

A Quadruple Coating of Probiotics for Enhancing Intestinal Adhesion and Competitive Exclusion of *Salmonella* Typhimurium

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ABSTRACT Previously, our group showed that a quadruple coating of probiotics resulted in higher survivability of probiotics under high acid, bile salt, and thermal stresses. In this study, we evaluated the effect of the quadruple coating of probiotics on adhesive properties as well as on competitive exclusion of *Salmonella* Typhimurium in Caco-2 cells. We found that the quadruple coating of probiotics exhibited an overall increased adhesion property (up to 10.8-fold) and increased competitive exclusion of *Salmonella* Typhimurium (up to 4.3-fold). Thus, this study has significant implications and can lead to the development of methods that can improve the adhesive ability of probiotics as well as the adhesive inhibition of pathogens.

KEYWORDS: • adhesion • microencapsulation • probiotics • quadruple coating • *Salmonella* Typhimurium

PROBIOTICS HAVE RECENTLY attracted considerable attention due to increasing scientific evidence concerning their health benefits.^{1,2} For example, the Korean market grew more than fourfold in size from \$140 million (USD) in 2015 to \$770 million in 2019. According to the Food and Agriculture Organization of the United Nations/World Health Organization in 2002, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host.³ The beneficial effects of probiotics include cholesterol reduction, immune boosting, and the prevention or alleviation of conditions such as lactose intolerance, acute infectious diarrhea, irritable bowel syndrome, inflammatory bowel disorder, atopic dermatitis, and food allergies.^{4,5}

These effects arise from having a well-balanced microbiota and from metabolites (*i.e.*, short-chain fatty acids, peptides, and vitamins) produced by probiotic bacteria (*e.g.*, genera *Bifidobacteria* and *Lactobacilli*) in the intestines.^{6,7} In this regard, orally administered probiotics must pass through harsh conditions, including gastric acid, bile salts, and degrading enzymes, before they can become well colonized in the gastrointestinal tract.⁸

To improve survivability, the probiotic microencapsulation technique (PET) has been introduced as a highly efficient method.⁹ Generally, the main component of PET should be nontoxic, bioavailable, biocompatible, and cost-effective.^{9,10} Until now, various polysaccharides and proteins (*e.g.*, alginate, chitosan, xanthan-gellan, and pectin) have been used as substances for encapsulation, and their effect on microbial survivability varies depending on their composition, number of coatings, and species of probiotic bacteria.^{11,12} In contrast, adhesion of probiotics to the mucus layer is also a requirement, as it allows their survivability and beneficial effects in the human gastrointestinal tract.^{13,14}

Recent studies showed that encapsulation increases the adhesive properties of probiotics to intestinal epithelial cells. For example, encapsulation of *Bacillus coagulans* with chitosan and alginate exhibited ~1.5-fold higher adherence compared with control.¹⁵ Mineral-coated *Lactobacillus rhamnosus* SKB1258 also showed increased adhesive ability in the mucin model due to increased hydrophobicity.¹⁶ Previously, we showed that quadruple-coated probiotics exhibited higher survivability under high acid, bile salt, and thermal stresses compared with uncoated controls.¹⁷

In this study, the effects of the quadruple coating on adherence of probiotics to Caco-2 cells as well as on competitive exclusion against *Salmonella* Typhimurium were investigated. Tested strains (Table 1) were anaerobically cultured overnight at 37°C in a one-ton pilot scale

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TABLE 1. PROBIOTIC BACTERIA USED IN THIS STUDY

Strain	Origin	ATCC number	NCBI accession number
<i>Enterococcus faecium</i> IDCC 2102	Breastfed infant's feces	BAA-3146	EF533988.1 (16S rDNA)
<i>Streptococcus thermophilus</i> IDCC 2201	Homemade yogurt	BAA-3150	CP035306.1 (Genome)
<i>Lactococcus lactis</i> IDCC 2301	Homemade cheese	BAA-2834	—
<i>Lactobacillus gasseri</i> IDCC 3101	Breast milk	BAA-2841	KP174108.1 (16S rDNA)
<i>Lactobacillus rhamnosus</i> IDCC 3201	Breastfed infant's feces	BAA-2836	EF533991.1 (16S rDNA)
<i>Lactobacillus acidophilus</i> IDCC 3302	Breastfed infant's feces	BAA-2845	KP325412.1 (16S rDNA)
<i>Lactobacillus paracasei</i> IDCC 3401	Breastfed infant's feces	BAA-2839	—
<i>Lactobacillus casei</i> IDCC 3451	Breastfed infant's feces	BAA-2843	KP420227.1 (16S rDNA)
<i>Lactobacillus plantarum</i> IDCC 3501	Kimchi	BAA-2838	KP174109.1 (16S rDNA)
<i>Lactobacillus salivarius</i> IDCC 3551	Healthy child saliva	BAA-2835	—
<i>Lactobacillus bulgaricus</i> IDCC 3601	Homemade yogurt	BAA-2844	—
<i>Lactobacillus reuteri</i> IDCC 3701	Breast milk	BAA-2837	KM453732.1 (16S rDNA)
<i>Lactobacillus helveticus</i> IDCC 3801	Breastfed infant's feces	BAA-2840	GU121903.1 (16S rDNA)
<i>Lactobacillus fermentum</i> IDCC 3901	Homemade cheese	BAA-2842	—
<i>Bifidobacterium longum</i> IDCC 4101	Breastfed infant's feces	BAA-2847, 3145	EF58911.1 (16S rDNA)
<i>Bifidobacterium bifidum</i> IDCC 4201	Breastfed infant's feces	BAA-2850	EF589113.1 (16S rDNA)
<i>Bifidobacterium lactis</i> IDCC 4301	Breastfed infant's feces	BAA-2848	CP031703.1 (Genome)
<i>Bifidobacterium breve</i> IDCC 4401	Breastfed infant's feces	BAA-2849	KP325411.1 (16S rDNA)
<i>Lactobacillus johnsonii</i> IDCC 9203	Breastfed infant's feces	BAA-3147	CP031701.1 (Genome)

ATCC, American Type Culture Collection; IDCC, ILDONG culture collection; NCBI, National Center for Biotechnology Information; rDNA, ribosomal DNA.

fermenter, except for *Lactococcus lactis* IDCC 2301 (aerobically at 200 rpm). Strains were freeze-dried, then sequentially mixed with 0.1% (w/w) water-soluble polymer (PVP K-30, Polyvinylpyrrolidone K30), 0.001% hyaluronic acid, 0.4% maltodextrin, and 6% milk serum protein.¹⁷ In order, components of the coating were expected to function in (1) blocking outside air, (2) maintaining water activity and adhesion, (3) blocking of foreign substances, and (4) protecting from harsh environments respectively.¹⁷ Furthermore, hyaluronic acid was further customized by mixing with postbiotics of *Lactobacillus acidophilus* IDCC 3302 and prepared by freeze-drying.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to evaluate the cytotoxicity of probiotics against Caco-2 cells. Caco-2 cells (Korean Cell Line Bank, Seoul, Korea) were maintained in Dulbecco's modified Eagle's medium-low glucose, supplemented with 10% fetal bovine serum, 100 U/mL streptomycin, 100 U/mL penicillin, and 29.2 mg/mL L-glutamine in a humidified atmosphere containing 5% CO₂ at 37°C. The Caco-2 cells (2 × 10⁴/well) were incubated in 96-well plates for 24 h, and treated with 10⁶–10⁸ CFU/mL of probiotic bacteria for 90 min.

Then, MTT solution was added and incubated for 2 h. After removal of MTT, 100 μL of dimethyl sulfoxide was added to dissolve the formazan. Finally, the color change was measured using a microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 540 nm. No significant reduction in Caco-2 cell proliferation was observed at 10⁶–10⁸ CFU/mL of probiotics for 90 min (data not shown), implying that tested probiotic strains did not show any cytotoxicity *in vitro*.

Next, the adhesion assay was sequentially tested with un-, double-, triple-, and quadruple-coated probiotics. For this,

Caco-2 cells were treated with the probiotic bacteria at 1 × 10⁸ for 90 min. As the number of coating layers increased, the adhesive ability of probiotics increased considerably (Fig. 1). Notably, the quadruple coating exhibited a 1.8- to 10.8-fold increase in the adhesion ability of probiotic bacteria in comparison with the uncoated control (Fig. 1d).

More specifically, *Lactobacillus bulgaricus* IDCC 3601 (9.4-fold), *Lactobacillus casei* IDCC 3451 (6.4-fold), *Lactobacillus fermentum* IDCC 3901 (10.8-fold), and *Lactobacillus salivarius* IDCC 3551 (8.8-fold) showed relatively high increase in adhesion, whereas *Lactobacillus johnsonii* IDCC 9203, *Lactobacillus paracasei* IDCC 3401, *Bifidobacterium lactis* IDCC 4301, *Enterococcus faecium* IDCC 2102, and *Lactococcus lactis* IDCC 2301 showed a slight increase in adhesion to the cells (Fig. 1d).

Overall adhesion (% of initial cell density) after quadruple coating ranged from 44% to 72%, whereas that of uncoated probiotics ranged from 5% to 35%. Hence, quadruple coating has a beneficial effect on adhesion of probiotic strains. Adhesions by quadruple coating with customized hyaluronic acids were similar to or lower than those of the existing quadruple coating (Fig. 1d). Considering that double coating with hyaluronic acid resulted in a dramatic increase in the adhesive ability of IDCC strains, hyaluronic acid might be the key component for adhesion (Fig. 1b).

A previous study showed that the increased bioavailability of drugs encapsulated with nanoparticles was due to hyaluronic acid, which promoted interaction between nanoparticles and mucus.¹⁸ In addition, it was revealed that the adhesion between hyaluronic acid and mucin is due to hydrogen bonding and physical interaction, and the adhesion is a proportional relation with concentration of hyaluronic acid.¹⁹

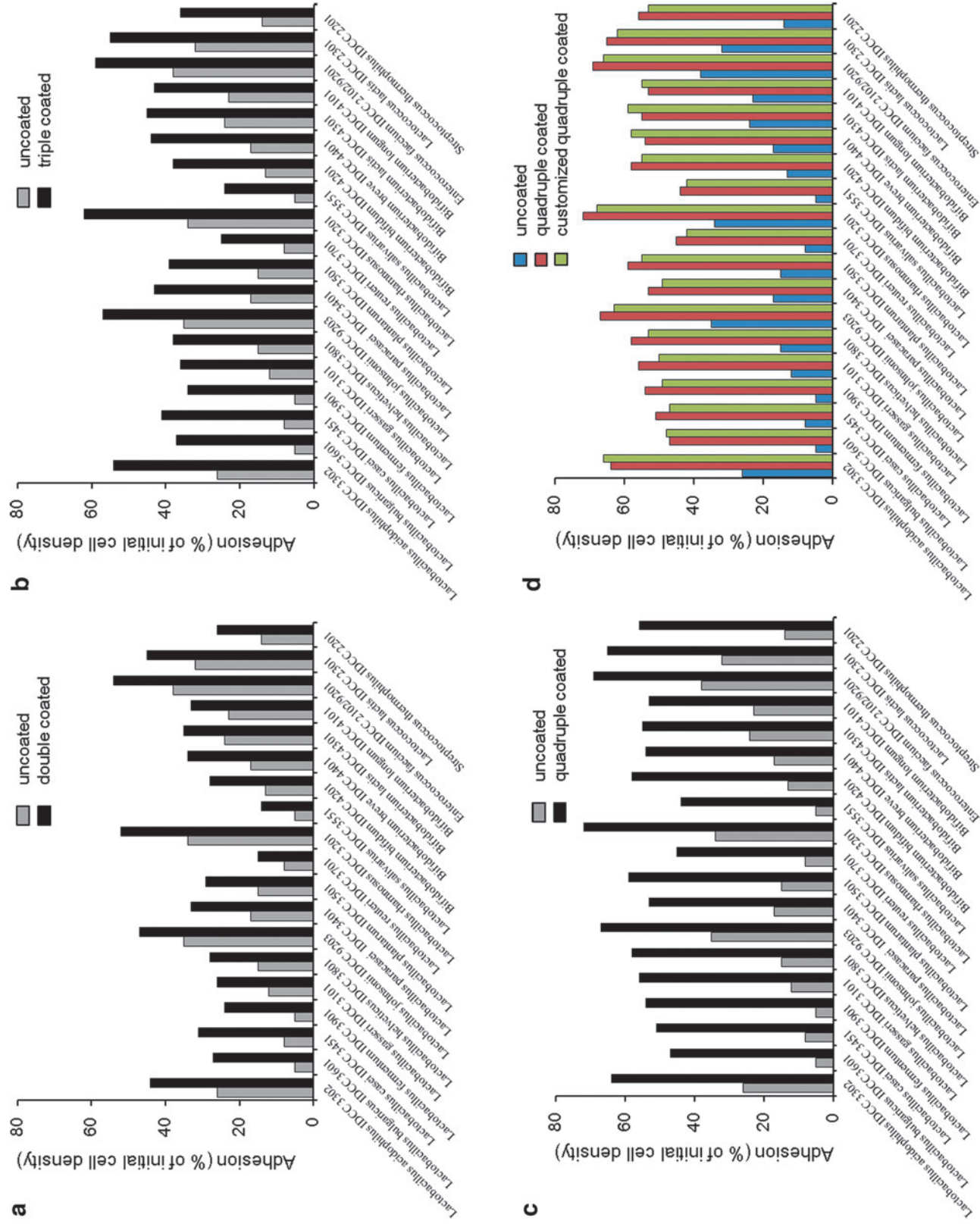


FIG. 1. Effects of coatings on adhesion in Caco-2 cells. (a) Double coated. (b) Triple coated. (c) Quadruple coated. (d) Customized quadruple coated. The experimental data are expressed as mean from three independent experiments. IDCC, ILDONG culture collection. Color images are available online.

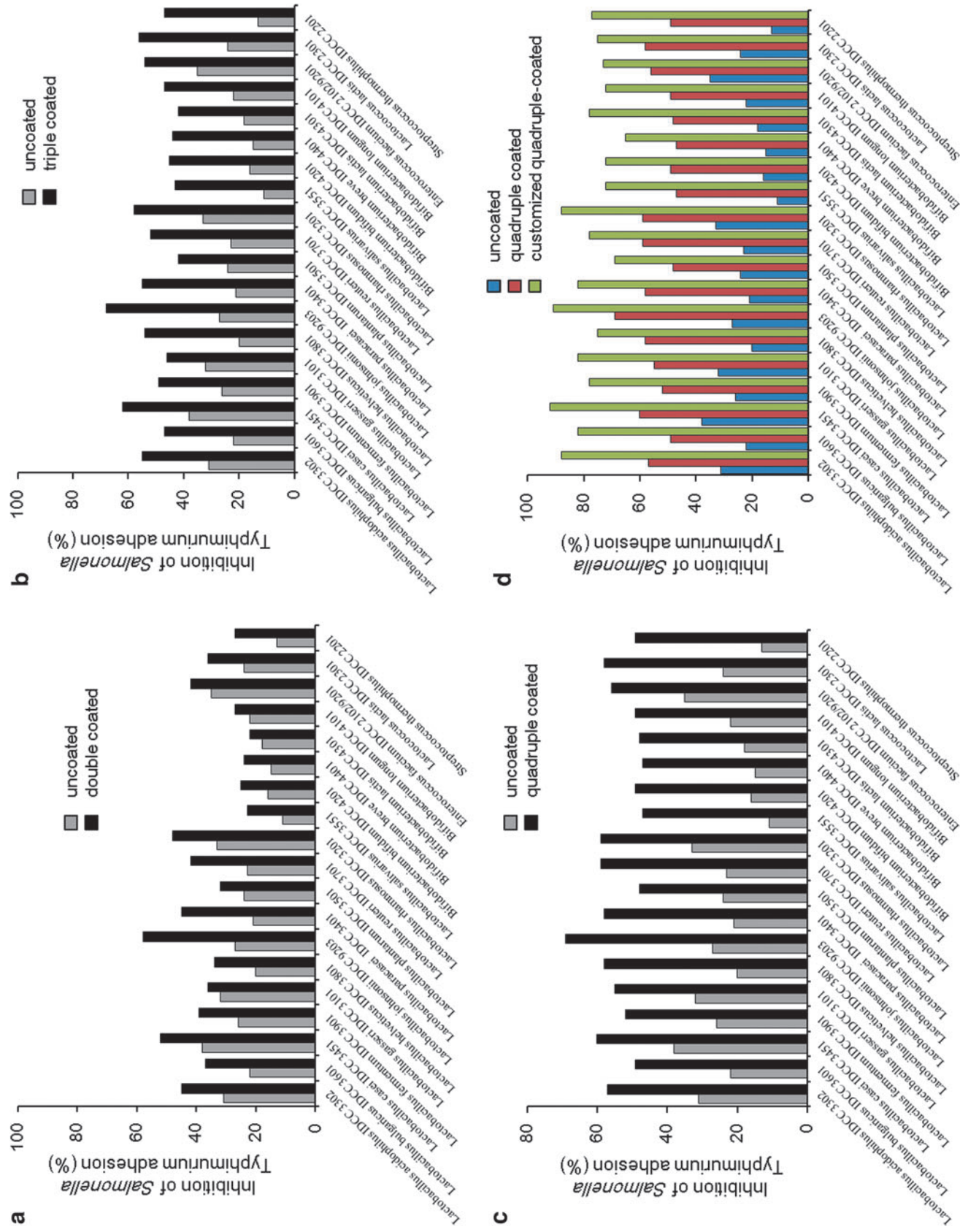


FIG. 2. Effects of coatings on competitive exclusion of *Salmonella Typhimurium*. (a) Double coated. (b) Triple coated. (c) Quadruple coated. (d) Customized quadruple coated. The experimental data are expressed as mean from three independent experiments. Color images are available online.

Diarrheagenic bacteria such as *Salmonella* share common mechanisms for host infection: adhesion, invasion, damage to the host, and survivability in the intestines.²⁰ Adhesion, which is typically facilitated by flagella or adhesion molecules is the first step and pathogens compete with intestinal microbiota to adhere to the intestinal epithelium. Beneficial microbes such as probiotics inhibit pathogens by secreting antipathogenic compounds or by competing for space to adhere, thereby preventing pathogen interaction with surface molecules of mucus layers.^{21,22} In this regard, the competitive exclusion properties of quadruple-coated probiotics against *Salmonella* Typhimurium were evaluated in Caco-2 cells. An one to one mix of *Salmonella* Typhimurium and probiotic bacteria (1×10^8 of each strain) was applied to Caco-2 cells.

Overall, quadruple coatings of probiotics showed a 1.5- to 4.3-fold increased inhibition of *Salmonella* Typhimurium adhesion compared with the uncoated control (Fig. 2d). The degree of inhibition observed was positively correlated with the overall adhesion ability of quadruple-coated probiotics to some degree (Figs. 1 and 2). In particular, *Lactobacillus salivarius* IDCC 3551 (4.3-fold), *Bifidobacterium bifidum* IDCC 4201 (3.1-fold), *Bifidobacterium breve* IDCC 4401 (3.1-fold), and *Streptococcus thermophilus* IDCC 2201 (3.8-fold) showed relatively high increases in inhibition of adhesion of *Salmonella* Typhimurium, whereas *Lactobacillus casei* IDCC 3451, *Lactobacillus gasseri* IDCC 3101, *Lactobacillus helveticus* IDCC 3801, and *Enterococcus faecium* IDCC 2102 showed only a slight increase.

The quadruple coating with customized hyaluronic acid containing postbiotic components of *Lactobacillus acidophilus* IDCC 3302 exhibited significant increases in the inhibition of adhesion of *Salmonella* Typhimurium (up to 5-fold) by competitive exclusion (Fig. 2d). This may be due to fermentation products of *Lactobacillus acidophilus* IDCC 3302 that might contain pathogenic inhibitors.²³ *Lactobacillus acidophilus* (i.e., strain CL 1285[®] and ATCC 4356) is known to inhibit pathogenic invasion, including *Staphylococcus aureus* and *Salmonella* Typhimurium through regulation of intestinal epithelium.²⁴

To verify this notion, we tested antibacterial activity of hyaluronic acid and customized hyaluronic acid (1–10%, w/v) against *Salmonella* Typhimurium ATCC 13311. The results showed that 4% of customized hyaluronic acid was found to have minimal growth inhibition concentration of *Salmonella* Typhimurium (data not shown). In conclusion, this study demonstrated that a selected encapsulation method of probiotics can improve the adhesive ability and competitive exclusion of pathogens.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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