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Survivability of Collagen-Peptide Microencapsulated Lactic Acid Bacteria during Storage and Simulated Gastrointestinal Conditions

Sun-Il Kim, Jin-Woo Kim, Ki-Tae Kim and Chang-Ho Kang *

Mediogen, Co., Ltd., Bio Valley 1-ro, Jecheon-si 27159, Korea; sunil_2003@naver.com (S.-I.K.); valdicava@naver.com (J.-W.K.); rlxo357@naver.com (K.-T.K.)

* Correspondence: changho-kang@naver.com; Tel.: +82-43-842-1940

Abstract: The intracellular homeostasis of lyophilized lactic acid bacteria (LAB) is destroyed by extreme cold stress, resulting in decreased stability. This study aimed to verify the validity of collagen as a potential protective agent for improving microbial stability deteriorated by freezing. The collagen types used in this study were low molecular weight collagen (LC) of less than 1000 Da and low molecular weight collagen-peptide (LCP) of less than 300 Da. By the accelerated stability test according to the addition of each collagen type, a 3% LCP displaying a protective effect on the viability of various LAB strains (*Lactoplantibacillus plantarum* MG989, *Lactococcus lactis* MG5125, *Enterococcus faecium* MG5232, *Bifidobacterium animalis* ssp. *lactis* MG741, and *Streptococcus thermophilus* MG5140) was finally selected. It was evaluated whether LCP enhances bacterial stability, survivability in the gastrointestinal (GI) tract, and heat resistance. LCP significantly improved the viability of all strains in the GI tract compared to sucrose and skim milk, which are conventional protective agents. Based on morphological observations, LCP was uniformly coated on the cell surface, resulting in protective effects against multiple external stress stimuli. Such findings indicate the applicability of LCP as an unprecedented protective agent, which can improve the stability of various probiotics with antifreeze effects.

Keywords: antifreeze; collagen-peptide; freeze-drying; gastrointestinal tract; lactic acid bacteria; probiotics; stability

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1. Introduction

Probiotics, such as lactic acid bacteria (LAB), are administered in appropriate amounts to a host to provide health benefits [1]. Probiotics have been studied not only to maintain the balance of intestinal microbes, but also to exert various therapeutic effects against human diseases, such as anticancer, immune regulation, improvement of the oral environment, and vaginitis [1–6]. The functions of probiotics are expanding from health promotion to disease prevention and improvement, and accordingly, commercial interest in probiotics is expanding not only in food, but also in cosmetics and pharmaceuticals [7].

Probiotics must survive in the extreme environment of the gastrointestinal (GI) tract in the human body [8]. To exert positive physiological activity, it is necessary to consume appropriate amounts of live bacteria [9]. A dry powder form is utilized rather than liquid or frozen forms to maintain the cost and stability of LAB during storage and distribution as commercial products [10]. Among various drying processes, freeze-drying is a protective method for safely dehydrating bacteria from a microbial culture to a dry powder [11,12]. However, during this process, the bacteria are exposed to inappropriate conditions, such as ice crystallization and low temperatures. These conditions lead to programmed cell death by denaturing cell membrane proteins and destroying cell homeostasis [12,13].

The cell membrane, the first layer that protects microbial cells, is the main target of abiotic stress during freezing. According to Chen et al. [14], membrane lipids are

transferred to the gel phase by cold stress stimulation. This action can disrupt the balance of intracellular ionic concentrations due to the denaturation of proteins, such as membrane-related enzymes and transporters [15]. Additionally, low temperature stress increases the microbial ability to utilize oxygen, which causes the accumulation of reactive oxygen species (ROS) in cells [16]. Accumulated ROS oxidizes membrane lipids, resulting in decreased membrane fluidity [17]. In addition, enzymes with antioxidant activity that protect against ROS are present in cells [13,15,17]. Therefore, the activities of enzymes, such as peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), need to be enhanced to protect bacterial strains from oxidative stress [18]. According to a report by Zhang et al. [15], the addition of glutathione significantly increased viability during cold stress by not only increasing the antioxidant enzyme activity of LAB, but also protecting ATPase as the membrane protein.

During drying and long-term storage, it is necessary both technically and economically to sustain the activity of LAB against the various external stress stimuli mentioned above [19,20]. To protect the survivability of LAB during freeze-drying, some cryoprotectants, such as trehalose, alginate, and skim milk (SM), have been commercially applied [12,20–22]. These cryoprotectants with different molecular weights have been shown to protect the viability of microorganisms by inhibiting ice recrystallization, cell membrane protection, and antioxidant activity, as reported in many studies [13,21–24]. Among the many cryoprotectant candidates, collagen is a component that can be obtained from fish scales or pig skin and is composed of a variety of molecular sizes [13,25,26].

Peptides, produced by collagen hydrolysis, act as antifreeze, are reported to have superior inhibitory effects against ice recrystallization, and are known to have antioxidant effects [27,28]. In addition, the collagen types obtained from various sources have been confirmed to have a similar cryoprotective effect by protecting the cell membrane [13,25]. However, studies comparing the effect of collagen according to its molecular weight type are insufficient, and it is necessary to confirm whether it is applicable to various probiotics, not just a specific strain. Therefore, in this study, collagen derived from fish scales with potential effects was employed, and low and high molecular weight collagens were compared to determine suitable conditions for various LAB.

2. Materials and Methods

2.1. Microbial Strains and Growth Conditions

Five bacterial strains (*Lactiplantibacillus plantarum* MG989, MG989; *Lactococcus lactis* MG5125, MG5125; *Enterococcus faecium* MG5232, MG5232; *Bifidobacterium animalis* ssp. *lactis* MG741, MG741; and *Streptococcus thermophilus* MG5140, MG5140) were obtained from MEDIOGEN Co. Ltd. (Jecheon, Korea). All bacterial strains at an inoculum size of 2% (*v*/*v*) were inoculated in de Man, Rogosa, and Sharpe medium (MRS, Difco, Sparks, MD, USA). The medium was sterilized for 15 min at 121 °C. MG5140 was incubated at 42 °C for 18 h and all other strains at 37 °C for 18 h. MG741 was incubated in a CO_2 incubator (Vision Scientific Co., Bucheon, Korea), and the rest of the LAB strains were incubated in a BOD incubator (Han Baek Scientific Co., Bucheon, Korea). All microbes were used in the experiments after more than two subcultures.

2.2. Cryoprotective Agent for Strains

This experiment was performed to investigate the cryoprotective effect and suitable concentration of low molecular weight collagen (LC) obtained from Geltech Co. (Seoul, Korea) and low molecular weight collagen peptide (LCP) from Hubei Nutratide Biotech Co. (Huanggang, Hubei, China). In the accelerated stability test, LC with a molecular weight of less than 1000 Da and LCP with a molecular weight of less than 300 Da were used at concentrations of 0, 1, 3, and 5% (*w*/*v*) compared to the basic drying medium containing 5% sucrose of food additive grade. The type of collagen and the concentration of addition that are effective for various LAB strains were established through a comparative analysis between collagen treatment groups based on the different concentrations added.

To confirm the microbial stability in the GI tract, a dry medium containing 5% sucrose was used as the basic composition, and 10% SM was added to the basic dry medium as a positive control. The verification of the effect of collagen was confirmed by applying the optimal condition sample selected by the accelerated stability test.

2.3. Conditions of the Freeze-Drying Process

The freeze-drying of LAB was carried out according to the method described by Nguyen et al. [11]. The bacterial subculture was inoculated in 4 L MRS medium and incubated at the same culture temperature using a 7 L fermenter (KoBioTech, Incheon, Korea) to carry out mass culture. After 18 h of cultivation, the bacterial cultures were centrifuged 4000× *g* for 20 min at 4 °C. The harvested cell pellets were mixed with each dry medium for 5 min at a ratio of 1:2.5 (*w*/*w*). The mixed samples were incubated at −80 °C for 1 h and freeze-dried.

2.4. Analysis of the Cell Viability of Strains

The viability of the strains was confirmed via spreading each strain on an agar plate. After one milliliter of the sample was serially diluted in sterilized 0.1% peptone water (Oxoid, Hampshire, UK), the diluted sample was plated on Transgalactosylated oligosaccharides-mupirocin lithium salt agar (TOS-MUP, MB cell, Seoul, Korea) for MG741 and MRS agar for MG989, MG5125, MG5232, and MG5140. Colony-forming units (CFU) were determined after plates inoculated with MG5140 were incubated at 42 °C for 48 h and all the other strains at 37 °C. Additionally, MG741 was anaerobically cultured in a CO_2 incubator, and the remaining strains were cultured in a BOD incubator. The bacterial survival rate was calculated as a percentage based on the initial number of viable cells in the freeze-dried bacteria using the following formula:

$$\text{Survival rate (\%)} = \text{CFU of freeze-dried bacteria under the test condition/CFU of freeze-dried bacteria before the test} \times 100 \quad (1)$$

2.5. Conditions for the Accelerated Stability Test Using the Different Strains

The freeze-dried LAB strains were subdivided, sealed at 10 g each and stored in an incubator (Memmert, Büchenbach, Germany) at 40 °C and 70% relative humidity for 1 week [29,30]. Thereafter, the viable cell count of the sample was determined. After stepwise dilution in sterile peptone water and spreading on MRS and TOS-MUP agar medium, culture was performed for 48 h. The number of viable cells was then measured to confirm the survival rate under accelerated stress conditions.

2.6. Survivability under Simulated GI Tract Conditions

Artificial gastric and intestinal juices were prepared and simulated to confirm the viability of bacterial strains in human GI tract. The condition of GI juice was designed by modifying the method described by Tokatlı et al. [31]. Prior to incubation, 1 g of freeze-dried cells was diluted in 9 mL of sterile phosphate-buffered saline (PBS) at pH 7. In the case of a simulated gastric fluid (SGF) condition, SGF was prepared using PBS adjusted to pH 3 using 1 N HCl (Daejung Chemicals Co., Siheung-si, Korea) with 3 g/L of pepsin (Sigma, St. Louis, MO, USA). To test the survivability under simulated intestinal fluid (SIF) condition, SIF was prepared by dissolving 1 mg/mL pancreatin (Sigma, St. Louis, MO, USA) in sterile PBS at pH 7. The diluted cells (1 mL) were mixed with sterile fluids (9 mL) and incubated at 37 °C. Samples under the SGF condition were incubated for 3 h, and samples under the SIF condition were cultured for 5 h to determine the number of viable cells in the initial and post-culture. The samples were analyzed by applying the method used for cell viability, as mentioned above.

2.7. Heat Tolerance of Freeze-Dried LAB

The heat tolerance of dried LAB was evaluated by modifying the method described by Halim et al. [32]. One gram of the freeze-dried strain was inoculated in 9 mL of sterile PBS at pH 7 and incubated in a water bath at 50 °C for 30 min. The viable cell count in the

initial sample was analyzed. After 30 min, the heat treatment was terminated by cooling at 4 °C, and the number of viable cells in the final sample was analyzed.

2.8. Determination of Microbial Morphological Characteristics

Scanning electron microscopy (SEM, Hitachi S-4300SE, Tokyo, Japan) was used to determine the morphological characteristics of the bacterial cells. The measurements of the samples were analyzed by modifying the methods of Nguyen et al. and Wang et al. [11,25]. The magnification used for cell observation was 10,000× *g*.

2.9. Statistical Analysis

All results are expressed as the mean ± SEM of triplicate experiments, and statistical analysis was performed using SPSS version 25.0 software (SPSS Inc., Chicago, IL, USA). Significant differences between groups were confirmed by Student's *t*-test and one-way ANOVA, followed by Tukey's post hoc test with a significance level at $p < 0.05$.

3. Results and Discussion

3.1. Stability under Accelerated Stress Condition Due to the Addition of Different Collagen Types

The experiment was performed to select the optimal molecular type and concentration of collagen as a protective agent that can improve the stability of the five probiotic strains. Lyophilized LAB powder was stored for 1 week under accelerated stress conditions (40 °C), while LC and LCP were treated with several concentrations in a basic drying medium. The result of the survival rates of LAB strains is presented in Table 1. Overall, the number of viable cells in all groups tended to decrease after one week of storage. Additionally, by comparing the survival rate for each collagen type, the LCP-added group was found to have no significant difference or a superior protective effect relative to the LC-added group. For MG5125 and MG741, there was no significant change in survival rate between groups; however, the numerically higher survival rates were 30.00 ± 3.40 and 91.89 ± 5.41% in the 3% LCP group, respectively. For the remaining three strains (MG989, MG5232, and MG5140), the LCP 3 and 5% addition groups showed the most significant survival rate maintenance effect ($p < 0.05$). Considering the viability protection effect for various strains and the economic aspect of the production process, the addition of 3% LCP was judged to be a suitable condition for the five probiotic strains. Therefore, 3% LCP was applied in subsequent experiments to confirm its effect of improving microbial stability. Collagen peptide, a substance with an antioxidant effect, is known to protect against programmed cell death by improving the microbial cell membrane imbalance caused by freezing and enhancing microbial intracellular antioxidant activity [13,14,25]. From this viewpoint, the potential of LCP to be applied as a protective agent for freeze-drying and long-term storage of various LAB was confirmed.

3.2. Survival of Probiotic Strains under Simulated GI Conditions

LAB used as probiotics are living bacteria and are defined as microorganisms that have positive effects on the health of the host [33]. However, the GI tract in the human body contains strong acids and various degrading enzymes. Accordingly, LAB must be resistant to the GI environment to serve as probiotics [34]. This experiment was conducted to determine whether the addition of 3% LCP, a potential protective agent, could have a positive effect on enhancing cell stability in the human GI tract (Table 2). To verify whether LCP is suitable as a potential stability enhancer, sucrose and SM, known as commercial cryoprotectants, were used for comparison [19,25,35]. The control groups of MG989, MG5125, MG5232, MG741, and MG5140 had survival rates of 63.64 ± 4.50, 0.18 ± 0.01, 56.15 ± 6.90, 47.46 ± 13.60, and 0.0003 ± 0.00%, respectively, after 3 h in SGF condition of pH 3. Compared to the control group, the survival rate of all strains in the 3% LCP group increased significantly ($p < 0.05$), and the addition of LCP showed a tendency to increase the survival rate compared to SM. In the SIF condition of pH 7, there was no significant

difference between each protective agent-treated group of all strains ($p > 0.05$); however, overall, a trend of high survival rate was confirmed in the order of LCP > SM > control.

Table 1. Changes in the survival rate of LAB strains by treatment with different concentrations of low molecular weight collagen and collagen-peptide for 1 week in the accelerated stability test.

Strain	Group	Concentration (%)	Viable Cell Counts (Log CFU/g)		Survival Rate (%)
			Time (Week)		
			0	1	
MG989	LC	1	11.59 ± 0.00 **	10.26 ± 0.03	4.81 ± 0.65 ^{cd}
		3	11.57 ± 0.01 ***	10.54 ± 0.01	9.33 ± 0.27 ^{bc}
		5	11.59 ± 0.01 ***	9.49 ± 0.03	0.79 ± 0.10 ^{d}
	LCP	1	11.63 ± 0.01 **	10.80 ± 0.02	12.87 ± 2.02 ^{b}
		3	11.60 ± 0.03 **	10.81 ± 0.00	14.67 ± 1.46 ^{ab}
		5	11.71 ± 0.00 ***	11.04 ± 0.02	21.42 ± 1.02 ^{a}
MG5125	LC	1	11.79 ± 0.04 **	11.20 ± 0.01	25.53 ± 0.80
		3	11.63 ± 0.01 ***	11.07 ± 0.01	27.21 ± 0.70
		5	11.67 ± 0.01 ***	11.11 ± 0.02	27.63 ± 2.16
	LCP	1	11.71 ± 0.01 ***	11.04 ± 0.01	21.67 ± 1.27
		3	11.70 ± 0.01 ***	11.17 ± 0.02	30.00 ± 3.40
		5	11.72 ± 0.03 **	11.07 ± 0.01	22.26 ± 0.57
MG5232	LC	1	11.92 ± 0.00 ***	11.69 ± 0.00	57.74 ± 0.60 ^{ab}
		3	11.90 ± 0.04 *	11.58 ± 0.01	48.13 ± 3.13 ^{b}
		5	11.79 ± 0.04	11.66 ± 0.00	73.60 ± 1.60 ^{a}
	LCP	1	11.87 ± 0.01 *	11.74 ± 0.00	73.62 ± 1.41 ^{a}
		3	11.83 ± 0.00 *	11.64 ± 0.01	64.71 ± 2.94 ^{ab}
		5	11.77 ± 0.00	11.63 ± 0.02	72.51 ± 6.75 ^{a}
MG741	LC	1	11.28 ± 0.02	11.16 ± 0.01	76.32 ± 2.63
		3	11.34 ± 0.01	11.17 ± 0.04	68.18 ± 13.64
		5	11.27 ± 0.03	10.96 ± 0.06	50.00 ± 13.16
	LCP	1	11.22 ± 0.01	11.19 ± 0.01	93.33 ± 3.64
		3	11.27 ± 0.01	11.23 ± 0.01	91.89 ± 5.41
		5	11.27 ± 0.02	11.12 ± 0.04	72.97 ± 13.51
MG5140	LC	1	11.58 ± 0.04 ***	8.74 ± 0.02	0.14 ± 0.01 ^{c}
		3	11.62 ± 0.06 **	9.55 ± 0.02	0.83 ± 0.07 ^{c}
		5	11.52 ± 0.01 ***	9.52 ± 0.03	1.02 ± 0.14 ^{c}
	LCP	1	11.60 ± 0.03 ***	9.12 ± 0.06	0.33 ± 0.09 ^{c}
		3	11.45 ± 0.02 ***	10.09 ± 0.01	4.29 ± 0.36 ^{b}
		5	11.61 ± 0.01 ***	10.52 ± 0.01	8.05 ± 0.49 ^{a}

LC: low molecular weight collagen, LCP: low molecular weight collagen-peptide. Bacterial survival rate was calculated as a percentage based on the initial number of viable cells (CFU/g) of the freeze-dried bacteria. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison of the viable cell counts between 0 and 1 week in each group, indicates statistical significance. Different letters (^{a}, ^{b}, ^{c}, ^{d}) indicate statistical significance in survival rate in the same collagen type groups for each strain.

Table 2. Cell viability of the probiotic bacterial strains under the simulated GI conditions at pH 3 and 7.

Strain	Group	Conditions of Simulated GI Juices	Viable Cell Counts (Log CFU/g)		Survival Rate (%)
			Initial	Final	
MG989	Control	pH 3	11.74 ± 0.04 *	11.54 ± 0.03	63.64 ± 4.50 ^{b}
	SM		11.79 ± 0.03	11.79 ± 0.01	100.82 ± 0.40 ^{a}
	LCP		11.64 ± 0.02	11.66 ± 0.01	103.13 ± 0.90 ^{a}
	Control	pH 7	11.74 ± 0.04	11.74 ± 0.06	100.00 ± 13.60
	SM		11.79 ± 0.03	11.79 ± 0.05	101.84 ± 11.60
	LCP		11.64 ± 0.02	11.67 ± 0.01	105.11 ± 2.80
MG5125	Control	pH 3	11.98 ± 0.04 ***	9.23 ± 0.02	0.18 ± 0.01 ^{b}
	SM		11.82 ± 0.02 ***	8.83 ± 0.00	0.10 ± 0.00 ^{b}
	LCP		11.60 ± 0.03 ***	9.19 ± 0.04	0.39 ± 0.03 ^{a}
	Control	pH 7	11.98 ± 0.04 *	11.76 ± 0.02	59.74 ± 2.60
	SM		11.82 ± 0.02	11.80 ± 0.04	96.23 ± 9.43
	LCP		11.60 ± 0.03	11.59 ± 0.04	96.88 ± 9.38
MG5232	Control	pH 3	12.21 ± 0.01 *	11.96 ± 0.05	56.15 ± 6.90 ^{b}
	SM		12.12 ± 0.02 *	11.98 ± 0.02	73.33 ± 2.90 ^{ab}
	LCP		11.64 ± 0.10	11.65 ± 0.01	100.00 ± 2.80 ^{a}
	Control	pH 7	12.21 ± 0.01	12.11 ± 0.03	80.00 ± 6.20
	SM		12.12 ± 0.02	12.13 ± 0.03	101.91 ± 6.70
	LCP		11.64 ± 0.10	11.67 ± 0.01	102.78 ± 2.80
MG741	Control	pH 3	11.87 ± 0.01	11.53 ± 0.13	47.46 ± 13.60
	SM		11.77 ± 0.03	11.70 ± 0.02	85.11 ± 4.30
	LCP		11.32 ± 0.15	11.21 ± 0.01	73.18 ± 0.60
	Control	pH 7	11.87 ± 0.01	11.75 ± 0.03	76.27 ± 5.10
	SM		11.77 ± 0.03	11.66 ± 0.04	77.45 ± 7.70
	LCP		11.32 ± 0.15	11.24 ± 0.14	81.01 ± 25.10
MG5140	Control	pH 3	11.56 ± 0.05 ***	6.06 ± 0.05	0.0003 ± 0.00 ^{c}
	SM		11.40 ± 0.03 ***	6.17 ± 0.01	0.0006 ± 0.00 ^{b}
	LCP		11.19 ± 0.02 ***	6.28 ± 0.01	0.0012 ± 0.00 ^{a}
	Control	pH 7	11.56 ± 0.05 **	11.04 ± 0.04	30.34 ± 2.80
	SM		11.40 ± 0.03 *	11.00 ± 0.05	39.41 ± 4.90
	LCP		11.19 ± 0.02 **	10.88 ± 0.01	49.59 ± 0.80

SM: skim milk, LCP: low molecular weight collagen-peptide. Bacterial survival rate was calculated as a percentage based on the initial number of viable cells (CFU/g) of the freeze-dried bacteria. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison between initial and final viable cell counts in each group indicates statistical significance. Different letters (^{a}, ^{b}, ^{c}) indicate statistical significance in survival rate at the same condition of GI juices of each strain.

Previously, we screened novel cryoprotectants containing natural food additives, such as pumpkin powder, which are effective for probiotics, by investigating bacterial cell stability and viability in the GI tract [11,29,36]. Nguyen et al. [11] verified that the acid resistance and bile resistance of five LAB species were enhanced by applying a dry medium containing pumpkin powder, which was due to the coating effect. In this study, LCP, applied as a potential protective agent, showed a significant cytoprotective effect in the GI tract containing acid and bile salts relative to SM and sucrose, which are known as general protective agents. Chen et al. [13] reported that fish-derived collagen

peptides exert a cryoprotective effect by maintaining cell homeostasis and enhancing the stability of *S. thermophilus*. In addition, Wang et al. [25] clarified that pig-derived collagen protects against cell membrane damage in *S. thermophilus* and enhances the enzyme activity of LAB. Thus, the stability enhancing effect of collagen-peptide has been identified by various mechanisms; however, verifying the protective effect of various LAB other than *S. thermophilus* is insufficient. From this viewpoint, it was confirmed that LCP, as a protective agent, displays an excellent stability enhancing effect in the GI tract, an essential property for various probiotics, similar to the previously verified pumpkin powder [11,29].

3.3. Heat Resistance of Probiotic Strains through the Addition of LCP

This experiment was conducted to confirm the additional advantage of improving stability from other external stresses by applying of LCP to improve stability suppressed in the freeze-drying process (Table 3). The LCP-added groups of MG989, MG5125, and MG5232 at 5.82 ± 1.24%, 0.11 ± 0.01%, and 59.84 ± 0.48%, respectively, showed significantly higher survival rates than the control and SM groups. Additionally, the survival rates of MG741 and MG5140 showed no significant difference between the groups after heat treatment but showed a tendency for better survival through the addition of LCP. LCP has been reported to increase cell stability in recent studies [13,25]. Chen et al. [13] reported that the application of fish-derived peptides can maintain the balance of cell membrane potential and fluidity of LAB and enhance the activity of intracellular antioxidant enzymes. High-temperature treatment causes cell membrane, protein, and intracellular molecular damage with mechanisms similar to microbial apoptosis due to freezing, thereby promoting the death of microorganisms [37]. In this regard, the mechanisms of the antifreeze effect can have a positive effect on improving the stability of cells from other stresses. Accordingly, as shown in Table 3, LCP is expected to have the additional advantage of supporting the heat resistance improvement of various strains by enhancing the stability of cells.

Table 3. Cell viability of freeze-dried lactic acid bacteria by heat treatment at 50 °C for 30 min.

Strain	Group	Viable Cell Counts (Log CFU/g)		Survival Rate (%)
		Initial	Final	
MG989	Control	11.88 ± 0.12 ***	7.40 ± 0.02	0.003 ± 0.00 [b]
	SM	11.84 ± 0.02 ***	8.44 ± 0.04	0.04 ± 0.01 [b]
	LCP	11.67 ± 0.05 **	10.43 ± 0.09	5.82 ± 1.24 [a]
MG5125	Control	11.98 ± 0.01 ***	7.79 ± 0.09	0.01 ± 0.00 [b]
	SM	11.95 ± 0.05 ***	8.78 ± 0.02	0.07 ± 0.00 [a]
	LCP	11.73 ± 0.03 ***	8.75 ± 0.05	0.11 ± 0.01 [a]
MG5232	Control	12.20 ± 0.01 **	11.64 ± 0.03	27.86 ± 1.83 [b]
	SM	12.20 ± 0.05 **	11.67 ± 0.01	29.38 ± 0.63 [b]
	LCP	11.89 ± 0.05 *	11.67 ± 0.01	59.84 ± 0.48 [a]
MG741	Control	11.88 ± 0.01 ***	11.19 ± 0.02	20.49 ± 0.82
	SM	11.77 ± 0.03 **	11.29 ± 0.03	33.40 ± 2.34
	LCP	11.39 ± 0.09 *	10.94 ± 0.07	35.00 ± 5.00
MG5140	Control	11.54 ± 0.03 ***	8.23 ± 0.04	0.05 ± 0.01
	SM	11.62 ± 0.02 ***	7.85 ± 0.01	0.02 ± 0.00
	LCP	11.44 ± 0.01 **	8.82 ± 0.12	0.25 ± 0.07

SM: skim milk, LCP: low molecular weight collagen-peptide. Bacterial survival rate was calculated as a percentage based on the initial number of viable cells (CFU/g) of the freeze-dried bacteria. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparison between initial and final viable cell counts in each group indicates statistical significance. Different letters ([a], [b]) indicate statistical significance in survival rate of the control, SM, and LCP groups of each strain.

3.4. Microbial Morphological Characteristics Based on SEM

A morphological characterization using SEM was performed to better understand the effect of LCP on freezing-induced changes in the cell membrane and to investigate the interaction between the cell membrane and LCP. If the cell surface is exposed to the outside, it is easily affected by external stress stimuli, and cell death is promoted as the stability of the cell membrane is destroyed [14]. In the 3% LCP-treated group, a thin layer of protective matrix was confirmed to uniformly cover the outside of the cells. Further, the spacing between the bacteria was constant, and the release of intracellular components was not confirmed (Figure 1). In contrast, in the free cell condition, the interval between bacteria was irregular, and the cell membrane exposed from the outside was confirmed (data not shown). Therefore, the storage stability and thermal stability of the probiotic strains in this study were significantly improved after lyophilization by LCP treatment. This finding indicates that the addition of LCP effectively maintains the formation and structural integrity of the cell membrane barrier and prevents the leakage of intracellular components following cell membrane disruption.

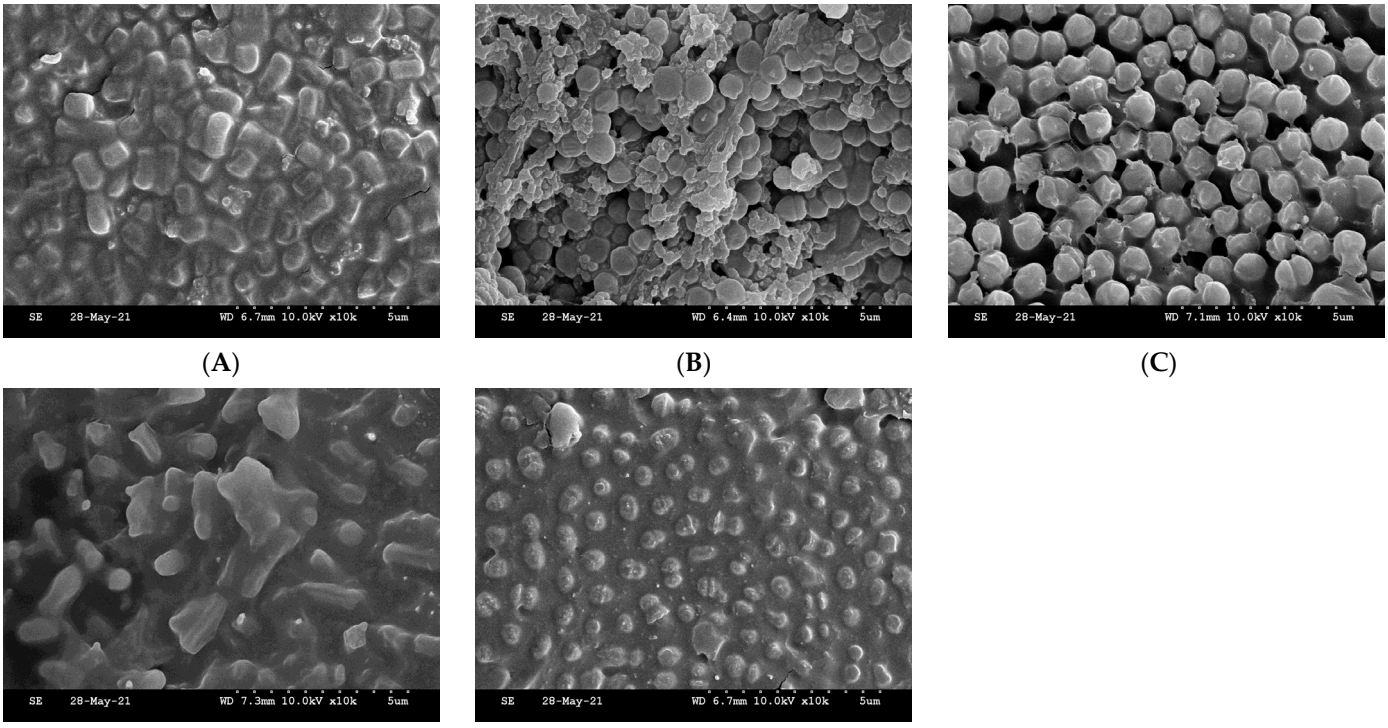

Figure 1. Representative scanning electron micrographs (SEM) at 10,000× of probiotic bacterial cells after freeze-drying owing to the addition of 3% low molecular weight collagen-peptide (LCP). (**A**): *Lactoplantibacillus plantarum* MG989, (**B**): *Lactococcus lactis* MG5125, (**C**): *Enterococcus faecium* MG5232, (**D**): *Bifidobacterium animalis* ssp. *lactis* MG741, and (**E**): *Streptococcus thermophilus* MG5140.

4. Conclusions

This study was conducted to investigate and identify the efficacy of a novel additive that aids in the cryoprotection and enhancement of intracellular stability. Based on the findings of this study, LCP is an unprecedented stability enhancer for various probiotics that causes excellent improvement in the viability of LCP-coated LAB strains after freeze-drying, simulated GI conditions, and heat treatment. Therefore, applying the LCP technology developed in this study can contribute to the enhanced stability of the production of several probiotic products and delivery systems for the human body.

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