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Oral Delivery of Probiotics Using pH-Sensitive Phthalyl Inulin Tablets

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Probiotics show low cell viability after oral administration because they have difficulty surviving in the stomach due to low pH and enzymes. For the oral delivery of probiotics, developing a formula that protects the probiotic bacteria from gastric acidity while providing living cells is mandatory. In this study, we developed tablets using a new pH-sensitive phthalyl inulin (PI) to protect probiotics from gastric conditions and investigated the effects of different compression forces on cell survival. We made three different tablets under different compression forces and measured survivability, disintegration time, and kinetics in simulated gastric-intestinal fluid. During tableting, there were no significant differences in probiotic viability among the different compression forces although disintegration time was affected by the compression force. A higher compression force resulted in higher viability in simulated gastric fluid. The swelling degree of the PI tablets in simulated intestinal fluid was higher than that of the tablets in simulated gastric fluid due to the pH sensitivity of the PI. The probiotic viability formulated in the tablets was also higher in acidic gastric conditions than that for probiotics in solution. Rapid release of the probiotics from the tablet occurred in the simulated intestinal fluid due to the pH sensitivity. After 6 months of refrigeration, the viability of the PI probiotics was kept. Overall, this is the first study to show the pH-sensitive properties of PI and one that may be useful for oral delivery of the probiotics.

Keywords: Probiotics, oral delivery, pH-sensitive tablet, phthalyl inulin

Introduction

Nowadays, probiotics are used as an alternative for antibiotics since they are generally considered as safe and confer health benefits to the host. Probiotics produce antimicrobial molecules (*e.g.*, lactic acid and bacteriocins) and enzymes, enabling them to function as an alternative to antibiotics owing to their antimicrobial effects toward pathogens [1]. Among probiotics, *Lactobacillus* is the most common probiotic because *Lactobacillus* has shown excellent antimicrobial activity against *Salmonella* spp. and *Escherichia coli*, which are major pathogens in livestock animals [2, 3]. *Lactobacillus* reduces weight loss, improves feed intake and improves the growth performance of animals [4]. In our previous study, we isolated *L. reuteri* LRT18 from porcine feces and selected the highest antimicrobial effect on K88-positive *Escherichia coli* and *Salmonella enterica* subsp. [5].

To deliver probiotics orally, many strategies target the intestine as a main location for the probiotics to provide the most beneficial effect to the host [6, 7]. Since the intestine has a neutral pH, long transit time and reduced host enzymatic activity, an intestine-specific drug delivery system increases the bioavailability of probiotics [8]. However, oral delivery of probiotics is extremely challenging

because they can get destroyed and/or cause cell death due to stomach acid [9]. Therefore, delivering probiotics to the intestine safely while passing through harsh gastric conditions is of utmost importance in enabling probiotics to provide their therapeutic effect to the host. Recently, polymeric delivery systems have been attracting attention as a means to deliver biological materials, proteins, genes, and chemotherapeutics because they can deliver drugs to target sites [10]. Among many strategies for orally delivering probiotics, pH-sensitive polymers, such as hydroxypropyl methylcellulose phthalate (HPMCP) [11], hydroxypropylmethyl cellulose acetated succinate [12], and cellulose acetate phthalate (CAP) [13] have been used to protect probiotics from harsh gastric conditions since probiotics loaded into pH-sensitive polymers cannot be released in an acidic pH environment due to the deprotonated carboxylic acids in the polymers [14]. However, because these pH-sensitive polymers only have the ability to protect probiotics from harsh gastric conditions, we designed a new type of pH-sensitive polymer using inulin as a prebiotic.

Inulin has been used as a prebiotic source in industrial applications because they are found in many natural sources (*e.g.*, chicory root, Jerusalem artichoke, leek, and onion) [15]. Inulin consists of fructose polymers linked by β (2 → 1) bonds containing glucosyl moieties at the chain terminal. Due to the β (2 → 1) linkages in inulin, it is not digested by pancreatic enzymes in the upper GI tract [15], although the gut microbiota can ferment inulin and produce short chain fatty acids (SCFAs), which induces the growth of beneficial microorganisms, thereby altering the composition of organisms in the gut microbiome and boosting the host immune system [16, 17]. Also, there has been a growing interest in the use of inulin as an adjuvant or drug delivery system. Interestingly, delta inulin in microparticle form showed adjuvanting ability for enhancing immune activity in vaccines against influenza, hepatitis B, etc. [18], although soluble inulin has less immunological activity [19].

In the health industry, there are several methods of formulating probiotics for use as food supplements and these include powder, liquid, and spray forms, although the most commonly used is powder. Although there have not been many reports, on creating a tablet form for oral delivery of probiotics one previous study did show that it is easy to formulate probiotics into tablet form with a pH-sensitive polymer that successfully protected probiotics from harsh stomach conditions [20].

In this study however, we aimed to develop a new pH-sensitive tablet using phthalyl inulin (PI) to protect the *L. reuteri* LRT18 (LR) from harsh gastric conditions. Moreover, we obtained promising results for further in vivo application. To the best of our knowledge, this is the first report to exhibit the pH-sensitive properties of PI that protect probiotics from harsh gastric conditions.

Materials and Methods

Materials

L. reuteri LRT18 (LR, KCTC3594) used in this study was isolated from a previous study [5]. All of the materials and chemicals used in this study were purchased from Sigma-Aldrich (USA) unless otherwise stated. De Man, Rogosa and Sharpe agar (MRS) broth and MRS agar were purchased from BD Difco (Sparks, USA) for the bacterial cultures.

Synthesis of Phthalyl Inulin (PI)

Phthalyl inulin (PI) was synthesized as described in a previous report [21]. Briefly, 1 g of inulin (MW: 5,000 g/mol) was dissolved in 5 ml of *N*,*N*-dimethylformamide and 2.0 g of phthalic anhydride were added in the above solution and 0.2 ml of 5% (w/v) sodium acetate was used as a catalyst. The reaction was conducted at 40°C for 24 h under nitrogen gas. And then, the PI was dialyzed in cold water for 24 h. The PI was lyophilized and stored at −20°C until use. The conjugation of phthalyl groups in PI was confirmed by 600 MHz ^{1}H-NMR spectroscopy (AVANCE600, Bruker, Germany).

Tablet Preparation

LR cultures were grown in MRS broth at 37°C for 24 h and collected by centrifugation. Harvested cells were washed 3 times in phosphate buffer solution and suspended in 10% skim milk. The cells were then frozen at −20°C for 12 h and lyophilized. The lyophilized probiotics were ground into a fine powder and stored at 4°C until use. The tablets were prepared at room temperature (RT) by direct compression using a single press. For the tablets, a mixture of LR and PI (weight ratio of LR to PI = 1:1) was filled into a 4 mm diameter die. The tablets were formed under different pressures ranging from 3 to 10 kilopascal (KP) with a plane surface according to Tao *et al.* [20].

Measurement of the Probiotic (LR) Viability and Disintegration Time of Tablets

The viability of LR in the tablet was expressed as colony forming units (CFU). Briefly, the tablets were broken and dispersed in 1 ml of phosphate buffer solution (PBS, pH 6.8). And then, the serial-diluted suspension was dropped into the MRS agar plate and incubated at 37°C to count the LR colonies according to the Tao *et al.* method [22]. The tablets were transferred into 5 ml PBS (pH 6.8) and the complete disintegration time was measured.

Measurement of the Swelling Ratio of Tablets

The tablets were transferred into 5 ml simulated gastric fluid (SGF) adjusted to pH 2 with pepsin (1,000 U/ml). The swelling ratio was calculated by the following equation [22].

$Q = (M_s\text{-}M_d)/M_d$

The swelling ratio is Q, the M_d is the tablet mass in the dried state and the M_s is the mass of the tablet in the swollen state. At the beginning of the experiment, the excess water outside the tablet was removed.

Stability of the Tablets in the SGF with or without Pepsin

The stability studies were performed as described in a previous method [20]. The SGF was prepared by PBS adjusted to pH 2.0 with or without pepsin (1,000 U/ml) by 1 M HCl. The tablets and LR powder were transferred into 5 ml of SGF with or without pepsin. The survivability of LR was observed as the CFU at the end of the incubation period (0, 30, 60, 90, 120 min) when incubated at 37°C with 100 rpm.

Viability of the Tablets in SGF and Simulated Intestinal Fluid (SIF) Medium in Sequential Exposure

The cell viability of LR in the tablets sequentially exposed to SGF and SIF was performed by the following method [20] with some modifications. Tablets were incubated in 5 ml SGF (pH 2, 1,000 U/ml pepsin) at 37°C with 100 rpm for 2 h. Then, the tablets were quickly transferred to 5 ml SIF and incubated at 37°C with 100 rpm for 4 h. SIF was prepared by PBS adjusted to pH 6.8 with 1.2% (w/v) bile salt. The viable cells were counted in the supernatant medium as were the non-disintegrated tablets at each incubation time.

Tablet Stability

The stability of the tablets was tested when stored at 4°C for up to 6 months. Every month the cell viability was counted as described above.

Statistical Analysis

Data are presented as the mean ± SEM of three independent experiments. The statistical significance was analyzed between each group by one-way ANOVA and Tukey's test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

Results

Synthesis and Characterization of PI

To develop a pHs sensitive polymer, the phthalic group was introduced to inulin by an ester bond between hydroxyl groups in inulin and carboxylic acids in phthalic acid. The reaction scheme of the synthesis of PI is shown in Fig. 1A. After synthesizing the PI, the degree of the phthalic groups in the PI was estimated by measurement of ^{1}H-NMR. The fifth protons of inulin appeared at 3.8 ppm

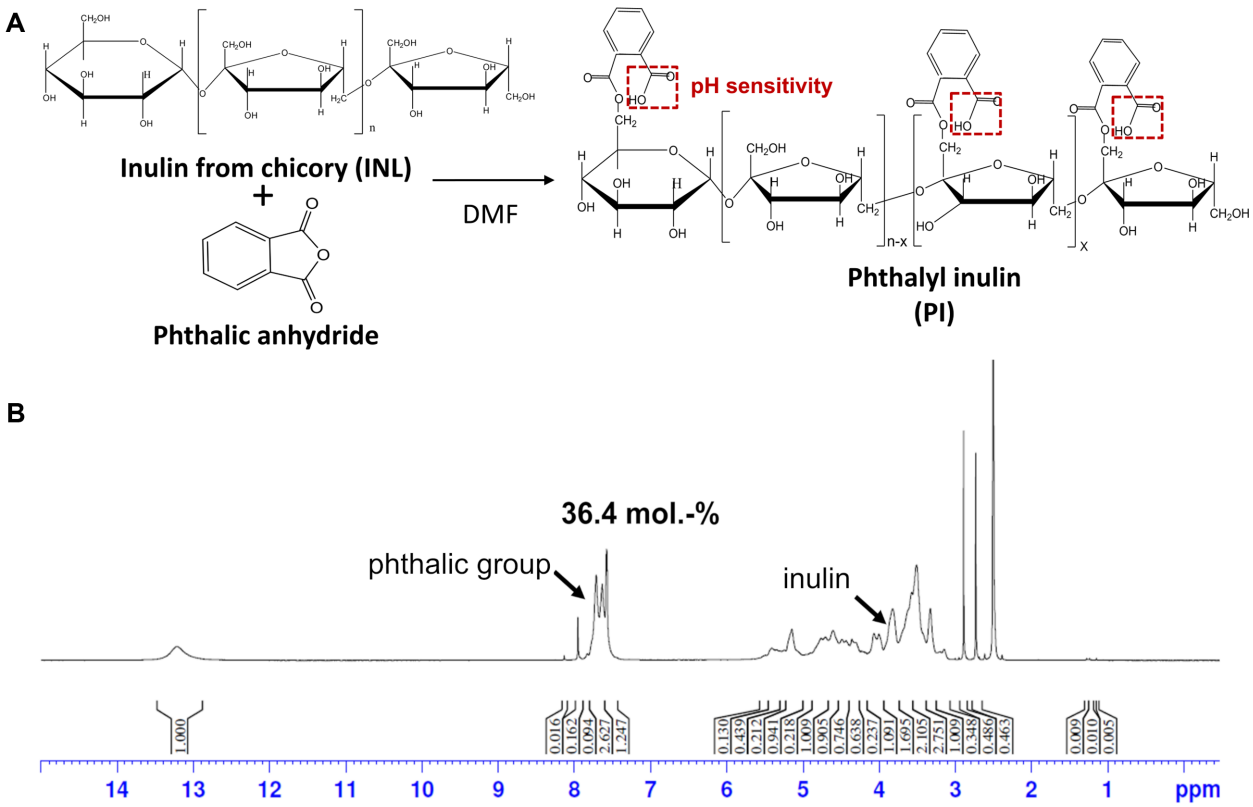

Fig. 1. Scheme and characterization of phthalyl inulin (PI). Chemical reaction scheme of PI (**A**) and NMR spectrum of PI (**B**).

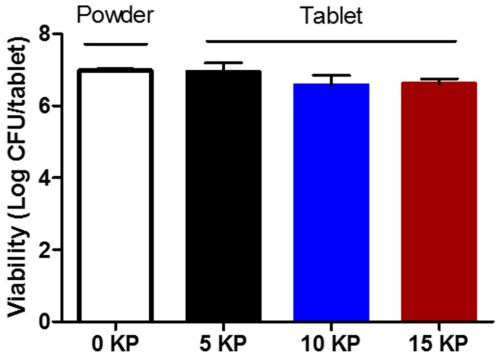

Fig. 2. Viability of *L. reuteri* (LR) after tableting under different compression forces (5, 10, and 15 KP) (means ± standard deviation, SD; $n = 3$).

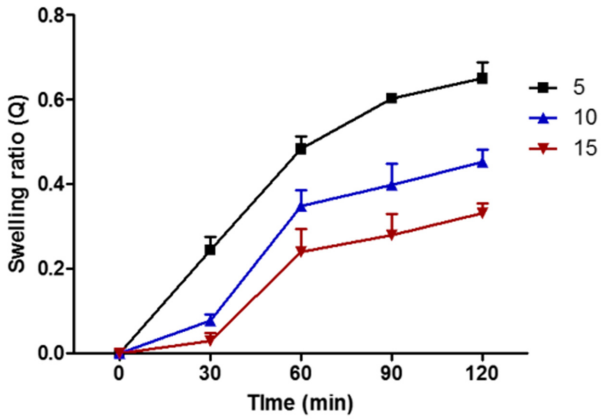

Fig. 3. Swelling ratio of LR-loaded PI tablets with different compression conditions (5, 10, and 15 KP) until 2-h incubation in SGF (means ± standard deviation, SD; $n = 3$).
(PI: phthalyl inulin, LR: *Lactobacillus reuteri*, and SGF: simulated gastric fluid)

and the protons of the phthalic groups in the PI appeared at 7.4–7.7 ppm as shown in Fig. 1B. Based on the integration of protons in both the inulin and phthalic groups, the degree of the phthalic groups in the PI was 36.4 mol.-%.

Effects of Compression Forces on the Viability of LR and Tablet Properties

To evaluate whether different compression force can affect the viability of LR, we measured it after tableting and found that there were no significant differences in the viability of LR in the tablets even with the use of varied compression forces (Fig. 2). To determine the protective effect of the LR in gastric conditions, the swelling ratio of the tablets and viability of the LR in SGF were measured. The swelling ratio of the tablets prepared according to different compression forces was very low in SGF conditions (Fig. 3). It was observed that the tablets were not completely disintegrated within 2 h in gastric conditions. In particular, among the groups, the highest compression force (15 KP) showed the lowest swelling ratio. The viability of LR in gastric conditions was then measured using the SGF conditions with or without pepsin (Fig. 4). The 5, 10, and 15 KP tablets and probiotics alone (powder) were loaded in SGF for 2 h. The results showed that the viability of the probiotics alone dramatically decreased in both the SGF conditions and especially in the presence of pepsin. However, LR-loaded PI tablets were able to protect probiotic death in the SGF regardless of the presence of pepsin. The viability of LR between loaded tablets and LR alone showed significant differences in SGF in the presence of pepsin after 2 h (Fig. 4B), suggesting that the PI tablets

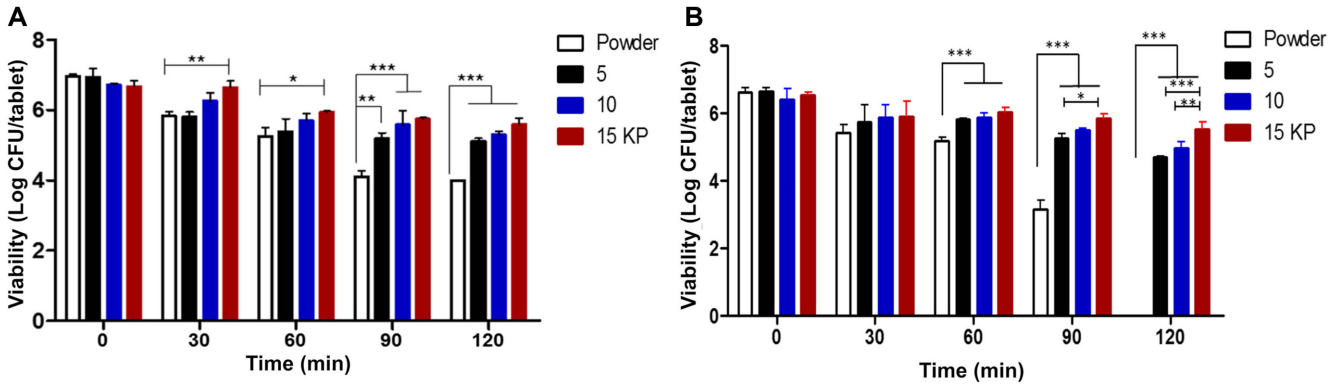

Fig. 4. Survivability of LR in LR-loaded PI tablets in SGF (pH 2.0) without pepsin (**A**) and with pepsin (**B**) until 2 h at 37°C (means ± standard deviation, SD; $n = 3$).
(PI: phthalyl inulin, LR: *Lactobacillus reuteri*, and SGF: simulated gastric fluid)

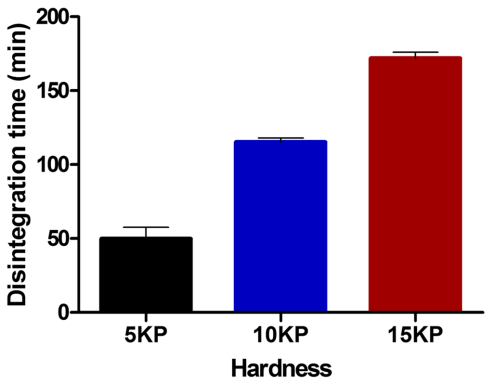

Fig. 5. Disintegration time of LR-loaded PI tablets with different compression forces in PBS (pH 6.8) (means ± standard deviation, SD; $n = 3$).
(PI: phthalyl inulin, LR: *Lactobacillus reuteri*, and PBS: phosphate buffer solution)

were able to protect LR from harsh stomach conditions. Furthermore, the higher compression force increased the viability of the LR in gastric conditions. The LR viability in the 15 KP tablets was significantly higher than LR alone in SGF without pepsin at 30 min (Fig. 4A). In SGF with pepsin, the difference in viability of LR between LR alone and the LR loaded into tablets, in case of the 15 KP tablet, was significantly higher at 60 min (Fig. 4B). Moreover, the viability of LR in the 15 KP tablet was significantly higher than LR in the 5 KP and 10 KP tablets after 2 h, especially in the presence of pepsin, indicating that higher compression force was able to protect the probiotics better in gastric conditions.

To investigate the swelling effect in intestinal fluid, times for complete disintegration in SIF were measured among the different compression forces. In Fig. 5, the disintegration time increased with an increase of the compression force. The disintegration time for 15 KP was 160 min; however, for 10 KP it was nearly 110 min and for 5 KP it was 50 min or less. This demonstrates that tablets fully disintegrate in intestinal conditions due to the pH sensitivity of the PI, and also that the compression force affected the disintegration ability of the pH-sensitive tablets.

Release and Viability of LR from LR-Loaded PI Tablets in SGF and SIF

The release and cell viability of LR from LR-loaded PI tablets in SGF and SIF were tested by sequentially immersing the tablets into both types of fluid. First, as shown in Fig. 6A, the release of LR from the tablets in SGF and SIF was analyzed. In SGF, the viability of the probiotics alone dramatically decreased and no viable cells were found after 2 h incubation. From the tablets, no viable released cells were found in SGF. In SIF, the 5 KP and 10 KP tablets released viable cells faster than the 15 KP tablets. The higher compression force tablet delayed the release of LR by comparison with the other two tablets. However, nearly all of the probiotics were released from all tablets of each type after 5 h of immersion in SIF. Next, the viability of LR was measured by sequentially exposing the tablets to SGF and SIF (Fig. 6B). The viability of the probiotics alone was non-existent after exposure to SGF for 2 h. When the tablets were exposed to SGF, the LR became less viable with time. Although the LR viability slightly changed in SIF after 5 h, more viable cells remained inside the tablets prepared with the higher compression force than with lower compression force. The viability of LR within the 15 KP tablets was significantly higher than the other two groups after 7 h in SGF and SIF conditions. Altogether, owing to the pH-sensitivity of the PI that was used, the PI tablets

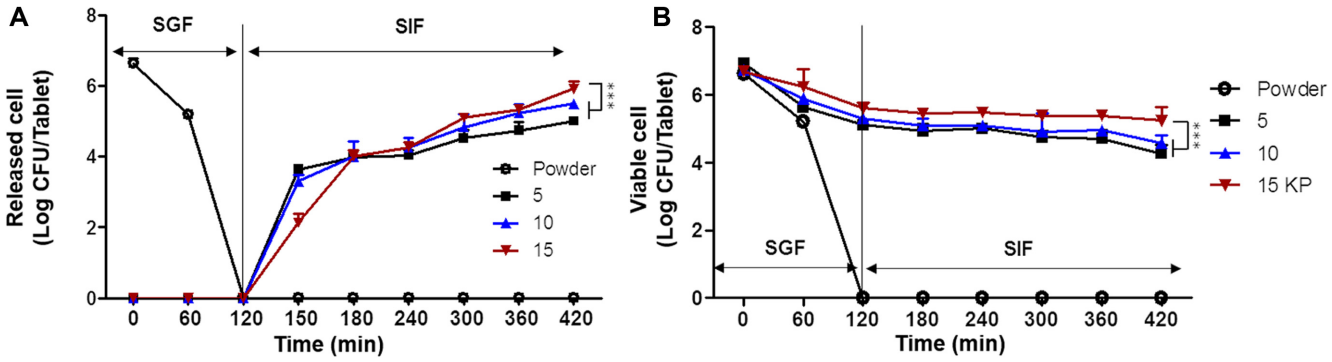

Fig. 6. Released (**A**) and viable cell numbers (**B**) of LR from powder and LR-loaded PI tablets sequentially exposed to SGF and SIF until 7 h at 37°C (means ± standard deviation, SD; $n = 3$).
(PI: phthalyl inulin, LR: *Lactobacillus reuteri*, SGF: simulated gastric fluid, and SIF: simulated intestinal fluid)

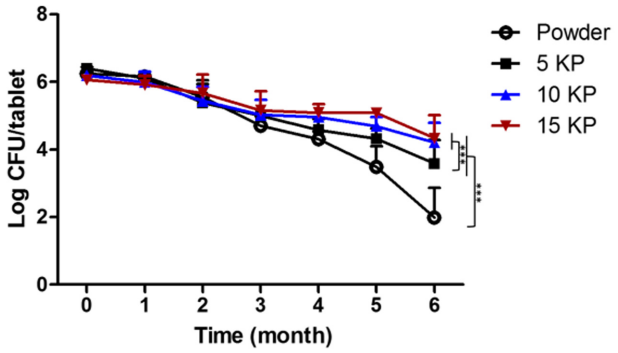

Fig. 7. Storage stability of LR in LR-loaded PI tablets during 6 months at 4°C (means ± standard deviation, SD; $n = 3$).
(PI: phthalyl inulin and LR: *Lactobacillus reuteri*)

were able to protect LR from harsh gastric conditions and release LR without the LR viability being affected by the PI tablets' disintegration.

LR Viability in LR-Loaded PI Tablets in Long-Term Storage

The stable viability of probiotics is a major index for the industrialization of probiotics. The stability of LR inside the tablets during storage was checked over a 6-month period at 4°C. The temperature was selected because most probiotic products recommend storage in a refrigerator. In Fig. 7, the stability of LR for 6 months was calculated by the viable CFU per one tablet. Probiotics in powder form dramatically decreased in viability after 3 months. However, the viability of the LR in tablet form was significantly more stable even after 6 months. In particular, higher compression force tablets (10 and 15 KP) showed a significantly higher number of viable cells at the end of 6 months than the 5 KP tablet. Overall, the viability of LR was more stable in PI-based tablets while higher compression force also allowed for higher cell stability.

Discussion

In this study, we developed a new pH-sensitive tablet using PI and examined its potential usage and effect on probiotic survivability and stability for oral delivery of probiotics. In delivering probiotics to the host gut, one of the most important aspects is to permit the probiotics to survive after passing through harsh gastric conditions [23]. Generally, most probiotic products on the market for human use are now sold in liquid or semi-liquid forms containing probiotics and prebiotics together [24]. Such products provide only low cell viability after oral ingestion because prebiotics cannot protect probiotics from harsh gastric conditions in mixture form [24]. Furthermore, the probiotics used for livestock animals are administered orally through feed although most probiotics are just added to feed without any protection and the amount of the probiotics used is very inconsistent from animal to animal [25]. Therefore, developing a dry form of probiotics that can protect the microorganisms from harsh stomach conditions and homogenous administration of the probiotics through the oral route are needed. For these reasons, we designed a new pH-sensitive PI to protect probiotics in low pH conditions and to form homogenous tablets as a dry form for oral administration. The PI was prepared by conjugation with phthalic anhydride with inulin through ester bond linkage because the remained carboxylic acid groups in phthalic acids after conjugation reaction have pH-sensitive properties due to the deprotonation at pH 7 and protonation in low pH (such as pH 2) [26], which is similar with CAP or HPMCP used for popular oral delivery systems [11, 27, 28].

Inulin has been widely used as a prebiotic for many decades because it promotes the growth and activity of probiotic *L. reuteri* [29]. It has also been used in drug delivery systems for intranasal, parenteral, intravenous, and subcutaneous routes of administration [30] although inulin itself is difficult to use as a carrier in an oral colonic drug delivery system because inulin is highly soluble in water. Therefore, many strategies have been tried to reduce the solubility of inulin in water by mixing hydrophobic coating materials such as Eudragit [31, 32] or by conjugating hydrophobic residues [33]. By conjugation of the phthalyl groups to inulin, we were able to reduce its water solubility. The pH-sensitive PI protected the probiotics from the low pH condition and released them after the dissolution of polymers in neutral pH condition. Moreover, in our previous study it was found that phthalyl inulin nanoparticles as a new type of prebiotics were able to enhance the antimicrobial activity of probiotics [21]. Therefore, we can assume that PI would have multi-functional properties when it is orally administered. Firstly, PI can be used as a tablet material to protect probiotics from harsh gastric conditions as previously mentioned. Secondly, PI may enhance the growth and activity of probiotics after the hydrolysis of PI into inulin and degradation of inulin into fructooligosaccharides (FOS) in the host gut. Thirdly, PI may also enhance the antimicrobial activity of probiotics when phthalyl inulin nanoparticles are mixed with the PI tablet. However, more study on this concept should be conducted in the near future.

In our previous result, we isolated *L. reuteri* LRT18 (LR) from porcine feces and it showed the highest antimicrobial

activities against both pathogenic bacteria K88-positive *E. coli* and *S. enterica* subsp [5], although it was very weak against acid stress, especially in the presence of pepsin (Fig. 4). Therefore, we used pH-sensitive PI to protect LR from harsh acidic conditions and deliver LR to the host gut efficiently. First, we investigated the LR survival during tableting under different compression forces. There were no significant differences in probiotic viability among the different compression forces, which was similar to previous results [20, 34]. The LR viability in the 15 KP tablet received the most protective effect even after 2 h of incubation in the SGF condition. For the incubation in SGF with or without pepsin, the survivability of LR in the 15 KP tablet was both approximately 7 Log CFU. Especially, the LR viability loaded in the 15 KP tablet was significantly higher than with other tablet groups in SGF with pepsin. The results suggest that when tableting probiotics, compression force is one of the important factors in protecting probiotics because a high compression force prohibits the fluid from physically coming inside the tablets. Moreover, the results were also consistent with the different disintegration times and swelling ratio between the groups. The disintegration and swelling ratio of the tablets in pH 2 are shown in Fig. 3. It was found that the swelling ratios of tablets were different according to the compression forces although the ratios in all three groups were very low in SGF conditions for 2 h incubation, suggesting that this was due to the pH sensitivity of the tablets. Furthermore, in Fig. 5, the disintegration times of tablets in SIF conditions differed from their compression forces. Higher compression forces meant more time for the tablets to disintegrate. The results were consistent with the release behavior in SIF conditions in Fig. 6, where the 15 KP tablet released LR more slowly than 5 or 10 KP tablets for more than 60 min after changing to SIF conditions. However, total viable cells released from the tablets were significantly higher in 15 KP than 5 or 10 KP because the survival rate of LR in 15KP tablets was higher after incubation in SGF conditions.

To check the pH-sensitivity of the PI, we incubated the PI tablets in the SGF media for 2 h with or without pepsin and compared them with LR alone (powder). Also, we exposed the PI tablets sequentially to SGF and SIF media. For identifying the survivability of probiotics in the gastrointestinal tract, most researchers choose pH 1.5–2 for gastric conditions and pH 6.8–7.2 for intestinal conditions because the presence of pepsin and low pH are required to represent the stomach environment when testing the survivability of probiotics [35, 36]. Although the pH of the stomach slowly declines from a neutral pH to pH 2 when food is ingested [37, 38], the pH of the stomach during fasting can decrease to 1.5, which suggests that an acidic environment is highly challenging for probiotic survival when administered orally [39]. Also, it has been generally reported that ingested food remains in the stomach for 2–3 h and transits to the intestinal tract where it then remains for 5–12 h [40], although bile salt may have an antimicrobial effect toward bacteria, intestinal pH is known to be pH 6.8–7.2, which is more suitable for bacteria to survive [39]. Therefore, tablets should be effective in protecting probiotics in gastric conditions throughout this time while releasing the probiotics in the intestine. The survivability of LR in the PI tablets was significantly higher than LR alone for 30 min incubation in SGF media with or without pepsin, indicating PI tablets were able to protect LR from acidic conditions. The swelling degree of PI tablet in SIF was higher than in SGF due to the pH sensitivity of the PI. Moreover, the viability and release behavior of LR in SGF and SIF were shown to be similar to other types of pH-sensitive tablets [41]. In SGF the viable released cells were not shown and the fast release of probiotics was shown in SIF as the swelling degree of PI increases at pH 6.8. Even though higher compression force delayed the release of LR more than 1 h in SIF, after 5 h incubation all tablets viable released cell counts had the same viable cell counts as Fig. 6B in SIF. This can indicate that LR is able to be delivered and work in the intestine since in ingested food generally remains there for up to 12 h. However, our study may have limitations in in vitro conditions because it is very difficult to mimic in vivo conditions by adjusting the pH with enzymes. The pH in the digestive system actually declines slowly from neutral to pH 2 over the course of 3 h after a meal. Therefore, a follow-up study on the protection and release of LR from the new type of pH-sensitive tablets should be conducted in vivo in the near future.

LR-loaded PI tablets also increased LR stability for long-term storage compared to LR alone. The results suggest that PI tablets can be used in industry since many probiotics are stored at 4°C for more than three months. In conclusion, PI is a suitable material for making probiotic tablets that can preserve cells in harsh gastric conditions, release easily in the intestinal condition and show long-term stable storage. To the best of our knowledge, this is the first report to suggest the possibility of PI as a tableting material and to be used as an alternative to antibiotics in industry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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