The effects of viable and non-viable *Lactobacillus gasseri* CP2305 cells on colonic ion transport and corticotropin releasing factor-induced diarrhea

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ABSTRACT

The effect of non-viable lactic acid bacteria on gastrointestinal physiology and dysfunction remains still unclear. Previous clinical trials have reported that *Lactobacillus gasseri* CP2305 (CP2305) exerts stress-relieving and anti-flatulent effects regardless of cell viability. In this study, we investigated the effect of viable and non-viable CP2305 cells on electrical field stimulation (EFS)-evoked increases in short-circuit current (Isc) using the Ussing chamber technique. In mucosal-submucosal preparations of rats, both viable and non-viable CP2305 cells significantly and acutely inhibited the EFS-evoked increases in Isc in the middle and distal colon and rectum but not in proximal colon. The inhibition of EFS-evoked Isc differed from strain to strain. Peripheral injection of corticotropin releasing factor (CRF) is known to mimic diarrhea symptoms in rats. Therefore, we examined the chronic effects of CP2305 cells on CRF-induced diarrhea in the rat model. Treatment with viable and non-viable CP2305 cells significantly improved CRF-induced diarrhea in the rat model. However, the treatment did not affect the fecal pellet output. These findings suggest that CP2305 has an important role in gastrointestinal physiology and dysfunction.
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The chronic effect of CP2305 on stress-induced diarrhea was investigated in the current study, using the animal model as one of the appropriate in vivo models for studying the chronic effect of CP2305 on stress-induced watery diarrhea. However, it is currently unclear how viable and non-viable CP2305 affect colonic ion transport, the dysfunction of which causes watery diarrhea. Physical and psychological stress can influence GI function. Particularly, peripheral administration of corticotropin releasing factor (CRF) is known to alter colonic secretory function (37). Furthermore, peripheral injection of CRF is known to mimic diarrhea symptoms in rats (54). Therefore, in the current study, we used this animal model as one of the appropriate in vivo models for studying the chronic effect of CP2305 on stress-induced watery diarrhea.

The aim of this study was to assess the acute effects of both viable and non-viable CP2305 cells on colonic ion transport in vitro and to study the chronic effect of CP2305 using CRF-induced diarrhea model in rats.

MATERIALS AND METHODS

Preparation of Lactic acid bacteria cells. CP2305 cells were cultured in an original, food ingredient-based medium containing glucose, casein, peptone, yeast extract, a pH adjuster, and an emulsifier at 37°C for 18 h under partially anaerobic atmosphere. The cells were harvested by centrifugation, washed three times by sterile 0.85% sodium chloride solution, and lyophilized. Non-viable cells were prepared by pasteurization at 95°C prior to lyophilizing. The strains Lactobacillus gasseri CP3238 (CP3238), Lactobacillus acidophilus CP1583, and Lactobacillus amylovorus CP1563 (CP1563) were prepared in the same manner as mentioned above.

Ussing flux chamber experiments. Adult male Sprague-Dawley rats (SLC, Inc., Shizuoka, Japan) weighing 380–400 g were used for this study. Animals were fed pellet diets with water ad libitum. All animals were anesthetized with isoflurane and decapitated; subsequently, colonic tissue preparation was performed according to a previous study (16). Segments of proximal, middle, and distal colon, and rectum were cut along the mesenteric border. Luminal contents were gently removed. Tissues were immediately placed in ice-cold glucose-free Krebs–Ringer solution (117 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, and 2.5 mM CaCl₂) saturated with 95% O₂ and 5% CO₂, and then attached on a silicone rubber-coated petri dish. The mucosal–submucosal preparations were made by removing the external muscle layers containing the myenteric nerve plexus with fine forceps under a stereomicroscope. These experiments were conducted in accordance with the ethical guidelines for the care and use of laboratory animals of the University of Shizuoka, and the study was approved by the University of Shizuoka animal ethics committee (Implementation period: June 2010–March 2013).

The mucosal–submucosal preparations of rat colon and rectum were mounted between the halves of an Ussing flux chamber (CHM2; World Precision Instruments, Inc., Sarasota, FL, USA), with a cross-sectional area of 0.64 cm². Both sides of the tissues were bathed with 10 mL of Krebs–Ringer solution maintained at 37°C by heated water. The solution was gassed with 95% O₂ and 5% CO₂ and buffered at pH 7.4. The tissues were stabilized for 50 min before the experiment. The short-circuit current (Iₛ) was continuously measured in voltage-clamp mode at zero potential (CEZ-9100; Nihon-Koden, Tokyo, Japan). Positive Iₛ indicated net transepithelial electrolyte transport of anions from the serosal to the mucosal side and transport of cations from the mucosal to the serosal side. To calculate tissue conductance, the clamped voltage was automatically and intermittently changed at 10 mV for 3 s per min. Under voltage-clamped conditions, transepithelial potential differences were calculated from Iₛ and tissue conductance (Gt) using Ohm’s law. The Iₛ responses were recorded on a chart recorder (Rectihoritz-8K; Nihon-Denki San-ei, Tokyo, Japan) and a Power Lab system 4/26 (ADInstruments, Cattle Hill, Australia). Tissue preparations were electrically stimulated by passing a current parallel to the plane of the tissue via a pair of aluminum foil ribbon electrodes placed on the submucosal surface. Rectangu-
lar electrical field stimulation (EFS) pulses of 25 V, 5 Hz, and 0.5-ms duration were applied for 120 s using an electronic stimulator (SEN-3301; Nihon-Koden, Tokyo, Japan). After tissues were equilibrated, 10 μL of silicone deforming agent (TSA7341; Momentive Performance Materials, Inc., NY, USA) was administrated to the mucosal side to remove air bubbles before the administration of viable or non-viable cells, since air bubbles were generated by the administration of viable or non-viable cell suspension in the chambers. No electrical parameters were affected by the administration of the deforming agent. After tissues were equilibrated, to test whether viable or non-viable cell suspension affected EFS-evoked changes in $I_{sc}$, cell suspensions with various cell concentrations were administrated to the mucosal side 10 min before stimulation.

**CRF-induced diarrhea model.** Male Sprague-Dawley rats (4 weeks old) were obtained from Charles River Japan, Inc. (Kanagawa, Japan). The rats were housed in an air-conditioned room maintained at 23 ± 1.5°C with a relative humidity of 55 ± 15% under a constant 12 h light/12 h dark cycle (with light from 8:00 a.m. to 8:00 p.m.) for acclimatization. The rats were fed a normal powder diet (Charles River Formula-1 (CRF-1); Oriental Yeast Co., Ltd. Tokyo, Japan) with water ad libitum. The rats were given three types of diet as follows: (1) normal powder diet containing freeze-dried viable CP2305 cells ($1.6 \times 10^{9}$ bacterial cells/g diet), (2) normal powder diet containing freeze-dried non-viable CP2305 cells ($1.6 \times 10^{9}$ bacterial cells/g diet), (3) only normal powder diet. The rats were divided into three groups as per the diet fed to them. The rats were fed their assigned diets for 3 weeks. CRF powder (AnaSpec, Inc., California, USA) was dissolved according to the manufacturer’s instructions in sterile saline for intraperitoneal injections. After feeding the assigned diets for 3 weeks, body weights of the rats were measured; subsequently, intraperitoneal injections of CRF (125 μg/kg body weight) were administered. After the injections, all feces samples were collected at intervals of 20 min for 100 min. Fecal weight, fecal pellet output number, and stool form characteristics were noted.

Stool form characteristics, such as color, form, and hardness were assessed using a modified version of the Bristol stool scale (25). The stool form characteristics were categorized into four classes: (1) normal solid stool with black color and cracks on the surface; (2) mild diarrhea with brown color, smooth and soft form; (3) diarrhea with brown color and soft blobs; (4) severe diarrhea with brown color and mushy stool. Diarrheal score at every 20 min interval after CRF injection was given by the equation: Diarrheal score = stool weight × stool form characteristic class. Total diarrheal score was calculated by summing up the diarrheal scores obtained at every 20 min intervals after CRF injection.

**Data analysis and statistics.** All data are expressed as means ± standard error of the mean (SEM). The $n$ values represent the numbers of animals. Paired $t$-test or Dunnett’s multiple comparison test followed by ANOVA were used to examine the effects of viable and non-viable cells. In addition, time-dependent changes in the diarrheal score and fecal pellet output numbers were analyzed by two-way ANOVA with repeated measures. The diarrheal score was analyzed by ANOVA, followed by Dunnett’s multiple comparison procedure. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using JMP v. 13.0 (SAS Japan, Tokyo, Japan).

**RESULTS**

**Effect of viable and non-viable CP2305 cells on the EFS-evoked increases in $I_{sc}$ in the colon and rectum.** We evaluated the effect of viable and non-viable CP2305 cells on EFS-evoked increases in $I_{sc}$ in rat colon and rectum using the Ussing chamber method. The whole bacterial cells were prepared by three times repeatedly washing so as not to include bacterial metabolites and medium components. Mucosal addition of viable CP2305 cells induced concentration-dependent decrease in EFS-evoked $I_{sc}$ in middle and distal colon and rectum (Fig. 1A). Particularly, the 500 μg/mL viable CP2305-induced inhibitory action on EFS-evoked $I_{sc}$ response was significantly reduced as compared with control to 57.8%, 60.5%, and 69.5% in middle colon, distal colon, and rectum, respectively. Likewise, non-viable CP2305 cells inhibited the EFS-evoked $I_{sc}$ responses in a concentration-dependent manner (Fig. 1B). In detail, the 500 μg/mL non-viable CP2305-induced $I_{sc}$ response was significantly reduced to 39.2%, 39.2%, and 44.9% in middle colon, distal colon, and rectum, respectively. However, in the proximal colon, both viable and non-viable CP2305 had no effect on the EFS-evoked increases in $I_{sc}$. Serosal addition of viable and non-viable CP2305 cells had no effect on EFS-evoked responses (data not shown). Thus, in the following experiments, 500 μg/mL non-viable CP2305 cells were used to analyze strain dependency.
Probiotic effects are known to be strain specific, and hence it is important to identify the best strains for further strain-dependent physiological studies (49). Thus, we used four *Lactobacillus* strains to evaluate the strain dependency of EFS-evoked intestinal ion transport. In the present experiments, all strains significantly reduced the EFS-evoked increases in $I_{sc}$ in the middle colon, distal colon, and rectum, except for CP1563 in the middle colon as shown in Fig. 2. Among the strains used in these experiments, CP2305 showed strongest inhibitory action on EFS-evoked $I_{sc}$ in the colon and rectum. In particular, for the purpose of evaluating the activity within the same *Lactobacillus gasseri* species, the $I_{sc}$ responses of the CP2305 and CP3238 strains were compared only in the distal colon having a strong inhibitory CP2305 response, other parts were not measured. On the contrary, in the proximal colon, no strains had any effect on EFS-evoked $I_{sc}$.

**Effect of viable and non-viable CP2305 cells on CRF-induced diarrhea**

Next, we evaluated the effect of viable and non-viable CP2305 cells on CRF-induced diarrhea associated with excess water secretion in the rat model. The

![Graph](image)

**Fig. 1** Segmental differences in the $I_{sc}$ changes induced by viable and non-viable CP2305 cells in the rat colon and rectum. (A) The effect of viable CP2305 cell addition to the mucosal side of the proximal colon, middle colon, distal colon, and rectum on EFS-evoked $I_{sc}$. (B) The effect of non-viable CP2305 cell addition to the mucosal side of the proximal colon, middle colon, distal colon, and rectum on EFS-evoked $I_{sc}$. Values indicate the means ± SEM, $n = 4$. Data were analyzed by paired t-test. *$P < 0.05$, **$P < 0.01$ (versus control).

![Graph](image)

**Fig. 2** Strain-dependent effects on EFS-evoked changes in $I_{sc}$ when different strains of lactic acid bacteria were added to the mucosal side of the rat distal colon. Values indicate the means ± SEM, $n = 3–4$. Data were analyzed by paired t-test. *$P < 0.05$ (versus control group). Within the strain, Dunnett's multiple-comparison test was used. *$P < 0.05$ (versus CP2305 group).
mune, endocrine, and metabolic functions (53) and neuronal firing behavior (22, 50). Further, gut microbiota like *Saccharomyces boulardii* significantly decreased the number of calbindin-28k expressing myenteric neurons in experimental animals (19).

Clinical efficacy of probiotics in treating various forms of diarrhea has been clearly established. Diarrhea is caused either by decreased absorption or increased secretion of electrolytes and solutes in the intestine. However, mechanisms underlying antidiarrheal effects of paraprobiotics are not completely understood. In this study, both viable and non-viable CP2305 cells inhibited the EFS-evoked increases in $I_{sc}$, reflecting the decrease of chloride and bicarbonate secretion in the middle colon, distal colon, and rectum of rats; the same inhibition was not observed in the proximal colon. This *in vivo* study also showed

**DISCUSSION**

It is now well known that ingesting certain species of gut-dwelling microorganisms can alter host immune, endocrine, and metabolic functions (53) and neuronal firing behavior (22, 50). Further, gut microbiota like *Saccharomyces boulardii* significantly decreased the number of calbindin-28k expressing myenteric neurons in experimental animals (19). Clinical efficacy of probiotics in treating various forms of diarrhea has been clearly established. Diarrhea is caused either by decreased absorption or increased secretion of electrolytes and solutes in the intestine. However, mechanisms underlying antidiarrheal effects of paraprobiotics are not completely understood. In this study, both viable and non-viable CP2305 cells inhibited the EFS-evoked increases in $I_{sc}$, reflecting the decrease of chloride and bicarbonate secretion in the middle colon, distal colon, and rectum of rats; the same inhibition was not observed in the proximal colon. This *in vivo* study also showed
that CP2305 cells ameliorated CRF-induced diarrheal symptoms in the rat model.

The ENS plays a critical role in maintaining normal gut function, including absorption and secretion. EFS-induced increases in $I_{sc}$ are due to the activation of enteric neurons, because Tetrodotoxin completely blocks EFS-evoked increases in $I_{sc}$ in human and other experimental animals (15, 21). Furthermore, ion substitution experiments and studies using a basolateral sodium-potassium-chloride cotransporter (NKCC1) blocker, bumetanide, and adenylate cyclase inhibitor showed that EFS-evoked increases in $I_{sc}$ are due to the stimulation of chloride and bicarbonate secretion and not amiloride-sensitive sodium absorption (21). Most secretomotor neurons have cell bodies in the submucosal nerve plexus and two pharmacologically distinct components of neurally evoked mucosal secretion have been widely identified (9, 10). One is mediated by acetylcholine (ACh) acting on $M_\text{3}$ muscarinic receptors on enterocytes and another is almost certainly mediated by vasoactive intestinal peptide (VIP). In the present study, mucosal but not serosal addition of viable and non-viable CP2305 cells significantly suppressed the EFS-evoked increases in $I_{sc}$ of colon and rectum. This result suggests that viable and non-viable CP2305 may acutely affect neuronal circuits related to the regulation of ion transport in the colon and rectum.

Enterendocrine cells found in the epithelial cell layer of the GI tract comprise transepithelial signal transduction routes that respond to luminal chemicals including nutrients (2, 12). Indeed, certain luminal molecules activate intrinsic primary afferent neurons (IPANs) and extrinsic afferent neurons through enteroendocrine cells that release gut peptides/amino acids to stimulate axon terminals to trigger action potentials (13). For example, direct mucosal application of butyrate activates after-hyperpolarization (AH) neurons of rat colonic myenteric nerve plexus (22). Therefore, similar to these observations, CP2305 cells might affect sensory neurons to modulate EFS-evoked electrolyte secretion, because serosal application of CP2305 did not affect the EFS-evoked electrolyte secretion. Taken together, it is reasonable to speculate that CP2305-derived chemicals might be detected by certain receptors or other sensory devices located on epithelial cells, and this might subsequently lead to the activation of IPANs that carry electrical signals to submucosal ganglia. Finally, information processing in the submucosal ganglia may suppress cholinergic and VIP secretomotor neurons to reduce EFS-evoked electrolyte secretion. At this moment, we could not identify the final target of CP2305 cells to inhibit EFS-evoked electrolyte secretion though acute exposure of another *Lactobacillus* strain, *L. salivarius* UCC118, is reported to selectively inhibit bethanechol-evoked colonic Cl secretion in mice (27). Thus, the precise mechanisms by which CP2305 cells exert such effector signals are not clearly known. One possibility is that CP2305 cells directly affect the muscarinic receptors on enterocytes to inhibit EFS-evoked electrolyte secretion. Further study is needed to prove this hypothesis since ACh receptors are located both on enterocytes and ENS.

Bacterial components are important to communicate between gut microbiota and host. For example, a lipopolysaccharide-free polysaccharide A completely mimics the neuronal effect of the parent organism *Lactobacillus reuteri* (30). In the current study, we used viable and non-viable CP2305 cells and both components showed similar effect on EFS-evoked increases in $I_{sc}$. Therefore, these findings suggest that CP2305 cell components including cell wall and membrane may be able to modulate neuronal circuit to influence ion transport in the colon. Furthermore, they suggest that the components responsible for the inhibitory action for EFS-evoked electrolyte secretion does not need to be synthesized in situ.

Many *Lactobacillus* species produce and release gamma-aminobutyric acid (GABA) (14, 43), a neurotransmitter that has established effects on intestinal motility and enteric AH cell excitability (20). However, GABA and a GABA$_\text{A}$ receptor agonist, 3-APS, evoked increases in $I_{sc}$ in the guinea pig ileum (28). Thus, it is unlikely that both viable and non-viable CP2305 cells contain GABA or GABA-like chemicals that act as signaling molecules to inhibit EFS-evoked electrolyte secretion in rat large intestine except proximal colon. In the ENS, peptide YY (PYY), neuropeptide Y (NPY), galanin, or somatostatin act as antisecretory neuropeptides (9, 10). Thus, CP2305-derived components may act on antisecretory neuropeptide receptors such as PYY, NPY, galanin, or somatostatin. Taken together, the present preliminary results suggest that CP2305 cells may be able to modulate ion transport via ENS or enterocytes. However, further studies are needed to prove this hypothesis and to understand the mechanism of action of CP2305 cells, since there are many CP2305 acting points in the intestine.

Various *Lactobacillus* strains can influence the activities of key regulators of intestinal ion transport, including cystic fibrosis transmembrane conductance regulator (CFTR), the downregulated in adenoma
CP2305 for colonic transport

Histamine evokes Cl− secretion in different parts of the intestine, including colon, in several species (41). Furthermore, histamine excites neurons in the human submucosal plexus through the activation of all histamine receptors (H1–H4) (4). These findings indicate another possible explanation of the inhibitory action of CP2305 on CRF-induced watery diarrhea: the action might be linked with CRF or histamine signaling pathways in vivo. Pharmacological studies have demonstrated that colonic propulsive movement enhanced by CRF or acute stress is abolished or dampened by peripheral injection of CRF antagonist (7, 29, 31).

In the present experiment, fecal pellet output measurement for colonic transit was used to evaluate the effect of chronic ingestion of CP2305 cells on CRF-induced enhanced colonic motility. In the results, there was no difference in the time-dependent changes in fecal pellet output between control and CP2305-fed groups (Fig. 3C). At this moment, we do not know why CP2305 cells could not ameliorate colonic transit similar to that of CRF-induced diarrhea. A recent study has shown that VIP signaling is involved in the CRF-induced, CRF1-mediated diarrhea and defecation (52). However, the precise mechanism of altered colonic motility induced by peripheral CRF has not been clarified. Thus, further studies are needed to elucidate these issues.

In summary, this preliminary study showed that both viable and non-viable CP2305 cells acutely inhibited EFS-evoked electrolyte secretion in rat large intestine, and chronic ingestion of CP2305 cells inhibited the CRF-induced diarrhea in rats. Moreover, the suppressing effect of CP2305 cells on ENS-evoked electrolyte secretion showed segmental and bacterial strain dependency. Overall, this preliminary study provides an interesting opportunity to better understand the effect of non-viable lactic acid bacteria on GI physiology. It also provides a rationale for the use of paraprobiotics in a range of stress-related symptoms in addition to GI disorders.

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