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Improving the Viability of Freeze-dried Probiotics Using a Lysine-based Rehydration Mixture

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The probiotic market is constantly continuing to grow, concomitantly with a widening in the range and diversity of probiotic products. Probiotics are defined as live microorganisms that provide a benefit to the host when consumed at a proper dose; the viability of a probiotic is therefore of crucial importance for its efficacy. Many products undergo lyophilization for maintaining their shelf-life. Unfortunately, this procedure may damage the integrity of the cells due to stress conditions during both the freezing and (vacuum-) drying process, thereby impacting their functionality. We propose a lysine-based mixture for rehydration of freeze-dried probiotics for improving their viability during *in vitro* simulated gastric and duodenum stress conditions. Measurement of the zeta potential served as an indicator of cell integrity and efficacy of this mixture, while functionality was estimated by adhesion to a human enterocyte-like Caco-2 cell-line. The freeze-dried bacteria exhibited a significantly different zeta potential compared to fresh cultures; however, this condition could be restored by rehydration with the lysine mixture. Recovery of the surface charge was found to influence adhesion ability to the Caco-2 cell-line. The optimum lysine concentration of the formulation, designated "Zeta-bio", was found to be 0.03 M for improving the viability of *Lactiplantibacillus plantarum* Lp-115 by up to 13.86% and a 7-strain mixture (400B) to 41.99% compared to the control rehydrated with distilled water. In addition, the lysine Zeta-bio formulation notably increased the adherence ability of lyophilized Lp-115 to the Caco-2 cell-line after subjected to the *in vitro* stress conditions of the simulated gastrointestinal tract passage.

Keywords: Zeta potential, probiotic, viability, cell adhesion, L-lysine

Introduction

Probiotics are defined to have a beneficial impact on host health when administered in adequate amounts. Scientific evidence is steadily accumulating on the beneficial impact of probiotics on human health in various ways including the alleviation of immune disorders, inflammatory bowel disease, type 2 diabetes and atherosclerosis [1–4]. Although recommendations tend to favor the consumption of high dose probiotics, neither the specific dosage nor the minimal viable numbers required for a putative probiotic strain are well-defined [5]. Strains with potential probiotic properties can be "naturally" obtained via fermented food such as fermented dairy products, yet the distribution of freeze-dried probiotic powders packaged in sachets or capsules are rapidly expanding in the market [6]. Marketed probiotics should be transportable, shelf-stable concentrates that guarantee the effects of intrinsic functional properties [7]. Com-

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mercialization of non-dairy probiotic products requires exact optimization of the final processing steps such as the harvesting, freezing and drying process [8]. The freeze-drying (a.k.a. lyophilization) process is known to be a stressful condition for live bacteria, yet this technology is still considered an appropriate approach to guarantee an extended shelf life for most probiotic products. The freeze-drying process is indeed a "challenge" to the viability of probiotic strains. Therefore, in order to maintain an effective dose, the number of bacteria in most products are generally three- to ten-fold higher compared to the numbers stated on the product label [5].

Key requirements for an effective probiotic include its survival under gastrointestinal stress conditions and its adhesion to the intestinal wall. Colon cell-lines may be used as an *in vitro* model for determining the adherence potential of a putative probiotic strain [9]. Adhesion properties of the bacterial cell envelope are determined by surface characteristics such as hydrophobicity, extracellular polymers (exopolysaccharides, adhesins) and the electric charge; at the same time the envelope plays a key role in the maintenance of cellular homeostasis and the support of intracellular functions [10–12]. During interactions with their environment bacteria are exposed to diverse physical forces that are transmitted by the specific surface structures to the cell [13]. Diverse functional acidic and basic groups such as phospholipids, lipopolysaccharides (LPS) on the membrane surface of Gram-negative bacteria and surface proteins, and hydrocarbon-like proteins such as lipoteichoic and teichoic acids on the cellular surface of Gram-positive bacteria will determine a strain's response to its environment [13, 14].

The electrostatic charge of the cell surface is considered to be a reflection of its functional groups. When in contact with a liquid the surface charge of a bacterial cell can be measured in millivolt units as zeta or electrokinetic potential; values for living cells are typically more negative than those for dead cells [15]. Both the cell surface composition and the properties of the surrounding medium (e.g., conductivity/ionic strength and pH) will determine the cell's zeta potential [16]. Changes in the zeta potential (caused, e.g., by cationic agents) reflect membrane damage and alterations in permeability and may thus serve to predict cell viability [17]. Composition and chemical characteristics of the cell surface play a key role in its response to the surrounding medium (e.g., conductivity/ionic strength and pH) and thereby in determining the electrostatic charge of the cell surface and its zeta potential [16]. Zeta potential can therefore be used as a measure of the electrical surface charge of a bacterial particle in a suspension [18]. It is determined by the nature of the groups exposed at the surface, and under normal physiological conditions bacteria are usually negatively charged due to the large amount of phosphate and carboxyl groups present on the cell surface [19]. Together with interfacial viscoelasticity and tension the zeta potential can serve to characterize the surface properties of intestinal bacteria and to predict their adhesion potential in the GI tract [20].

The zeta potential around bacteria can thus serve as an indicator of their viability, integrity and efficacy especially in terms of overall and physiological potential [21]. We therefore propose a new approach for sachet packaged probiotics containing the probiotic together with a special lysine-based formulation for supporting the re-activation of a probiotic strain in water. We have named this mixture "Zeta-bio" since the zeta potential was used as an initial screening factor for 33 different chemicals using three representative strains [(*Lactiplantibacillus (Lp.) plantarum* Lp-115, *Lacticaseibacillus. (Lc.) casei* Lc-11 and *Bifidobacterium longum* Bl-05)] from a commercial 7-strain mixture (400B). This mixture also contained *Lactobacillus acidophilus* La-14, *Streptococcus thermophilus* St-2, *Bifidobacterium animalis* subsp. *lactis* Bl-04 and *B. breve* Bb-03. Optimization of the lysine concentration for the 7-strain mixture (400B) and the commercial strain *Lp. plantarum* LP-115 was further studied using the simulated stomach duodenum passage (SSDP) *in vitro* model and cell adhesion as the key parameters.

Material and Methods

Microorganisms

Lp. plantarum Lp-115 and *Lc. casei* Lc-11 were cultivated in MRS broth (BD, USA) at 37℃ for 18 h. *B. longum* Bl-05 was cultivated in BL broth (BD) with 5% bovine blood at 37℃ for 18 h under anaerobic conditions (Anaerobic Chamber Whitley DG250, Don Whitley Scientific, UK). Initially, 1% (v/v) of each strain was activated in either 5 ml MRS broth or BL broth and propagated by two sub-culturing steps in MRS broth

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Table 1. Concentrations of ingredients combined for the potential Zeta-bio formulation.

Ingredients (Brand)	LP/Mix-1	LP/Mix-2	LP/Mix-3	LP/Mix-4	LP/Mix-5
L-lysine hydrochloride (Samin)	0.182 g (0.01 M)	0.364 g (0.02 M)	0.546 g (0.03 M)	0.728 g (0.04 M)	0.910 g (0.05 M)
FOS (Hi-tech)	3.5 g	3.5 g	3.5 g	3.5 g	3.5 g
Microorganism	0.15 g (2×10^{11} CFU/g)	0.15 g (2×10^{11} CFU/g)	0.15 g (2×10^{11} CFU/g)	0.15 g (2×10^{11} CFU/g)	0.15 g (2×10^{11} CFU/g)
Dextrin (Daesang)	6.204 g	6.130 g	5.912 g	5.766 g	5.62 g
Total	10 g	10 g	10 g	10 g	10 g

before each experiment.

All strains and freeze-dried probiotics including COSMAX NBT (400B) 7-strain HRB and its materials (Table 1) were provided by COSMAX NBT Inc. (Korea). Each probiotics sachet contained 3×10^{10} CFU of the freeze-dried microorganism mix with 3.5 g of fructooligosaccharides (FOS) and dextrin (Table 1).

Zeta potential screening test

The lyophilized probiotic powders of *Lp. plantarum* Lp-115, *Lc. casei* Lc-11 and *B. longum* Bl-05 were respectively transferred at a concentration of 1×10^9 CFU/g to a 50 ml tube and mixed with 0.1 g of each 33 different chemicals in the ingredient list (Table 2). These three strains were the major representatives of the mixture and comprised > 60% of the total viable numbers (data provided by COSMAX NBT Inc.). The weight of each formulation varied according to the composition when matched to the final concentration 0.1 M. The 33 chemicals were selected according to the "Food Additive Status List" provided by the Food and Drug Administration (FDA, USA). One milliliter of deionized distilled water (DDW) was added to the freeze-dried strain and the single chemical mixture and rehydrated for 1 min at 25℃. Subsequently 9 ml of DDW at pH 2.5 were added to each sample and the pH re-adjusted using 0.1 N HCl, and 800 µl of the calibrated sample were transferred to DTS1070 cuvettes. The electrophoretic mobility was measured by the Zetasizer Nano ZEN 3600 (Malvern Panalytica, UK) after 2 min of equilibration time and the Smoluchowski equation was used to convert the data into zeta potential values. Each value represented the average of three reads.

Simulated stomach duodenum passage (SSDP) test

The potential of Zeta-bio formulations (Table 1) for the activation, adhesion and protection of freeze-dried *Lp. plantarum* Lp-115 and the 7-strain mixture (400B) was evaluated using the *in vitro* SSDP according to Ji *et al.* [22] with some modifications. The screening of the formulations showed superior protection by lysine to be this was further analyzed for determining the optimal lysine concentration for supporting bacterial cell viability within the of 0.01 M to 0.05 M, also considering possible adverse taste-effects in the product. The sample mixture was prepared according to Table 1 in 1 ml of distilled water at 25℃ and mixed for 1 min. After 1 min 9 ml of sterile phosphate buffer saline 1X (PBS, USA) at pH 2.5 were added to each sample. The tubes were incubated at 37℃ and subjected to low pH gastric stress for 1 h. This was directly followed by exposure to 4 ml of a bile salts solution (10% oxgall; Becton, Dickinson and Company – BD) and 17 ml of synthetic duodenum juice at pH 6.0 ($NaHCO_3$: 6.4 g/l, KCl: 0.239 g/l and NaCl: 1.28 g/l) thereby simulating the small intestinal passage for two hours. During the GI-tract assay samples were taken at 0, 1, and 3 h incubation time (t = 0, 1 and 2) to calculate the probiotic survivability after gastric stress and bile stress, respectively, by plate counting viable colonies on MRS and BL (BD) agar. The plates were incubated under anaerobic conditions at 37℃ for 48 h.

Cell adhesion for the validation of the effect of lysine

Lp. plantarum Lp-115, *Lc. casei* Lc-11, *B. longum* Bl-05 and the 7-strain mixture (400B) were tested for their ability to adhere to the human enterocyte-like Caco-2 cell-line (distributed by the Korean Cell Line Bank). The cells were grown and maintained in Minimal Essential Medium (MEM, USA, SH30024.01) with 10% Fetal Bovine Serum (Gibco, Thermo Fisher Scientific, USA), 1% non-essential amino acids (Gibco), and 1% of antibiotics (Antibiotic-antimycotic, Gibco) at 36.5℃ and

Table 2. List of ingredients tested for their zeta potential activity.

Ingredient	Chemical formula	Molecular weight (g/mol)	Purity (%)	Brand	Lot no.
		Carbohydrates			
Arabinose	$C_5H_{10}O_5$	150.13	100	USB	70047
Xylose	$C_5H_{10}O_5$	150.1	99	Sigma	053K00131
Rhamnose	$C_6H_{12}O_5.H_2O$	182.2	99	Sigma	058K0695
Fructose	$C_6H_{12}O_6$	180.16	100	Sigma	125K01611
Mannitol	$C_6H_{14}O_6$	182.17	100	Sigma	034K0061
Sucrose	$C_{12}H_{22}O_{11}$	342.3	100	Daejung	S0784RE1
Sorbitol	$C_6H_{14}O_6$	182.17	97	Daejung	S0503QC1
Glucose	$C_6H_{12}O_6$	180.16	99.5	Sigma	SLBS2877V
Maltose	$C_{12}H_{22}O_{11}.H_2O$	360.31	100	USB	109469
Trehalose	$C_{12}H_{22}O_{11}.2H_2O$	378.33	99	Sigma	SLBV3123
Fructooligosaccharide	$C_6H_{12}O_6$	-	95	Qhtbio	10002428
		Amino acids			
L-Arginine	$C_6H_{14}N_4O_2$	174.2	98	Sigma	017K0664
L-Tryptophan	$C_{11}H_{12}N_2O_2$	204.23	99	Kanto chemical co.	403N2187
L-Phenylalanine	$C_9H_{11}NO_2$	165.19	99	Junsei	2012L1433
L-Ornithine	$C_5H_{12}N_2O_2.ClH$	168.62	99	Sigma	109K1468
L-Glutamic acid	$C_5H_8NNaO_4.xH_2O$	169.11	99	Sigma	SLBF7449V
L-Proline	$C_5H_9N0_2$	115.1	100	Sigma	72H0774
L-Lysine hydrochloride	$C_6H_{14}N_2O_2.HCl$	182.65	98	Sigma	108K1321
L-Serine	$C_3H_7NO_3$	105.09	99	Georgiachem	S454621R
L-Threonine	$C_4H_9NO_3$	119	99	Georgiachem	T262958H
L-Aspartic acid	$C_4H_7NO_4$	133.1	99	Georgiachem	A977123A
L-Tyrosine	$C_9H_{11}NO_3$	181.19	99	Samchun chemicals	101917
L-Histidine	$C_6H_9N_3O_2$	209.64	99	Daejung	H2821RL1
		Salts			
Sodium phosphate	Na_2HPO_4	141.96	99	Sigma	075K2520
Sodium L-tartrate dihydrate	$C_4H_4Na_2O_6.2H_2O$	230.08	99	Sigma	07425BC
Sodium bicarbonate	$NaHCO_3$	84	99.8	Yakuri	312132812
		Organic acids			
Malic acid	$C_4H_6O_5$	134	98	Sigma	SLBS7651
Pyruvic acid	$C_3H_4O_3$	80.06	100	Daejung	P3470SG1
		Osmolytes			
Betaine	$C_5H_{11}NO_2$	117	98	Daejung	B1549PE1
Taurine	$C_2H_7NO_3S$	125.15	99	Sigma	12515DU
		Vitamins			
Riboflavin	$C_{17}H_{20}N_4O_6$	376.37	100	Sigma	069k1585
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS.HCl$	337.27	98	Daejung	T0027QL1
L-Ascorbic acid	$C_6H_8O_6$	176.12	99	TCI	KNBGE

5% CO_2. The bacterial adhesion assay was performed according to Botes *et al.* [23] with some modifications. The lyophilizates (3×10^8 CFU) of the 3 strains and the 7-strain bacterial mixture were reactivated in 10 ml of

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0.03 M lysine or proline for 5 min. Hundred microliter of each bacterial suspension were incubated together with a Caco-2 monolayer at 10:1 (bacteria: cell ratio) for 1.5 h. After incubation, the bacteria were withdrawn by suctioning, and the cells washed two times with ice-cold PBS to remove the non-attached bacteria and then lysed with 400 μl of TrypLE (Gibco) for 15 min at 37℃ and 600 μl of MEM that were subsequently added. To quantify the number of bacteria associated with the Caco-2/TC-7 cells the samples were serially diluted and counted on MRS agar plates after 48 h at 37℃. All the experimental results were triplicated. For Lp-115 and the 7-strain mixture (400B), the products were adjusted to 2×10^8 CFU/g and re-activated in the Zeta-bio formulation with the different concentrations of lysine (0.03 M–0.05 M) resuspended in 1 ml of distilled water (DW). All strains were subjected to the SSDP test and harvested at 3000 *g* for 20 min to remove the components related to the stomach and duodenum conditions plus bile salts. The pellets were washed three times with 1x PBS and resuspended in 10 ml MEM cell culture media with 20% FBS, 2 mM glutamine and 1% non-essential amino acids. The rest of the process were identical to the initial lysine cell adhesion experiment given above.

Results

Screening of 33 chemical ingredients based on zeta potential

Measurement of the zeta potential was used to screen the effect of 33 chemical ingredients on three representative bacterial strains of the 7-strain mixture (400B): *Lp. plantarum* Lp-115, *Lc. casei* Lc-11 and *B. longum* Bl-05. Lyophilization appeared to result in a significant depolarization of the *B. longum* Bl-05 cells compared to the freshly cultivated cells (Fig. 1A). However, changes in the zeta potential charge were not observed in the *Lp. plantarum* Lp-115 and *Lc. casei* Lc-11 strains and opposite tendencies were detected (Figs. 1B and C). Most of the amino acids had a significant impact on the positive change of the zeta potential of all three strains (Fig. 1). However, only lysine produced a negative zeta potential for all three strains including *B. longum* Bl-05 (Fig. 1 A). Therefore, the effect of lysine was further validated by the cell adhesion test and proline which portrayed the opposite effect of lysine or in the case of *Lp. plantarum* Lp-115 showed extremely negative zeta potential (mV) values was also included to determine whether the zeta potential would affect the cell adhesion capability.

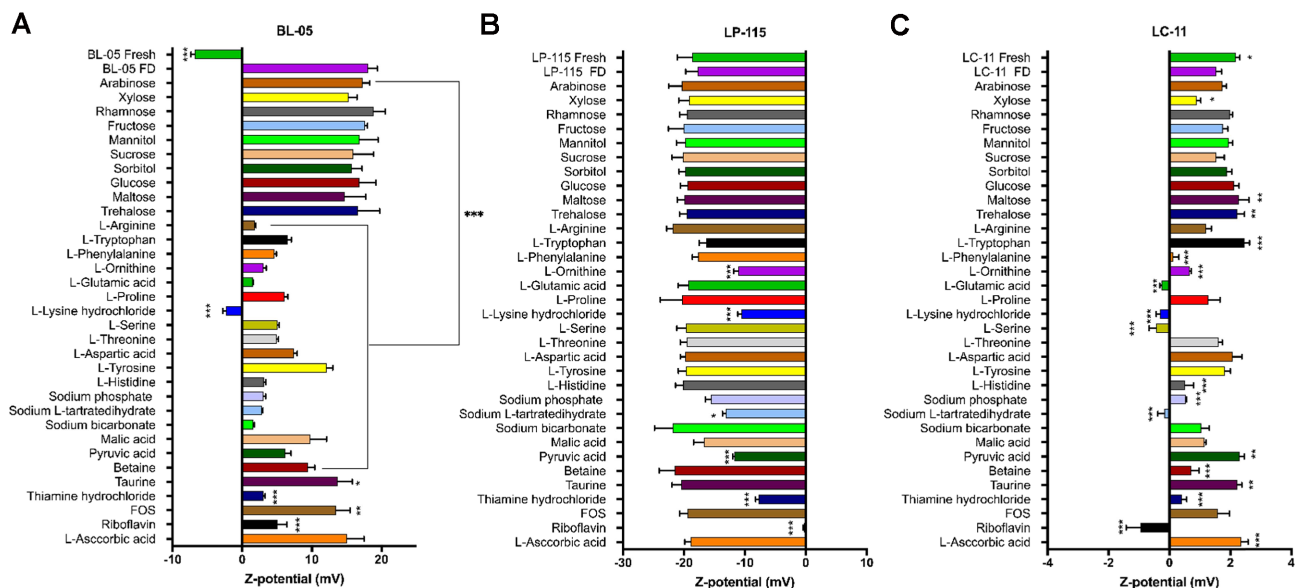

Fig. 1. Effect of 33 ingredients on the zeta potential of different probiotic strains within the 7-strain mixture (400B). Three representative strains of the 7-strain mixture (400B) were used to screen for the most optimal ingredient for the hyperpolarization of the zeta potential. (A) BL-05: *Bifidobacterium longum* Bl-05, (B) LP 115: *Lactiplantibacillus plantarum* Lp-115, (C) LC-11: *Lacticaseibacillus casei* Lc-11. Statistical analysis was performed using one-way ANOVA compared to each strain's freeze-dried (FD) sample. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Dunnett's test.

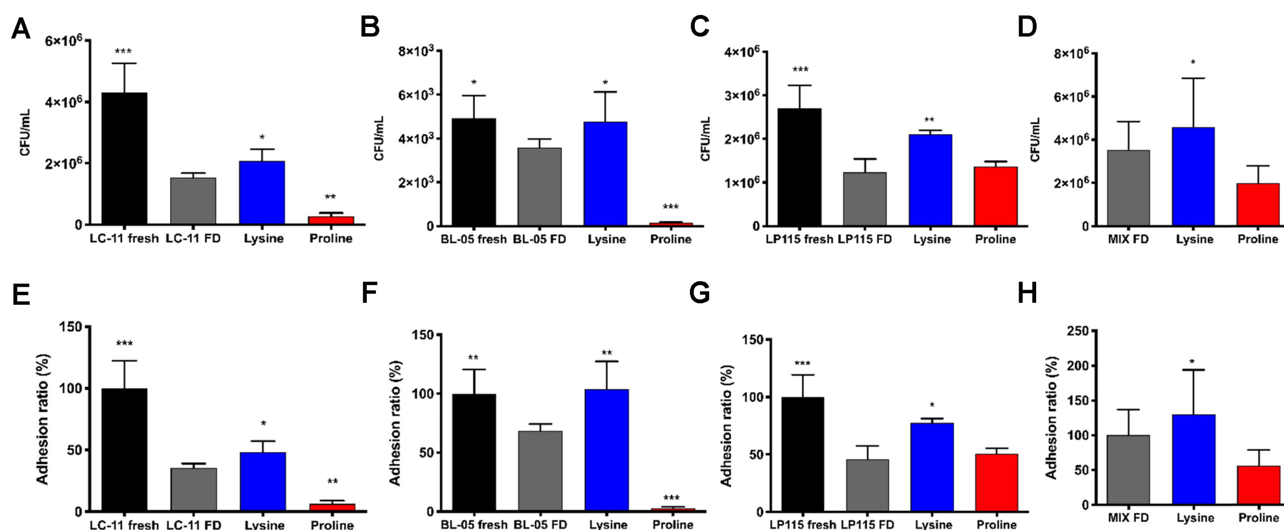

Fig. 2. Cell adhesion results of fresh, freeze-dried and freeze-dried cells following reactivation in 10 ml of 0.03 M lysine or proline for 5 min. (A, E) *Lacticaseibacillus casei* Lc-11; (B, F) *Bifidobacterium longum* Bl-05; (C, G) *Lactiplantibacillus plantarum* Lp-115 (D, H) 7-strain mixture (400B). Statistical analysis was performed using one-way ANOVA compared to each strain's freeze-dried (FD) sample. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Fisher's LSD test.

Freshly cultivated and freeze-dried bacterial suspensions of each strain were used as positive and negative controls to compare the effect of lysine and proline on the cell adhesion of the freeze-dried bacteria. Freeze-dried *Lc. casei* Lc-11 (Figs. 2A and E), *Lp. plantarum* Lp-115 (Figs. 2C and G) and *B. longum* Bl-05 (Figs. 2B and F) showed a significantly lower attachment to the Caco-2/TC-7 cell-line compared to the fresh cells. However, the selected chemical, lysine, significantly recovered the cell adhesive property of the bacterial cells when compared to the freeze-dried form (Fig. 2) whereas proline had a significantly opposite effect (Fig. 2). Proline, which induced an extremely negative zeta potential in *Lp. plantarum* Lp-115 did not have any significant affect in the adhesion ability of its freeze-dried cells (Figs. 2C and G). This result was also reflected in the 7-strain mix (400B) (Figs. 2D and H).

Selection of optimal lysine concentration through SSDP and cell adhesion

Based on the analysis of viability and cell adhesion, the two key parameters that are known to be influenced by the zeta potential, the optimal concentration of lysine was selected. The concentration of the lysine-based Zeta-bio formula was reduced to 0.01–0.05 M, considering the taste effect for the final product formulation (Table 1). Further analysis was conducted using the actual commercial products: 7-strain mixture (400B) and the single cell probiotic, *Lp. plantarum* Lp-115.

SSDP was performed to check the effect of the different concentrations of lysine in the Zeta-bio formulation on the viability of the freeze-dried bacteria after simulated passage of the stomach and duodenum. The viability of the freeze-dried form of *Lp. plantarum* Lp-115 significantly decreased after SSDP compared to the fresh control. Compared to the control, a lysine concentration of ≥0.03 M in the Zeta-bio formulation supported a significantly higher viability both after the stomach (>43%) and duodenum stress (>11%) for *Lp. plantarum* Lp-115. This tendency was also found to be true for the 7-strain mixture (400B) in which the survival rate after the stomach and duodenum stress was higher at 0.03 M lysine concentration with respective values was above 50.09% and 41.99%, respectively (Table 3). For the cell adhesion test the three concentrations of lysine: 0.03 M, 0.04 M, and 0.05 M, were therefore chosen according to the final viability after the SSDP procedure.

The different lysine concentrations strongly influenced the adherence ability of the freeze-dried bacteria to the intestinal Caco-2 cell-line. Compared to the freshly cultivated culture the freeze-dried cells of *Lp. plantarum* Lp-115 showed significantly lower adhesion. However, the

Table 3. Effects of lysine concentration in the Zeta-bio formulation on survival during simulated stomach-duodenum passage (SSDP).

Sample name	Strain	Lysine concentration	Initial	Stomach		Duodenum	
			log CFU/ml	log CFU/ml	Survival (%)	log CFU/ml	Survival (%)
Lp-115 fresh	*Lactiplantibacillus plantarum* Lp-115	none	8.21 ± 0.10	8.05 ± 0.26	64.45	7.61 ± 0.15	25.76***
Lp-115 FD		none	8.04 ± 0.16	4.15 ± 0.16	0.01	4.02 ± 0.66	0.02
LP-1		0.01M	7.05 ± 0.21	5.63 ± 0.05	5.07	4.29 ± 0.28	0.17***
LP-2		0.02M	8.46 ± 0.04	6.97 ± 0.01	4.25	6.26 ± 0.01	0.63***
LP-3		0.03M	8.59 ± 0.03	8.28 ± 0.08	48.64	7.73 ± 0.01	13.86***
LP-4		0.04M	8.62 ± 0.02	8.30 ± 0.00	48.01	7.69 ± 0.00	11.93***
LP-5		0.05M	8.63 ± 0.09	8.26 ± 0.01	43.20	7.72 ± 0.03	12.17***
Mix FD	7 strain mixture (400B)	none	8.75 ± 0.02	7.52 ± 0.05	6.01	4.96 ± 0.07	0.02
Mix-1		0.01M	8.20 ± 0.08	6.43 ± 0.00	1.74	5.88 ± 0.06	0.48***
Mix-2		0.02M	8.42 ± 0.01	7.79 ± 0.01	23.02	7.69 ± 0.04	18.70**
Mix-3		0.03M	8.32 ± 0.01	8.02 ± 0.01	50.09	7.94 ± 0.05	41.99**
Mix-4		0.04M	8.33 ± 0.03	7.95 ± 0.03	41.93	7.90 ± 0.01	37.65*
Mix-5		0.05M	8.37 ± 0.01	8.10 ± 0.01	53.33	7.82 ± 0.14	28.72*

The data are shown as mean ± standard deviation. Statistical analysis for the Lp-115 was performed by student's t-test compared to each product's freeze-dried (FD) sample. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cell adhesion ratio for *Lp. plantarum* Lp-115 was best recovered through the 0.03 M lysine Zeta-bio formulation. As the concentration of lysine was increased beyond 0.03 M the cell adhesion ratio decreased accordingly for *Lp. plantarum* Lp-115 (Figs. 3A and B). This tendency was also portrayed in the SSDP test of *Lp. plantarum* Lp-115 with 0.03 M showing the highest viability of 48.64% and 13.86% after both the stomach stress and bile stress (Table 3), respectively. The 7-strain mixture (400B), on the other hand, gave significantly higher adhesion ratios for both 0.03 M and 0.04 M lysine Zeta-bio formulations (Figs. 3C and D).

Discussion

Preserving the integrity of the cell envelope, and particularly of the plasma membrane, is important in maintaining cell viability and homeostasis. Adhesive potential may be considered as a criterion for reflecting functionality of an effective potential probiotic. Successful survival after gastric stress and proper attachment to the intestinal cells will promote immunomodulatory and metabolic functions, and thereby serve to strengthen the gut barrier and competitively inhibit the adhesion of pathogens [24]. Key adhesive factors on the cell surface of Gram-positive bacteria include lipoteichoic acid, surface layer proteins and mucous binding proteins [25]. Although the process of lyophilization helps to maintain bacterial shelf-life by the reduction of water activity, it also may disrupt the cell membrane thereby leading to the loss of its original functionality [26]. Recovering the integrity of the cell membrane may help reactivate its functionality through increase in viability and cell adhesive properties.

The depolarization of the zeta potential has been suggested as a positive indicator of bacterial cell membrane damage [17, 21]. Therefore, 33 ingredients were tested for their effect on the zeta potential of all three different strains. Deepika *et al.* [27] observed the decrease of the zeta potential of *Lactobacillus rhamnosus* GG with the increase of pH showing the highest zeta potential of the cells over a range of pH 2–3. The strongly acidic environment of the stomach is one of the initial hurdles in which the survivability of the bacteria is drastically reduced. The zeta potential detection of the freeze-dried bacterial cells was conducted at pH 2.5 which mimics the acid environment of the stomach. Out of the 33 ingredients, including carbohydrates, amino acids and proteins that may play a role in preserving the cell membrane [8], L-lysine was the only component showing polarization for

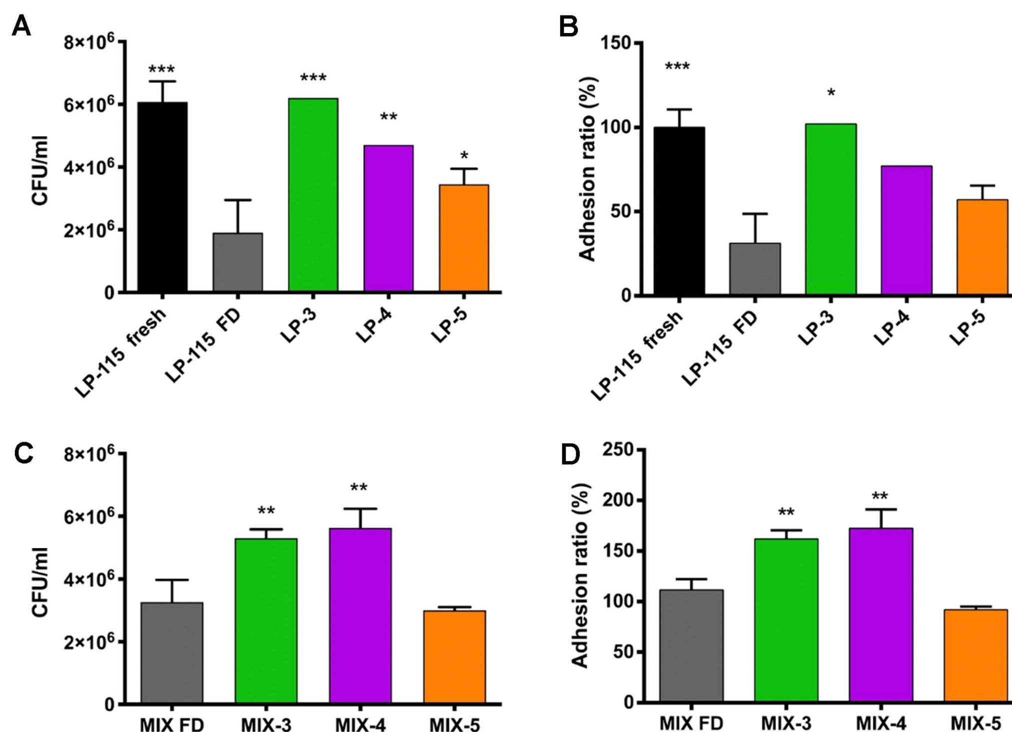

Fig. 3. Cell adhesion results to the human enterocyte-like Caco-2 cell-line as influenced by the concentration of lysine in the Zeta-bio formulation. (A, B) *Lactiplantibacillus plantarum* Lp-115, (C, D) 7-strain mixture (400B). Fresh: freshly (18 h) grown bacterial cells, FD: freeze-dried bacterial cells; 3: 0.03 M of lysine; 4: 0.04 M of lysine; 5: 0.05 M of lysine. Statistical analysis was performed using one-way ANOVA compared to each strain's freeze-dried (FD) sample. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by Fisher's LSD test.

all three strains that were tested (Fig. 1). Furthermore, L-lysine recovered the cell adhesion ratio that was decreased due to the freeze-drying process of each strain and mixture (Fig. 2). Proline is being used in some commercial mixtures in the market. However, when comparing the data obtained with proline, a slight polarization rather than hyperpolarization or depolarization of the zeta potential appears to be essential for better cell adhesion (Fig. 2), thereby serving as strong justification for the implementation of lysine rather than proline. This was also reported by de Wouters *et al.* [20] when finding a negative zeta potential closer to 0 mV to reflect a higher hydrophobicity and thereby leading to improved cell adhesion. Increased lysine concentrations resulted in higher hydrophobicity values of the cultures, suggesting a possible improvement in their adhesion potential. Therefore, achieving a negative zeta potential to maintain cell integrity and viability [17] and a slight polarization for higher cell adhesion [20] appears to be crucial for improving its functionality as a probiotic. Poly-L-lysine which is a homopolymer of L-lysine, an essential amino acid, has been proposed as a microencapsulating agent for bacterial coating that helps increase the bacterial cell viability in gastric acid conditions [28]. Besides L-lysine, other amino acids such as L-glutamic acid have also been investigated for their potential as encapsulating agents for the protection of probiotics [29].

Further research was performed to find the optimal concentration of L-lysine for the Zeta-bio formulation which is mixed with the prebiotic fructooligosaccharides (FOS). Besides enhancing the taste of the product, FOS is a well-known and commonly used prebiotic added for its prebiotic advantage and for improving the viability of probiotics due to its resistance to gastric stress [30–33]. Moreover, FOS is also known to provide beneficial health effects through the stimulation of colon bacteria producing short chain fatty acids, and for reducing weight-gain and preventing intestinal diseases [34]. FOS is also known to modulate the microbiome to a healthy state by increasing the ratio of the commensal bacteria in the human gut [35]. Furthermore, the addi-

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tion of L-lysine to FOS is known to help enhance the resistance of a strain under unbalanced osmotic conditions [36].

When the Zeta-bio formulation with different lysine concentrations (0.01 M–0.05 M) was applied to the commercial probiotic powders *L. plantarum* Lp-115 and the 7-strain mixture (400B), both the viability after SSDP and cell adhesion ratios were significantly higher in 0.03 M of lysine compared to the controls and other concentrations (Table 3 and Fig. 3). The rehydration of the probiotic lyophilizates helped reactivate cells by altering the zeta potential and increasing their viability. Complex rehydration media may play a role in promoting repair of damaged cells by providing additional nutrients, energy and essential cell components required for injured cells [37]. Furthermore, the increase in the viability and alteration of the zeta potential of the cells may improve the total cell adhesion ratio of the bacteria to the intestinal cells.

In conclusion, the application of the 0.03 M lysine-based Zeta-bio formulation for freeze-dried probiotic products, as exemplified by *Lp. plantarum* Lp-115 and the 7-strain mixture (400B), can improve the viability after gastric and duodenum stress conditions and restore their functional potential by increasing of adherence to intestinal cells.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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