Propionibacterium freudenreichii ET-3で培養した乳清発酵物は特異的に遠位大腸の収縮頻度を充進させる

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Milk Whey Culture with *Propionibacterium freudenreichii* ET-3 Exclusively Enhances Contractile Frequency of the Distal Colon: An In Vitro Evaluation using Rats

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**Abstract**

Constipation is a serious problem to decrease the quality of life following socioeconomic loss. Milk whey culture with *Propionibacterium freudenreichii* ET-3 (milk whey culture) has been reported to improve the constipation by enhancing the growth of *Bifidobacterium* in clinical study. However, the effect of milk whey culture on the colonic motility has not been clarified. Therefore, this study aimed to clarify this point using the rat colon in vitro. To clarify the regional specificity, longitudinal specimens of the proximal, middle and distal colon were used. After obtaining the constant spontaneous contraction, milk whey culture was added in a concentration-dependent manner. The contractile frequency and force were used as end-point. Milk whey culture significantly enhanced the contractile frequency of the distal colon in a concentration-dependent manner, but not the contractile force. On the contrary, in the proximal and middle colon, milk whey culture did not show any significant effect in both contractile frequency and force. Milk whey culture contains 1,4-dihydroxy-2-naphthoic acid (DHNA) enhancing the growth of *Bifidobacterium*. DHNA did not influence the contractile frequency and force. Also propionic acid and acetic acid contained in milk whey culture did not show any significant effect. Above results show that milk whey culture may relief the constipation by exclusively enhancing the spontaneous contraction of the distal colon, which plays an important role for defecation, although active substance was not clarified. No effect on the contractile force may be a merit, because the enhancement of contractile force may lead to the abdominal pain.

**Key words:** colonic motility in rat, *Propionibacterium freudenreichii* ET-3, short chain fatty acid

**Introduction**

Intestinal microflora has been known to play an important role in the pathogenesis of constipation. Probiotics, especially *Bifidobacterium*, have been reported to improve the constipation in human\(^1,2\). Milk whey culture with *Propionibacterium freudenreichii* ET-3 (milk whey culture) is a prebiotics reported to enhance the growth of *Bifidobacterium\(^3,4\) and to be effective on the constipation by improving the intestinal flora. Amano *et al.*\(^5\) reported the efficacy of milk whey culture even on the constipation dominant irritable bowel syndrome. In addition, Ouwehand *et al.*\(^6\) also reported the efficacy of the juice supplemented with *Propionibacterium freudenreichii* on the constipation, although *Lactobacillus rhamnosus* was also used as combined supplement with *Propionibacterium freudenreichii*. However, the pathogenesis of constipation has not been completely clarified as yet especially in relation to the bowel flora. For example, 5-hydroxytryptamine (serotonin, 5-HT) exists in the gastrointestinal tract and plays an important role for the contraction of it. Short chain fatty acid also plays an important role for the small and large intestine. Therefore, the increase of *Bifidobacterium* may be one of the causes of the improvement of the constipation.

Ichikawa *et al.*\(^7\) investigated the efficacy of gastric instillation of short-chain fatty acids on the
epithelial cell proliferation of small and large intestine in rats. Ferreira et al.\(^8\) reported that oral administration of butyrate showed the beneficial effects on intestinal mucositis induced by 5-fluorouracil not only of the colon, but also of the small intestine. Vieira et al.\(^9\) also found that oral administration of sodium butyrate attenuated acute ulcerative colitis. On the contrary, Kien and Blauwinkel\(^10\) reported that the cecal infusion of butyrate did not alter cecal concentration of butyrate in piglets fed inulin. These reports suggest that oral administration of short chain fatty acid may influence on the colonic function, although the mechanisms have not been clarified.

Therefore, this study aimed to evaluate whether milk whey culture has a direct effect on the colonic motility in rats or not, although the increase of \(Bifidobacterium\) has been observed in the clinical studies\(^3,4\). In addition, the effects of propionic acid, acetic acid and 1,4-dihydroxy-2-naphthoic acid (DHNA) was investigated on the colonic motility from the finding that milk whey culture contains propionic acid and acetic acid, and DHNA which plays an important role to enhance the growth of \(Bifidobacterium\)^3,4).

Materials and Methods

All animal experiments were performed according to the “Guiding Principles for the Care and Use of Laboratory Animals” approved by the Japanese Pharmacological Society.

Animals: Male Sprague-Dawley rats weighing from 200 to 250 g were purchased from SLC (Shizuoka, Japan), and housed for 1 week prior to the commencement of the experiments under a constant temperature of 21±2°C and humidity of 55±15%. Light and dark cycle was 12 hr and light cycle was 7:00 to 19:00.

Experimental protocol: Following a midline incision after killing by a sharp blow to the head and exsanguinations from the axillary artery, the colon was carefully excised into the Krebs solution bubbled continuously with 95% \(O_2\) and 5% \(CO_2\). Three specimens of the longitudinal proximal (from 2 to 3 cm from the cecum), middle (7 to 8 cm from the anus) and distal (1 to 2 cm from the anus) colon were trimmed from the other mesenteric tissue. End of the specimen (1 cm length) was sustained in the organ bath (10 ml) and the other end was connected to the force transducer. Organ bath contained 10 ml of Krebs solution contained (g/L): NaCl 6.92, KCl 0.35, MgSO\(_4\) 7H\(_2\)O 0.29, NaHCO\(_3\) 2.1, CaCl\(_2\) 0.28, KH\(_2\)PO\(_4\) 0.16, glucose 2.1 and bubbled continuously with 95% \(O_2\) and 5% \(CO_2\) at 30°C. Static tension of 1 g was lured to the specimen. Spontaneous contractions were recorded for 20 min to obtain the constant contractile response. The contractile response was recorded for 5 min after addition of the test sample (10 to 100 \(\mu\)l in volume). Contractile force and frequency were used as criteria of the motility of the colon and calculated from the recorded chart.

Effect of milk whey culture: The effect on the motility of the colon was evaluated by the addition of milk whey culture dissolved in distilled water for injection at a final concentration from 1 to 10 \(\mu\)g/ml. In the control, distilled water for injection was added instead of milk whey culture.

Effects of propionic acid, acetic acid and DHNA: To clarify the active substance, the effects of propionic acid and acetic acid were investigated on distal colonic motility from the finding that milk whey culture contained propionic acid and acetic acid at a concentration of 0.1 mM. Moreover, the effect of DHNA was also evaluated, because milk whey culture contains DHNA at concentration of 4.4 \(\mu\)g/ml. In the control, distilled water for injection was added instead of propionic acid, acetic acid and DHNA.

Agents: Milk whey culture produced by Meiji Co., Ltd. (Tokyo, Japan) contained solid content. Contents of propionic acid, acetic acid and DHNA containing in milk whey culture were shown in Table 1. Sodium propionic acid and sodium acetic acid were purchased from Sigma (St. Louis, MO, USA). DHNA was purchased from Wako Pure Chemical (Tokyo, Japan). Distilled water for injection was obtained from Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan).

Statistical analysis: The results were expressed as the mean±standard error (S.E.). Statistical analysis was performed by using Student’s \(t\)-test and trend test. \(P<0.05\) was treated as statistical significance.
Table 1 Contents of propionic acid, acetic acid and DHNA containing in milk whey culture.

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<td>Acetic acid</td>
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<tr>
<td>DHNA</td>
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DHNA: 1,4-dihydroxy-2-naphthoic acid

Results

Effect of milk whey culture: Figure 1 shows the effect of milk whey culture on the proximal colon. In the control rats, the addition of distilled water for injection did not affect on the contractile force and frequency. Milk whey culture also did not affect on the contractile force and frequency of the proximal colon (Fig. 1).

Figure 2 shows the effect of milk whey culture on the middle colon. In the control rats, addition of the distilled water for injection did not affect on the contractile force and frequency. Milk whey culture also did not affect on the contractile force and frequency of the middle colon (Fig. 2).

Milk whey culture and distilled water for injection did not affect on the contractile force of the distal colon (Fig. 3). However, milk whey culture significantly and concentration-dependently enhanced the frequency of the distal colon, although the addition of the distilled water for injection did not affect on the contractile frequency (Fig. 3).

Moreover, it became clear that there is a significant difference in the inclination of two regression curves by the analysis of trend test (p<0.05).

Effects of propionic acid, acetic acid and DHNA: Propionic acid and acetic acid at a concentration contained in milk whey culture did not have any effect on the contractile force and frequency of the proximal, middle and distal colon (data not shown). Moreover, DHNA containing in milk whey culture also did not affect on the motility of the distal colon, contractile force and frequency (data not shown).

Discussion

Milk whey culture does not contain viable
Propionibacterium freudenreichii ET-3 in the milk whey culture, but contains dead one. Therefore, the materials in the colon produced by Propionibacterium freudenreichii ET-3 are excluded as the active substances on the colonic functions. However, many reports suggest that oral administration of short chain fatty acids affected on the colonic function\(^7\)~\(^9\). These reports may suggest that absorbed short chain fatty acids influence on the colonic function. Therefore, in this study we evaluated the effects of propionic acid, acetic acid and DHNA containing in milk whey culture.

In this study, we already investigated the effect of milk whey (culture medium) on the colonic motility and found that milk whey showed no effects on the colonic motility. In addition, there was no difference between milk whey and distilled water for injection. Therefore, in the control we used distilled water for injection to simplify the system for finding active substance.

The treatment with milk whey culture significantly and exclusively enhanced the frequency of the distal colon in a concentration-dependent manner, although the contractile force was not affected. However, the short chain acid and DHNA contained in milk whey culture did not influence on the colonic motility.

The present author previously reported the efficacy of milk whey culture against 2,4,6-trinitrobenzene sulfonyl acid-induced colitis in rats, and that propionic acid contained in the milk whey culture also effective on this colitis model\(^1\)~\(^3\). Short chain fatty acid, such as butyric acid, propionic acid and acetic acid, has been known to induce the contraction on the distal colon\(^13\). In these short chain fatty acids, propionic acid has the strongest contractile activity on the colon. Milk whey culture has been known to improve constipation in human study by increasing Bifidobacterium and active substance of bifidogenic growth factor reported to be DHNA\(^3\)~\(^4\). Then in this study, we tested the effect of DHNA on the colonic motility, but no effect was observed.

Suthamnatpong et al.\(^14\) reported that vasoactive intestinal peptide is the most likely candidate as a non-adrenergic non-cholinergic transmitter in the distal colon. Moreover, Appleyard et al.\(^15\) investigated strips of the proximal, middle, and distal colonic muscularis mucosae for measurement of short-circuit current and found that the greatest responses to substance P were found in the proximal region. Okishio et al.\(^16\) reported that participation of pituitary adenylate cyclase activating peptide in nonadrenergic noncholinergic relaxation was suggested only in the distal colon. These mechanisms were involved in the contractile force. However, in this study the contractile force was not affected by the addition of milk whey culture.

On the frequency of the colonic motility, Grider and Piland\(^17\) reported that chemical stimulation of the mucosa by short chain fatty acids triggers a peristaltic reflex mediated by the release of 5-HT from mucosal cells and activation of 5-HT\(_4\) receptors on sensory calcitonin gene–related peptide containing nerve terminals. Mitsui et al.\(^18\) reported that propionate modulates spontaneous contractions via enteric nerves and prostaglandin release in the circular muscle strips of the rat distal colon.
with the mucosa attached, and suggested that propionate increases the frequency of spontaneous giant contractions by an activation of cholinergic motor neurons and decreases the mean amplitude by a prostaglandin release. However, propionic acid contained in milk whey culture did not influence the frequency of the contraction at a concentration used in this study. In addition, Ono et al.\textsuperscript{19} reported that short chain fatty acids in rat distal colon decrease the frequency of spontaneous contractions of the longitudinal muscle, and that short chain fatty acids may contribute to colonic motility, including the peristaltic reflex, by regulating the frequency of spontaneous contractions of the longitudinal muscle through the enteric nervous system. However, no effect on the contractile frequency was observed by the addition of milk whey culture in this study. In addition, short chain fatty acid also did not show the change in contractile frequency. These findings may suggest that the other ingredient contained in the milk whey or combination of the ingredients tested in the present study plays an important role in the increase of the contractile frequency, because the additive or synergistic effect may be induce the enhancement of the contractile frequency observed in the present study.

On the contraction of the colon, interstitial cells of Cajal (ICC) has been known to play an important role also in rats. Indeed, Albertí et al.\textsuperscript{20} reported that the loss of ICC markedly affected pacemaker and motor activities of the rat colon and nitrergic innervation was reduced possibly related to the loss of ICC-mediated relaxation. Takahashi et al.\textsuperscript{21} found that ICC related to the occurrence of a normal number of colonic contractions and that the effect of nitric oxide on the occurrence of contractions appeared to be mediated by ICC. Therefore, milk whey culture might influence the ICC or nitrergic innervation. Moreover, Albertí et al.\textsuperscript{22} reported that neuronal nitric oxide synthase positive cells were found at the level of the Auerbach's plexus and the major density was found in the middle colon, although the regional difference was observed in the distal colon in the present study. Further studies would be needed to clarify the regional difference on the colon by milk whey culture.

In conclusion, it was found that milk whey culture may show a useful effect on the constipation by enhancing the spontaneous frequency of the distal colonic contraction in addition to the prebiotic effect enhancing the growth of \textit{Bifidobacterium}. However, \textit{in vivo} study would be needed to certify the effect of milk whey culture on the spontaneous frequency of the distal colon because the present results were obtained by \textit{in vitro} study.

References


Propionibacterium freudenreichii ET-3で培養した乳清発酵物は特異的に
遠位大腸の収縮頻度を亢進させる：ラットを用いたin vitroでの評価

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便秘は著しく生活の質を低下させるだけではなく社会経済的にも大きな損失となっている。乳清をPropionibacterium freudenreichii ET-3で培養した発酵物（乳清発酵物）はビフィズス菌を増加させて便秘を改善する作用を持っており、臨床試験で明らかにされている。しかし、大腸の運動に対しどのような作用を示すかについては明らかになっていない。そこで、今回の試験では部位差についても評価するため、ラットの近位、中位および遠位大腸について収縮力および収縮頻度に与える乳清発酵物の作用をin vitroで評価した。乳清発酵物は遠位大腸に対し濃度依存的に収縮頻度を有意に増加させた。しかし、収縮力には影響しなかった。一方、近位および中位大腸の収縮力および収縮頻度に対しては何ら影響を示さなかった。乳清発酵物に含まれる1,4-dihydroxy-2-naphthoic acid (DHNA)はビフィズス菌の増殖作用を示すことが明らかになっているが、大腸の収縮力および収縮頻度には作用を示さなかった。さらに乳清発酵物に含まれるプロピオン酸および酢酸も何ら作用を示さなかった。以上の結果から、乳清発酵物は排便に重要な役割を果たしている遠位大腸に対し特異的に収縮頻度を増加させることが明らかになった。また、収縮力を増加させないことは収縮力の増加による腹痛を引き起こすことから便秘に対する乳清発酵物の有用性を示す結果と考えられた。