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Lactic acid bacteria-containing chocolate as a practical probiotic product with increased acid tolerance

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ABSTRACT

This study shows an evaluation of the method to improve acid tolerance of probiotic bacteria aimed at enhancement of the probiotic effects in the intestinal canal. In the tolerance test of simulated digestive juice *in vitro*, the viable rate of *Lactobacillus brevis* subsp. *coagulans* (*Labre*) processed with chocolate (*Labre*-in-chocolate) is significantly increased, approximately one hundred times higher, compared to those of freeze-dried *Labre* powder and *Labre* contained in beverage. This protective effect depends on the content of water in chocolate for the probiotics processing. Differences in tolerance of metabolic activity were investigated with *Labre*-in-chocolate and freeze-dried *Labre* powder, both treated with simulated digestive juice *in vitro*. The enzyme activity of *Labre*-in-chocolate remained significantly even though that of the freeze-dried *Labre* powder was inactivated. These results indicate that chocolate processing is an effective method making probiotics to be delivered to the intestine in a viable condition and to be effective for host health.

1. Introduction

A bacterium that provides specific health benefits to the human body is called a probiotic, which is expected to have various benefits for health maintenance when consumed as a food component or supplement. *Lactobacillus brevis* subsp. *coagulans* called *Labre* is a lactic acid bacterium that belongs to probiotics.

Labre is effective to improve the immune system through inducement of interferon-α production and enhancement of natural killer (NK) cell activity (Kishi et al., 1996; Kishida., 1997; U.S.P.5662900). In other cases, it has been reported that *Labre* has various benefits for the human body such as early intervention in irritable bowel syndrome (Murakami et al., 2012), activation the availability of medical herb flavonoids (Sakurama et al., 2014). Moreover, there is a report that *Labre* could reduce the risk of infection in children during influenza season (Waki et al., 2014). As discussed above, the effectiveness of probiotics such as *Labre* is now widely accepted by consumers, and this may play a part in the growth element of the functional food market.

Lactic acid bacteria, however, are damaged by gastrointestinal stresses in the case of oral intake, and the viable rate is dramatically decreased in the intestine. It is expected to deliver live bacteria into the intestine to maintain the activities of the probiotics themselves or to protect their metabolisms generating effective compounds. Therefore, a live-bacteria-containing pharmaceutical preparation is coated with an enteric coating agent to prevent the pharmaceutical from being exposed to gastric acid aimed at enhancement of the probiotic effects in the intestine. Lactic acid bacteria are usually taken with yogurts and beverages, but they are easily exposed to gastric acid. In this case, the probiotic effects may not be expressed adequately in the intestine.

The aim of this study is to establish the practical methods to deliver bacteria to the intestine in a viable condition in the form of general food through the verification of increased acid tolerance of *Labre*, which is coated with chocolate against simulated digestive juice in vitro. Furthermore, we confirmed sustained enzyme activity in the lactic acid bacteria-in-chocolate after the treatment with simulated digestive juice with nucleoside-hydrolyzing activity as an indicator.

2. Materials and methods

2.1. Lactic acid bacteria

Lactobacillus brevis subsp. *coagulans* (*Labre*) FERM BP-4693

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used in this study is isolated from sugukizuke (a regional pickle of Japan made from a turnip-like vegetable) in Kyoto, Japan, and the probiotic powder was produced at Nitto Pharmaceutical Industries, Ltd. (Kyoto, Japan). *Labre* was incubated at 30 °C for 24–48 h in a growth medium mainly composed of 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, and 10 g/L meat extract. The culture supernatant was removed by centrifugation. The resultant bacterial cells were freeze-dried and used as *Labre* powder.

Lactobacillus brevis NTM003 (*NTM003*) NITE BP-1634 used in this study is isolated from nanohanazuke (a regional pickle of Japan made from the flower of cole) in Kyoto, Japan, and the probiotic powder was produced at Nitto Pharmaceutical Industries, Ltd.. *NTM003* was incubated at 37 °C for 24–48 h in a growth medium mainly composed of 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, and 10 g/L meat extract. The culture supernatant was removed by centrifugation. The resultant bacterial cells were freeze-dried and used as *NTM003* powder.

Commercial probiotics used in this study are as follows: Product A is a probiotic beverage that contains *Lactobacillus brevis*. Product B is a probiotic beverage that contains *Lactobacillus casei*. Product C is a probiotic yogurt that contains some kind of lactic acid bacteria and is certified as a Food for Specified Health Uses in Japan. Product D is a world-wide consumed probiotic yogurt that contains some kinds of lactic acid bacteria.

2.2. Preparation of Labre-in-chocolate and NTM003-in-chocolate

Labre or *NTM003* powder was added to melted chocolate, and then this highly concentrated lactic acid bacteria-in-chocolate was taken into tempered chocolate, keeping the temperature around 32 °C. This chocolate liquid was poured into a mold, cooled, and used as probiotics-in-chocolate. Final concentration of *Labre* or *NTM003* powder in the chocolate was 0.5% (w/w). The used chocolate was a commercial chocolate (LOTTE Co., Ltd. Saitama, Japan) composed of 30% (w/w) cocoa components (cocoa mass and cocoa butter), 20% (w/w) milk component, 9% (w/w) vegetable fat component, 40% (w/w) sugar component, and 0.5% (w/w) emulsifier and natural vanilla flavoring components.

2.3. Tolerance of lactic acid bacteria against simulated digestive juice in vitro

A simulated digestive juice was made as described previously (Azuma et al., 2001) with modifications. Briefly, Lactobacilli MRS broth (Becton Dickinson, and Company, NJ, USA) with 0.04% (w/v) pepsin (Wako, Osaka, Japan) was adjusted to pH 2.5 using HCl. Four gram of each probiotics-in-chocolate were added to 300 ml of simulated digestive juice and shaken at 37 °C for a few hours at 70 rpm; the liquid surface was gently shaken by the revolutions per minute. Then the total viable count of probiotics was analyzed after 0.5 h, 1 h, and 2 h incubations in simulated digestive juice by sampling 1 ml of the solution, diluting adequately, and plating on MRS broth plates with 1.5% (w/v) agar. For the treatment of probiotics powders or commercial probiotics products, the initial viable count before the treatment was adjusted as many as it of probiotics-in-chocolate.

2.4. Probiotics-in-chocolate with fresh cream or freeze-dried cream

Probiotics-in-chocolate with fresh cream was made as follows. Boiled fresh cream (20 g) was added to chopped chocolate (LOTTE Co., Ltd., Saitama, Japan) (50 g), and melted by heating at 40 °C. Then 0.5% (w/w) probiotics powder was added and mixed quickly. This chocolate liquid was poured into a tray and cooled at 4 °C for 0.5 h.

Probiotics-in-chocolate with freeze-dried cream was made as follows. A chopped chocolate (50 g) was melted by heating at 40 °C. Then freeze-dried cream (10.4 g) and 0.5% (w/w) probiotics powder was added and mixed quickly. This chocolate liquid was poured into a tray and cooled at 4 °C for 1 h.

2.5. Activity analysis of the enzyme converting purine nucleosides into purine bases

The enzyme activity of probiotics converting purine nucleosides into purine bases was assayed as described previously (Tsuboi et al., 2012; J.P.5149305) with modifications. Briefly, 50 mg of probiotics powder or 1 g of probiotics-in-chocolate which contained 50 mg of probiotics powder after treatment with 0.85% NaCl or simulated digestive juice was washed twice with 0.85% NaCl. After washing, 4.0 ml of the nucleoside solutions containing 2 mM guanosine and 4 mM inosine in 0.1 M potassium phosphate buffer (pH 7.0) were added into the resulting probiotics precipitation. The mixture was incubated for 1 h at 37 °C, and then the reaction was terminated by adding 0.5 ml of 2.0 N HCl. Guanine and hypoxanthine in the reaction mixtures were quantified with high-performance liquid chromatography (HPLC). The HPLC analysis was performed with a Shimadzu LC 10 A-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C18-PAQ column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) at 40 °C. 10 μl of the filtered supernatant of the reaction mixture was injected to HPLC. The mobile phase was 50 mM KH_2PO_4 (pH 3.0). The flow rate was 1.0 ml/min. The effluents were monitored at 254 nm using diode array detector (SPD-M20A, Shimadzu Kyoto, Japan).

2.6. Statistics

Each result is expressed as the mean ± standard deviation (SD).

Tukey's multiple comparison tests or Student's *t*-test were used to compare each group. Statistical analyses were done with Excel Toukei 2010 (Social Survey Research Information, Tokyo, Japan).

3. Results

3.1. Tolerance of differently processed probiotics against simulated digestive juice

The acid tolerances of various probiotics in different preparations, i.e., *Labre* powder, *Labre*-in-chocolate, and *Labre*-in-beverage (Product A), were evaluated. Fig. 1 shows the tolerance of lactic acid bacteria against simulated digestive juice in vitro. The viable count of *Labre* powder was not stable; it immediately decreased from 10^6 cfu/ml to 10^3 cfu/ml in 0.5 h, notably decreased to 10 cfu/ml in 1 h, and finally was non-detected (marker not shown) in 2 h after adding into simulated digestive juice. The viable count of *Labre*-in-beverage (Product A) was also not stable: it immediately decreased from 10^6 cfu/ml to 10^3 cfu/ml in 0.5 h, notably decreased to 10 cfu/ml in 1 h, and finally decreased to 10 cfu/ml in 2 h after adding to simulated digestive juice. However, the viable count of *Labre*-in-chocolate was more stable for at least 2 h, it decreased slightly to 10^5 cfu/ml in 2 h after adding to simulated digestive juice. These results showed that the viable rate of *Labre*-in-chocolate is significantly increased, by at least one hundred times, compared to those of the *Labre* powder and *Labre*-in-beverage.

3.2. Effects of chocolate processing on different lactobacillus brevis strains

Viability-enhancing effects of chocolate processing were investigated with two representative *Lactobacillus brevis* strains.