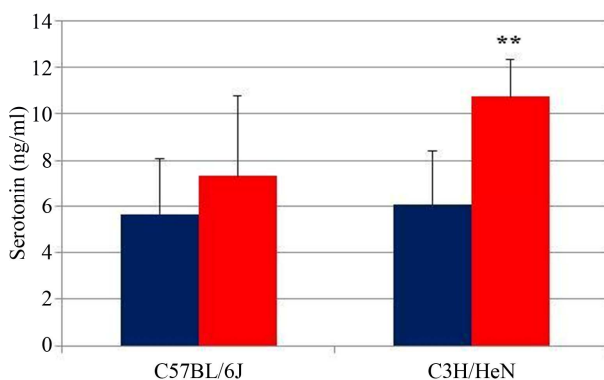


Figure 5. 5-HT release from the small intestine of mice. Effect of heat-killed *L. brevis* SBC8803 on 5-HT release from the small intestine was evaluated using the intestinal loop, which is composed of the duodenum and part of the jejunum, from male C3H/HeN and C57BL/6 J mice. Results are represented as mean \pm SD (n = 5). ^{**}P < 0.01 for SBC8803 vs. sterile water.

higher concentrations of 5-HT from the intestinal cells compared to that released after sterile water injection (SBC8803 vs. sterile water; P < 0.01).

4. Discussion

EC cells have been suggested to have important functions in bacterial recognition in afferent neural pathways from the gut to the CNS. For example, short-chain fatty acids, which can be derived from bacteria, stimulate intraluminal 5-HT release from EC cells [11]. Release of 5-HT is known to stimulate 5-HT₃ receptors on the vagal sensory fibers, and this sensory information is subsequently transferred to the CNS through the afferent and efferent vagal nerve [12]. In addition, EC cells are known to express functional Toll-like receptors [13], which play

important roles in bacterial recognition in the intestine.

The results of our experiments using the intestinal loops, which are composed of duodenum and part of the jejunum, from male C3H/HeN and C57BL/6J mice showed that heat-killed *L. brevis* SBC8803 significantly stimulates 5-HT release from the loops of C57BL/6J mice and effectively stimulates 5-HT release from loops of C3H/HeN mice (SBC8803 vs. sterile water; P < 0.01). In addition, heat-killed *L. brevis* SBC8803 stimulates 5-HT release from the rat endocrine RIN-14B cells (SBC8803 vs. sterile water; P < 0.01). From these results, heat-killed *L. brevis* SBC8803 may be considered to stimulate the bacterial recognition system in intestinal cells such as EC cells. Although short-chain fatty acids were reported to stimulate intraluminal 5-HT release from EC cells [11], acetate, butyrate, and propionate at 10 ppm had no influence on 5-HT release from RIN-14B cells (data not shown).

A schematic diagram depicting the sequence of events in gut mucosal 5-HT signaling has been proposed by Costedio *et al.* [14]. The sequence is as follows: at rest, 5-HT is stored in secretory vesicles at the basal half of EC cells; following mucosal stimulation, 5-HT is released from EC cells and activates 5-HT receptors on nearby afferent nerve fibers, 5-HT signaling is terminated by removal of 5-HT from the interstitial space, most of the 5-HT is transported into epithelial cells, and the remainder enters the blood stream from where it is taken up by platelets.

Our results for the *in vitro* cell-based study, *in vivo* plasma study, and *ex vivo* intestinal loop study using heat-killed *L. brevis* SBC8803 seem to agree with this proposed sequence of events in gut mucosal 5-HT signaling.

On the basis of our results, we propose that heat-killed *L. brevis* SBC8803 induces 5-HT release from intestinal cells, such as EC cells and this released 5-HT stimulates afferent-IVNA through 5-HT₃ receptors. This information is subsequently transmitted to the CNS, which then promotes the changes in autonomic nerve activity such as efferent-GVNA.

Studies on the active components of *L. brevis* SBC8803 and the mode of action involved in SBC8803-induced 5-HT release from intestinal cells are in progress.

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