

## Oral Delivery of Probiotics in Poultry Using pH-Sensitive Tablets

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As alternatives to antibiotics in livestock, probiotics have been used, although most of them in the form of liquid or semisolid formulations, which show low cell viability after oral administration. Therefore, suitable dry dosage forms should be developed for livestock to protect probiotics against the low pH in the stomach such that the products have higher probiotics survivability. Here, in order to develop a dry dosage form of probiotics for poultry, we used hydroxypropyl methylcellulose phthalate 55 (HPMCP 55) as a tablet-forming matrix to develop probiotics in a tablet form for poultry. Here, we made three different kinds of probiotics-loaded tablet under different compression forces and investigated their characteristics based on their survivability, morphology, disintegration time, and kinetics in simulated gastrointestinal fluid. The results indicated that the probiotics formulated in the tablets displayed higher survival rates in acidic gastric conditions than probiotics in solution. Rapid release of the probiotics from the tablets occurred in simulated intestinal fluid because of fast swelling of the tablets in neutral pH. As a matrix of tablet, HPMCP 55 provided good viability of probiotics after 6 months under refrigeration. Moreover, after oral administration of probiotics-loaded tablets to chicken, more viable probiotics were observed, than with solution type, through several digestive areas of chicken by the tablets.

**Keywords:** Probiotics, hydroxypropyl methylcellulose phthalate, oral delivery, poultry, pH-sensitive tablet

### Introduction

Recently, many developed countries have abolished the use of antibiotics to treat and prevent animal diseases, and to promote growth of livestock [1], because resistant pathogens related with human and animal diseases have appeared owing to the antibiotics used [2]. Therefore, other alternatives to antibiotics in livestock are urgently needed although it is not easy to find them owing to the complexity of the gastrointestinal (GI) ecosystem [1].

In Korea, battery farming of chicken is a hot issue to be solved because it causes many communicable diseases like

diarrhea and constitutes a considerable economic problem for poultry growers. *Salmonella enterica* serovar Gallinarum (*Salmonella gallinarum*) is the etiological agent of fowl typhoid (FT), a severe systemic disease of chickens that results in high mortality [3]. FT has become one of the most serious bacterial diseases in poultry in Korean farms since 1992 [4].

Probiotics as the alternatives of the antibiotics have been used because they inhibit colonization of pathogens on the intestinal receptor, they are generally regarded as safe, they are extensively used as foods, and they survive passage through stomach acid, although survival of the probiotics

is strain-dependent [5]. Among the probiotics, *Pediococcus acidilactici* (PA) has been found to have antimicrobial activity against *Salmonella Gallinarum* referred to as one of the major pathogens of poultry [6]. In our previous study [7], we constructed genome-shuffled (GS1)-PA for improving antimicrobial activity, through homologous recombination of two different PA genes having higher antimicrobial activity [8, 9].

Oral delivery of probiotics as one of therapeutics in animals is extremely challenging and should satisfy the following conditions. It should survive at the acidic pH and abundant enzymes conditions of the stomach with easy handling, reduced time, low cost, and reduced labor [10]. The luminal in the poultry pH varies from highly acidic in the proventriculus (pH 2.0–5.0) to the slightly basic in the small intestine (pH 5.0–7.0). All foods ingested to the chicken must be subjected to gastric pH in the range of 2.0 to 5.0, which results in a 10–100-fold killing of bacteria in the foods digested in the upper part of the gastrointestinal tract [11], which is different from bovine and sheep. The intestinal tract is one of the determining sites of probiotics against pathogens. Therefore, efficient delivery of probiotics to the intestinal site and safe passage through the acidic condition with higher cell viability are very important for getting the therapeutic effect of the probiotics. It has been reported that pH-sensitive polymers such as hydroxypropylmethylcellulose phthalate (HPMCP) [10], hydroxypropylmethyl cellulose acetate succinate [12], cellulose acetate phthalate [13], and carboxymethyl high-amylase starch [14] were used to deliver probiotics because the probiotics in the pH-sensitive polymers can be protected from gastric pH due to the unswelling of polymers at acidic pH condition [15]. There are many different methods for administering probiotics to broiler chickens: through feed, water, gavage including droplet or inoculations, and spray, although adding of the probiotics to feed is the most commonly used method in poultry farms [16]. However, there are few dry dosage forms for delivery of probiotics to poultry through feed, although the dosage forms of commercial probiotics for human use have various kinds of dry dosage forms such as enteric-coated granules and capsules.

Therefore, in this study, we aimed to develop pH-sensitive tablets for chickens and bring out promising results in oral delivery of probiotics to poultry using pH-sensitive tablets.

## Materials and Methods

### Materials

Genome-shuffled *Pediococcus acidilactici* (GS1) was constructed

[7] to improve the antimicrobial activity of wild-type PA. HPMCP 55 was kindly provided by Shin-Etsu Chemicals Ltd. (Japan). Difco lactobacilli MRS broth and lactobacilli MRS agar were purchased from BD (USA). Novobiocin, nystatin, vancomycin and other chemicals were purchased from Sigma-Aldrich (USA).

### Preparation of Tablets

Cultures of GS1 were grown in MRS broth at 37°C, and cultures were harvested at the beginning of the stationary phase and collected by centrifugation. The harvested cells were suspended in 10% skim milk solution following 3 times of PBS washing. The cultures were then frozen at –20°C for about 12 h and subsequently freeze-dried for 24 h. The lyophilized probiotics (GS1) were carefully ground into fine powders and stored at 4°C in closed containers for further experiments. [10, 17]. The number of GS1 cells in the powder of skim milk was controlled between  $10^{10}$  and  $10^{11}$  CFU/g. Tablets were prepared by direct compression using a single tablet press at room temperature. An exactly weighed powder mixture (25 mg) of GS1 and HPMCP 55 (weight ratio of GS1 to HPMCP 55 = 1:1) was filled into a die of 4 mm diameter, and the tablets were formed under a determined pressure ranging from 3 to 10 kilopond (KP) with a plane surface.

### Measurement of Probiotics (GS1) Viability in Tablets

According to a previously described method [18], each tablet was broken and dispersed in 1 ml of phosphate buffer solution (PBS, pH 7.2). The serially diluted suspension was then spread onto pre-dried MRS agar plates. Then, the plates were incubated at 37°C for 24–36 h. Colonies of GS1 were counted and converted to log CFU (colony-forming units).

### Exposure of Tablets to SGF Medium with or without Pepsin

The powder and tableted probiotics in stimulated gastric fluids (SGF) of pH 2.0 with or without pepsin (1,000 unit/ml) were studied. According to a previously described method [19], the SGF was prepared by adjusting the pH of a phosphate buffer solution (PBS) to 6.8 by addition of HCl. The GS1 powder and GS1-loaded tablets were transferred into 5 ml of SGF, with or without pepsin. To determine the survival of probiotics during exposure to the SGF, the end of the incubation period (0, 30, 60, 90, 120 min) in the incubator (100 rpm at 37°C), the viable cells in the non-disintegrated tablets were determined by the same method described above. The viability of probiotic cells was calculated by the following equation.

$$\text{Viability (\%)} = [\text{CFU after exposure to the test medium} / \text{CFU before exposure to the test medium}] \times 100$$

### Disintegration Time of GS1-Loaded Tablets in PBS against Compression Force

The test tablets prepared by different compression force were transferred into 5 ml of PBS (pH 6.8), and the complete disintegration time of GS1-loaded tablets was measured.

### Sequential Exposure of Tablet to SGF and SIF Media

To measure the cell viability of GS1 sequential exposure of tablets to SGF and simulated intestinal fluid (SIF) media, a certain number of each different compression force of GS1-containing tablets were individually immersed into 5 ml of SGF at 37°C for 1 h with continuous rotation (100 rpm). Then, the tablets were quickly shifted from the SGF to the SIF (PBS pH 6.8), and incubated for 4 h more. The viable GS1 in each indicated time released into the SGF and SIF media was determined by spreading of serial-diluted supernatant media onto pre-dried MRS agar plates. To measure the cell viability during sequential exposure to SGF and SIF media, the viable cells in the supernatant and non-disintegrated tablets were measured at regular intervals. The colonies of GS1 were counted and converted to the percentage of initial content of GS1 in the tablet.

### Stability of GS1-Loaded Tablets

For the stability test during storage of the tablets, the GS1-loaded tablets were kept in tight light-resistant containers at 4°C and room temperature for up to 6 months. The stability of GS1 in terms of cell viability in the tablet was monitored as described above for 6 consecutive months.

### Oral Administration of Probiotics in Chickens

Eleven-day-old broiler chickens (Ross 308, mixed sex) were used for oral administration. The chickens were provided with free access to water and feeds during the experiments. The chickens were used in accordance with the guidelines for the care and use of laboratory animals (Seoul National University, Korea). The chickens were divided into three cohorts (6 chickens per cohort). Per-oral administration in chickens was performed with a dose of probiotics containing  $2 \times 10^8$  CFU suspended in the appropriate volume of peidococci selective medium [20] of 1 ml, administered

by intragastric inoculation using a feeding needle, or one tablet containing  $2 \times 10^8$  CFU. Only PBS was used in the control group. All cohorts received a total of five doses of the probiotics (or PBS in control) on the hours of 0, 4, 24, 28, and 48.

### Sample Collection from Chickens

To investigate the viable cells in the GI tract of chickens after oral administration of tablet, the contents of the muscular stomach, small intestines, and cecum of the chicken were immediately collected after sacrifice. The contents of the collected samples were homogenized in an appropriate volume of ice-cold PBS, and the viable cells in the medium were determined by the same method described above.

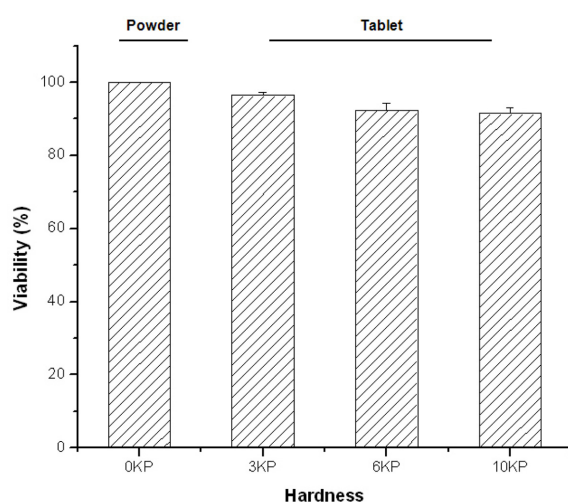
### Statistical Analysis

All results are expressed as the mean  $\pm$  SD. Differences between means were tested for statistical significance by an ANOVA and Duncan's new multiple range test. All statistical analyses were carried out with the SPSS 13 program (SPSS, USA).

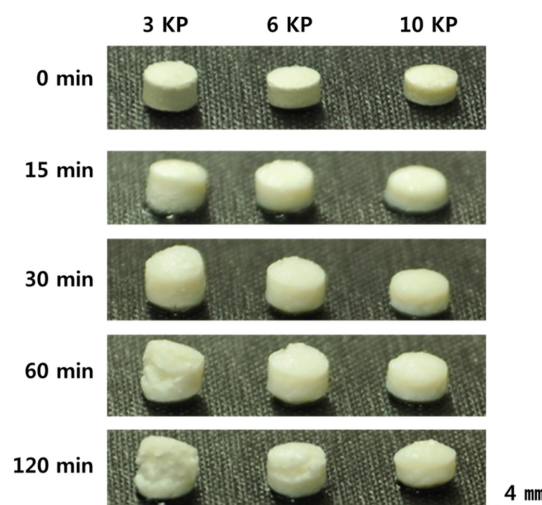
## Results

### Effects of Compression Force and Tablet Properties on Cell Viability

The effect of compression force during tableting on the viability of GS1 was investigated. It was found that the viability of GS1 was slightly decreased with an increase of compression force during tableting, as shown in Fig. 1, although there were no significant differences. The change in the morphology of tablets (compression force: 3 KP,

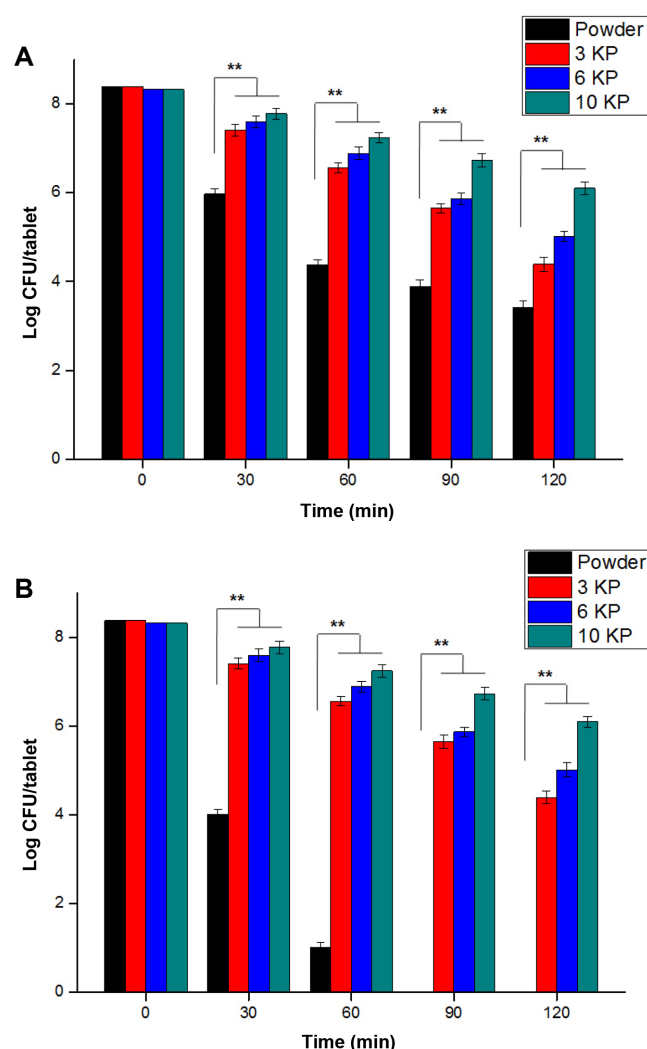


**Fig. 1.** Viability of genome-shuffled (GS1)-PA after tableting under different pressure conditions (3 KP, 6 KP, and 10 KP) (mean  $\pm$  SD,  $n = 3$ ).



**Fig. 2.** Observed morphology change of genome-shuffled (GS1)-PA-loaded tablet made under different pressures (3 KP, 6 KP, and 10 KP) after incubated in simulated gastrointestinal fluid for 0, 15, 30, 60, and 120 min (mean  $\pm$  SD,  $n = 3$ ).

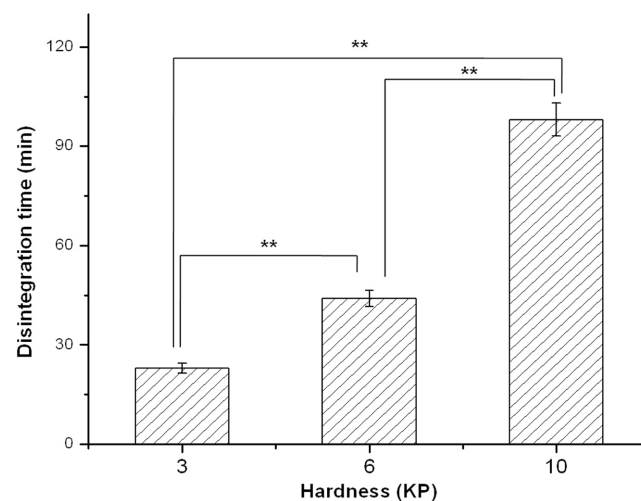
6 KP, 10 KP) was investigated by immersing the tablet in SGF (Fig. 2). It was observed that the tablets showed no complete disintegration within 2 h in the gastric-mimicking acidic medium; however, the disintegration degrees of the tablets under different compression forces were different. The viability of GS1 inside the non-disintegrated tablets after SGF immersion was then determined. The results showed that the viability of GS1 in the tablets had decreased with the increase of immersion time and increased with the increase of compression force, as shown Fig. 3; however, the powder type of free probiotics was dramatically decreased in the SGF, especially in the SGF with pepsin. The effect of



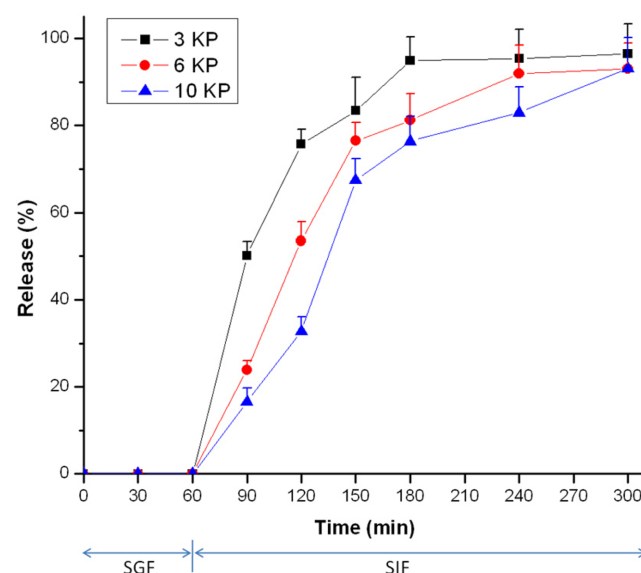
**Fig. 3.** Survivability of genome-shuffled (GS1)-PA in GS1-PA-loaded tablets in simulated gastrointestinal fluid (pH 2.0) without (A) or with pepsin (B).

The Y axis presents the total amount of survived GS1-PA per tablet (mean  $\pm$  SD,  $n = 3$ ) (\*\* $p < 0.01$ ).

compression force on the time of complete disintegration of GS1-loaded tablets in PBS (pH 6.8) was also investigated. As shown in Fig. 4, the disintegration time of the GS1-loaded tablets was increased with the increase of compression force. Whereas the GS1-loaded tablets prepared with the highest compression force of 10 KP was hardly disintegrated within 1 h, those prepared from the compression force of 3 KP was completely disintegrated within 30 min.



**Fig. 4.** Disintegration time of tablets made under different pressures (3 KP, 6 KP, and 10 KP) in PBS (pH 6.8) (mean  $\pm$  SD,  $n = 3$ ) (\*\* $p < 0.01$ ).



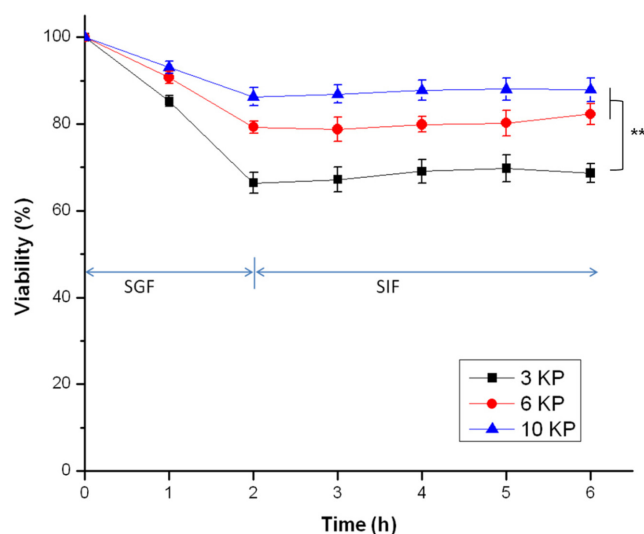
**Fig. 5.** Release efficiency of GS1-PA from GS1-PA-loaded tablets during sequential exposure to simulated gastrointestinal fluid and simulated intestinal fluid (mean  $\pm$  SD,  $n = 3$ ).

### Release of GS1 from GS1-Loaded Tablets in SGF and SIF

A release test was done by sequentially immersing the GS1-loaded tablets into SGF and SIF, and the released probiotics were calculated. As shown in Fig. 5, initially, after 1 h in SGF, no viable released probiotics from the GS1-loaded tablets were found in the gastric medium. The GS1-loaded tablets partially liberated probiotics during the first 30 min in SIF. The higher compression force during tableting provided delayed liberation of the probiotics from the GS1-loaded tablets. Almost 100% of probiotics were released from the tablet in approximately 180 min for the compression force of 3 KP, 240 min for 6 KP, and 300 min for 10 KP. Fig. 6 shows the cell viability of GS1 in the GS1-loaded tablets after sequential immersing in fluid media of SGF and SIF. The results indicated that cell viability inside the GS1-loaded tablets in SGF was slowly decreased with time, and the cell viability in SIF was slightly changed after 6 h, indicating that HPMCP 55 does not affect the cell viability of the released probiotics from the GS1-loaded tablets in SIF.

### Stability of the GS1-Loaded Tablets

The stability of probiotics in terms of cell viability is one of the major indexes that indicates the possibility of poultry excipients and dosage forms to protect the probiotics with a long shelf-life. Two different temperatures (4°C and room temperature) were selected to evaluate the stability of the GS1-loaded tablets, because the selected temperatures represent the common cool storage, as in a household

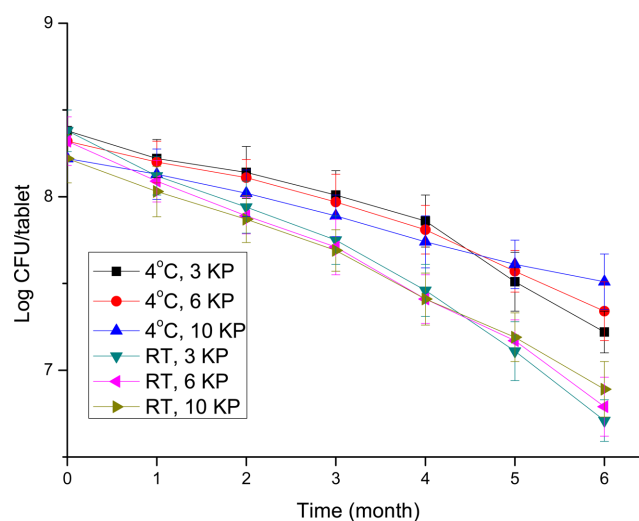


**Fig. 6.** Cell viability of the GS1-PA from GS1-PA-loaded tablets after immersing in media of simulated gastrointestinal fluid and simulated intestinal fluid (mean  $\pm$  SD,  $n = 3$ ).

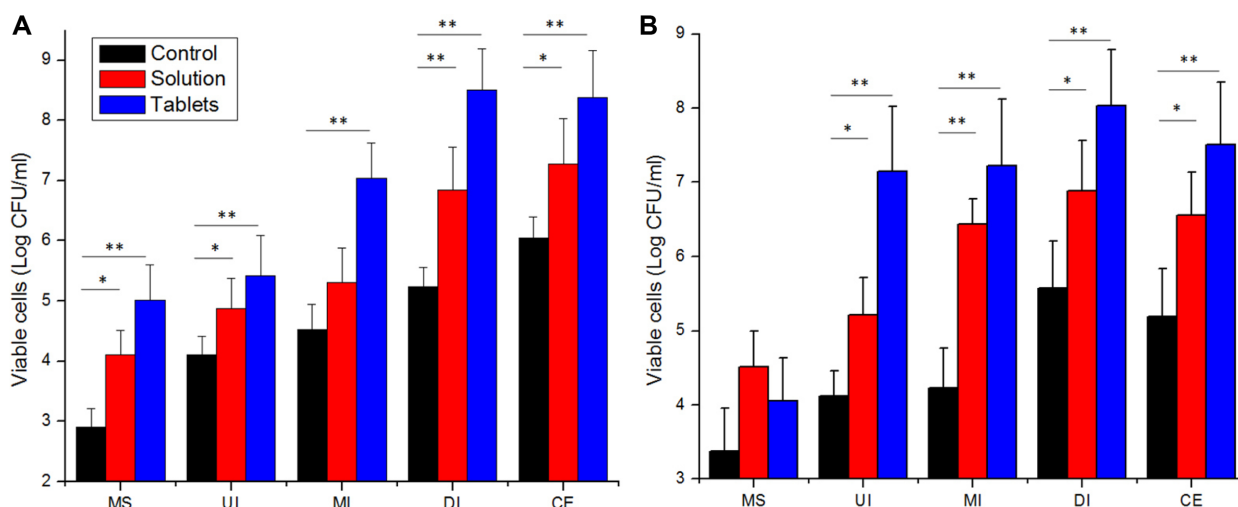
refrigerator and ambient room temperature, respectively. Fig. 7 shows the cell viability inside the GS1-loaded tablets after storage at 4°C and room temperature. The results indicated that storage at room temperature caused more significant decreases in cell viability than storage at 4°C. After 6 months of storage at 4°C, the loss of cell viability in the GS1-loaded tablets obtained at compression force of 10 KP was observed to be less than 1 log unit, indicating that storage temperature affects the cell stability.

### Oral Administration in Chickens

Since the probiotics were orally given to chickens as either tablet (10 KP) or solution form, the viability of probiotics in six digestive areas, such as muscular stomach (MS), upper portion of small intestine (UI), middle portion of small intestine (MI), lower portion of small intestine (DI), and cecum, at 3 and 6 h after oral administration was investigated. As shown in Fig. 8A, more viable cells were observed by oral administration of the tablets (10 KP) than of the solution in several digestive areas of chickens at 3 h after oral administration, suggesting that the tablets could protect probiotics in the digestive areas of chickens. In particular, the survivability of probiotics was 1.3, 1.3, and 1.25 log folds higher in the MS, MI, and DI respectively, by delivery in tablets than by solution. After 6 h of administration (Fig. 8B), the survivability of probiotics was also higher from the tablets than from solution in several digestive areas, except the MS. In particular, the survivability of probiotics was 1.5-fold higher in the UI by tablet than by solution delivery.



**Fig. 7.** Survivability of GS1-PA from GS1-PA-loaded tablets during 6 months of storage at 4°C and RT (mean  $\pm$  SD,  $n = 3$ ).



**Fig. 8.** Viable cells in the digestive systems of chickens post 3 h (A) and 6 h (B) of final administration.

MS: muscular stomach; UI: upper portion of small intestine; MI: middle portion of small intestine; DI: lower portion of small intestine; and CE: cecum (mean  $\pm$  SD,  $n = 6$ ) (\* $p < 0.05$ , \*\* $p < 0.01$ ).

## Discussion

In this study, the effect of probiotic survivability to chickens after delivering the probiotics using a pH-sensitive polymer tablet form was investigated. One of the most important things in delivering probiotics to a host is to ensure the probiotics survive and pass through the stomach with low pH and the upper GI tract with high bile salts [21]. Generally, the probiotic products available in the market for livestock animals are mostly in the form of liquid or semi-solid formulations, which show low cell viability after oral administration because the bacteria do not survive the harsh conditions in the stomach [22]. However, there are a few dry dosage forms for delivery of probiotics to poultry through feed, although the dosage forms of commercial probiotics for human use have various kinds of dry dosage forms such as enteric-coated granules and capsules. Therefore, the development of suitable dry dosage forms of probiotics allowing higher bacterial survival in the poultry is the main aim of the present study, because the formulation of probiotics into tablets is a promising method to reduce cell death during GI passage, as well as an opportunity to control release of these cells across the intestinal tract as a safe and effective oral delivery for feed supplement applications.

In this study, we used HPMCP 55, a pH-sensitive enteric coating polymer usually applied for human health as an excipient to make probiotics-loaded tablet. In particular, we selected HPMCP 55 for coating of probiotics because it is relatively cheaper compared with other enteric coating

material, such as the Eudragit series, although there are not much differences of pH-sensitivity between the cellulose series and poly(methacrylic acid) ones. First, the effects on bacterial survival during tableting were investigated. The results suggested that the compression force used during tableting does not much affect the viability of GS1, which is similar to the previous result [23]. In different compression forces, the viability of GS1 was slightly decreased with an increase of compression force during tableting; however, the viability of GS1-loaded tablets immersed in SGF after 2 h indicated that increasing the compression force to 10 KP improved the tablet efficacy in protecting bacterial cells against acidic challenge. In particular, the survivability of GS1 in GS1-loaded tablets obtained from compression force of 10 KP without pepsin and with pepsin after 2 h was 80% and 75%, respectively, suggesting that the compression force is one of the important tablet properties to protect the cells inside the tablet from the contact fluid. The disintegration time of the GS1-loaded tablets indicated that pH-sensitive HPMCP 55, as a tablet excipient with sufficient compression force, allowed the preparation of GS1-loaded tablets with suitable properties in terms of long disintegration time and high probiotic cell viability.

The main goal of this work was to check the possibility of pH-sensitive tablets for efficient oral delivery of probiotics as an alternative to antibiotics in poultry feed, because there is not report on probiotics-loaded tablets as animal feed by far, although many pH-sensitive polymers have been used for enteric coating of human drugs. Generally, it has been reported that the transient time of the ingested

food from the stomach to the intestinal tract for the chicken is about 3 h [24]. Therefore, the tablets should ideally protect probiotics throughout this time and then should release viable probiotics in the intestinal tract. The release of the probiotics is closely related to tablet swelling, because the HPMCP 55 is insoluble in SGF and soluble SIF owing to the pH-sensitive property of the polymer. Moreover, the swelling degree of HPMCP 55 increases at pH 6.8 than at pH 2.0. Here, HPMCP 55 tablets prepared with compression force of 10 KP fully protected probiotics survival and were disintegrated within 2 h in the SIF. The release behavior of GS1 from the GS1-loaded tablets (represented in Fig. 6) suggested that the release of probiotics can be controlled by the compression force. The release behavior of probiotics in the SIF, which was made under the compression force 10 KP, was slower than the probiotics made under lower compression force 3 KP and 6 KP, showing that it would affect the retention time of the probiotics in the intestinal tract, the effector site against pathogens [25].

From the in vivo experiment of oral administration of GS1-containing tablet, it was observed that the survivability of probiotics was significantly higher in several digestive areas of the chicken at 3 and 6 h after oral administration of tablet (10 KP) than solution. It means that the tablet form of GS1 can be protected efficiently in the stomach, with controlled release of the probiotics in the digestive areas of chicken, enhancing the real delivery efficiency of live probiotics in the intestinal tracts.

This research showed that the extent of cell survival depended on the compression force of the tablets using pH-sensitive HPMCP 55 as a matrix-forming material during tableting, and the probiotics formulated in the tablets were protected at the harsh conditions of the stomach in vitro and in digestive areas of the chicken. Almost all probiotics were preserved as viable cells when the tablets were stored at 4°C for 6 months. Moreover, through in vivo oral administration of probiotics-containing tablets in chickens, we found that the probiotics were efficiently protected in the stomach and delivered to the intestinal tract, which was effector site against pathogens. These results suggest the possibility of probiotics-loaded tablets as alternatives to antibiotics in poultry farms.

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