

Journal of MEDICINAL FOOD

Journal of Medicinal Food: <http://mc.manuscriptcentral.com/jmfkorea>

A quadruple coating of probiotics for enhancing intestinal adhesion and competitive exclusion of Salmonella Typhimurium

Journal:	<i>Journal of Medicinal Food</i>
Manuscript ID	JMF-2021-K-0117.R1
Manuscript Type:	Short Communication
Date Submitted by the Author:	28-Sep-2021
Complete List of Authors:	Bang, Won Yeong; Ildong Bioscience Kim, Hayoung; Ildong Bioscience Chae, Seung A; Ildong Bioscience Yang, Soo-Yeon; Ildong Bioscience Ban, O-Hyun; Ildong Bioscience; Kyungpook National University Kim, Tae-Yoon ; Ildong Pharmaceutical Co Ltd Central Research Institute, Kwon, Hyuk-Sang; Ildong Pharmaceutical Co Ltd Central Research Institute Jung, Young Hoon; Kyungpook National University Yang, Jungwoo; Ildong Bioscience,
Keywords:	adhesion, antimicrobial, probiotics
Manuscript Keywords (Search Terms):	Adhesion, microencapsulation, probiotics, Salmonella Typhimurium, quadruple coating

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Submitted to Journal of Medicinal Food (Short communication)

A quadruple coating of probiotics for enhancing intestinal adhesion and competitive exclusion of *Salmonella* Typhimurium

Won Yeong Bang¹, Hayoung Kim¹, Seung A Chae¹, Soo-Yeon Yang¹, O-Hyun Ban^{1,2}, Tae-Yoon Kim³, Hyuk-Sang Kwon³, Young Hoon Jung^{2*}, Jungwoo Yang^{1*}

¹Ildong Bioscience, 17 Poseunggongdan-ro, Pyeongtaek-si, Gyeonggi-do, 17957, Republic of Korea

²School of Food Science and Biotechnology, Kyungpook National University, Daegu, 41566, Republic of Korea

³Ildong Pharmaceutical, 20 Samsung 1-ro 1-gil, Hwaseong-si, Gyeonggi-do, 18449, Republic of Korea

*For correspondence. E-mail: yjw@ildong.com; Tel. (+82) 31 6463180; Fax (+82) 70 7500 2467 (J. Yang) & E-mail: younghoonjung@knu.ac.kr; Tel. (+82) 53 950 5777; Fax (+82) 53 950 6772 (YH Jung)

ABSTRACT

Previously, our group showed that a quadruple coating of probiotics resulted in higher survivability of probiotics under 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 implication 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* • *Salmonella* Typhimurium • quadruple coating

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 four-fold in size from \$140 million (USD) in 2015 to \$770 million in 2019. According to the FAO/WHO 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, and immune boost, and the prevention or alleviation of conditions such as lactose intolerance, acute infectious diarrhea, irritable bowel syndrome (IBS), inflammatory bowel disorder (IBD), 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, and they are 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 non-toxic, bioavailable, biocompatible, and cost-effective.^{9,10} Until now, various polysaccharides and proteins (e.g., alginate, chitosan, xanthan-gelan, 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} On the other hand, 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 approx. 1.5-fold higher adherence compared to 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 the quadruple coating of probiotics exhibited higher survivability of probiotics under acid, bile salt, and thermal stresses compared to 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 fermentor, except for *Lactococcus lactis* IDCC 2301 (aerobically at 200 rpm). Strains were freeze-dried, then sequentially mixed with 0.1% (w/v) water-soluble polymer (PVPK-30, polyvinylpyrrolidone K30), 0.001% hyaluronic acid, 0.4% maltodextrin, and 6% milk serum protein.¹⁷ In order, components of the coating was 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 by filling the pores of the triple coating, respectively.¹⁷ Meanwhile, hyaluronic acid was further customized by mixing with postbiotics of *L. acidophilus* IDCC 3302 and prepared by freeze-drying.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium 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 (DMEM)-low glucose, supplemented with 10% fetal bovine serum (FBS), 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 (2×10^4 /well) were incubated in 96-well plates for 24 h, and treated with $10^6 - 10^8$ CFU/mL of probiotic bacteria for 90 min. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and incubated for 2 h. After removal of MTT, 100 μ L of dimethyl sulfoxide (DMSO)

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 $10^6 - 10^8$ 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, probiotic bacteria at 1×10^8 were treated to Caco-2 cells 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 to the uncoated control (Fig. 1d). More specifically, *L. bulgaricus* IDCC 3601 (9.4-fold), *L. casei* IDCC 3451 (6.4-fold), *L. fermentum* IDCC 3901 (10.8-fold), and *L. salivarius* IDCC 3551 (8.8-fold) showed relatively high increase in adhesion, whereas *L. johnsonni* IDCC 9203, *L. paracasei* IDCC 3401, *B. lactis* IDCC 4301, *E. faecium* IDCC 9201, and *L. 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 %, while that of uncoated probiotics ranged from 5% to 35%. Hence, quadruple-coating have a beneficial effect on adhesion of probiotic strains. Meanwhile, 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 exhibited 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 Additionally, It was revealed that the adhesion between hyaluronic acid and mucin is due to hydrogen bond

and physical interaction, and the adhesion is proportional relation with concentration of hyaluronic acid.¹⁹

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, 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. A one to one mix of *S. 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 *S. Typhimurium* adhesion compared to 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 (Fig. 1 and 2). In particular, *L. salivarius* IDCC 3551 (4.3-fold), *B. bifidum* IDCC 4201 (3.1-fold), *B. breve* IDCC 4401 (3.1-fold), and *S. thermophilus* IDCC 2201 (3.8-fold) showed relatively high increases in inhibition of adhesion of *S. Typhimurium*, whereas *L. casei* IDCC 3451, *L. gasseri* IDCC 3101, *L. helveticus* IDCC 3801, and *E. faecium* IDCC 9201 showed only a slight increase. The quadruple-coating with customized hyaluronic acid containing postbiotic components of *L. acidophilus* IDCC 3302, exhibited significant increases in the inhibition of adhesion of *S. 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 *S. Typhimurium* ATCC 13311. The results showed that 4% of customized hyaluronic acid was found to have minimal growth inhibition concentration of *S. 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.

FUNDING INFORMATION

This work was supported by Ildong Bioscience and Ildong Pharmaceutical, and by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through the High Value-added Food Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (321033-3).

REFERENCES

1611. Kechagia M, Basoulis D, Konstantopoulou S, *et al.* Health benefits of probiotics: a review. *Int Sch Res Notices* 2013.
1632. Goldin BR. Health benefits of probiotics. *Br J Nutr* 1998;80:S203-S207.
1643. FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization). Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada. April 30 and May 1, 2002.
1674. Butel MJ. Probiotics, gut microbiota and health. *Med Mal Infect* 2014;44:1-8.
1685. Masood MI, Qadir MI, Shirazi JH, Khan IU. Beneficial effects of lactic acid bacteria on human beings. *Crit Rev Microbiol* 2011;37:91-98.
1706. Sun Q, Jia Q, Song L, Duan L. Alterations in fecal short-chain fatty acids in patients with irritable bowel syndrome: A systematic review and meta-analysis. *Med* 2019;98.
1727. Hill MJ. Intestinal flora and endogenous vitamin synthesis. *Eur J Cancer Prev: the official journal of the European Cancer Prevention Organisation (ECP)* 1997;6:S43-5.
1748. Pop OL, Dulf FV, Cuibus L, *et al.* Characterization of a sea buckthorn extract and its effect on free and encapsulated *Lactobacillus casei*. *Int J Mol Sci* 2017;18:2513.
1769. Gbassi GK, Vandamme T. Probiotic encapsulation technology: from microencapsulation to release into the gut. *Pharmaceutics* 2012;4:149-163.

17810. Călinoiu LF, Ștefănescu BE, Pop ID, Muntean L, Vodnar DC. Chitosan coating applications
in probiotic microencapsulation. *Coatings* 2019;9:194.
18011. Shori AB. Microencapsulation improved probiotics survival during gastric transit. *Hayati J
Biosciences* 2017;24:1-5.
18212. Pech-Canul ADLC, Ortega D, García-Triana A, González-Silva N, Solis-Oviedo RL. A brief
review of edible coating materials for the microencapsulation of probiotics. *Coatings*
2020;10:197.
18513. Fernández MF, Boris S, Barbes C. Probiotic properties of human lactobacilli strains to be
used in the gastrointestinal tract. *J Appl Microbiol* 2003;94:449-455.
18714. Dudík B, Kiňová Sepová H, Bilka F, Pašková L, Bilková A. Mucin pre-cultivated
Lactobacillus reuteri E shows enhanced adhesion and increases mucin expression in HT-29
cells. *Antonie Van Leeuwenhoek* 2020;113.
19015. Anselmo AC, McHugh KJ, Webster J, Langer R, Jaklenec A. Layer-by-layer encapsulation
of probiotics for delivery to the microbiome. *Adv Mater* 2016;28:9486– 9490.
19216. Kim KM, Yang SJ, Lee CW, *et al.* Probiotic properties and immune-stimulating effect of the
Jeju lava seawater mineral-coated probiotics. *LWT* 2020;126:109299.
19417. Kim MC, Moon JS, Kim Y, Kim M, Kim T, Park H. Analysis of quadruple coating for
improvement in improving gastrointestinal stability and survivability of probiotics. *KSBB J*
2019;34:107-113.
19718. Aguilera-Garrido A, Molina-Bolívar JA, Gálvez-Ruiz MJ, Galisteo-González F.

- 198 Mucoadhesive properties of liquid lipid nanocapsules enhanced by hyaluronic acid. *J Mol Liq*
199 2019;296:111965.
20019. Graça A, Gonçalves LM, Raposo S, Ribeiro HM, Marto J. Useful in vitro techniques to
201 evaluate the mucoadhesive properties of hyaluronic acid-based ocular delivery systems.
202 *Pharmaceutics* 2018;10:110.
20320. Viswanathan VK, Hodges K, Hecht G. Enteric infection meets function: how bacterial
204 pathogens cause diarrhoea. *Nat Rev Microbiol* 2009;7:110–119.
20521. Mohanty D, Panda S, Kumar S, Ray P. *In vitro* evaluation of adherence and anti-infective
206 property of probiotic *Lactobacillus plantarum* DM 69 against *Salmonella enterica*. *Microb*
207 *Pathog* 2019;126: 212-217.
20822. Collado MC, Melriluoto S, Salminen S. Role of commercial probiotic strains against human
209 pathogen adhesion to intestinal mucus. *Lett Appl Microbiol* 2007;45:454–460.
21023. Karska-Wysocki B, Bazo M, Smoragiewicz W. Antibacterial activity of *Lactobacillus*
211 *acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus*
212 (MRSA). *Microbiol Res* 2010;165:674-686.
21324. Li P, Ye X, Yang Q. Antagonistic activity of *Lactobacillus acidophilus* ATCC 4356 S-layer
214 protein on *Salmonella enterica* subsp. *enterica* serovar Typhimurium in Caco-2 cells. *Ann*
215 *Microbiol* 2012;62:905-909.

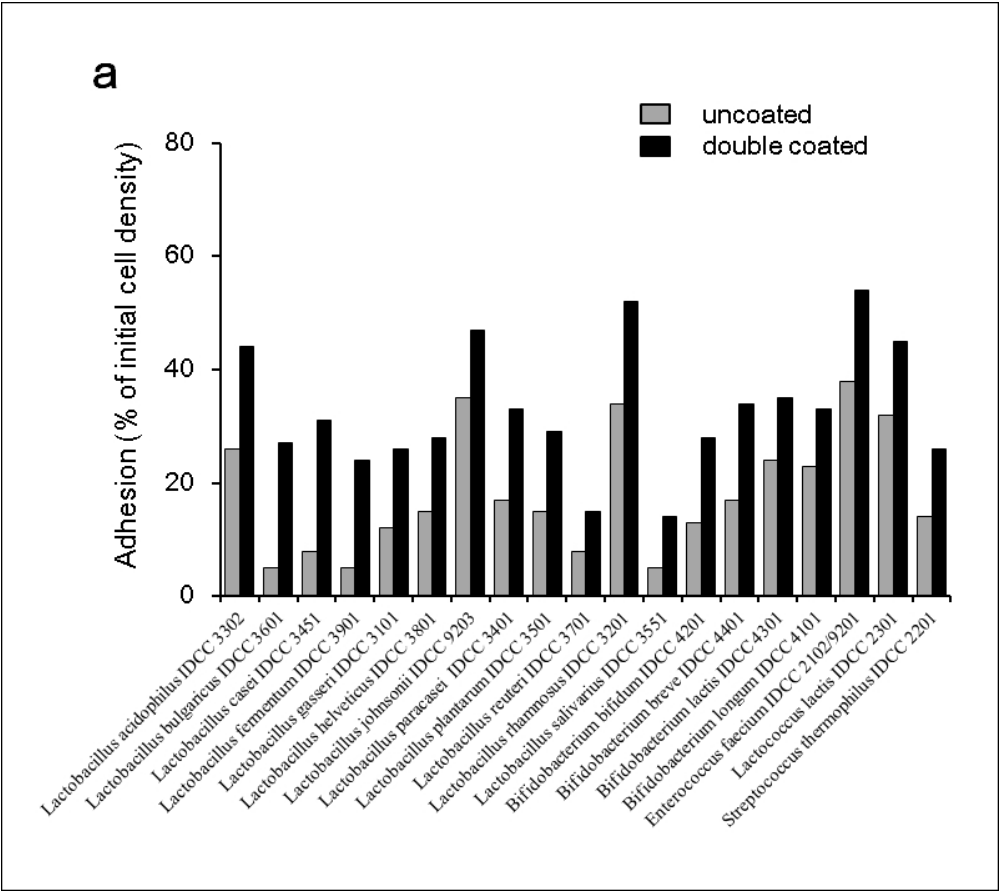
TABLE 1. PROBIOTIC BACTERIA USED IN THIS STUDY

Strain	Origin	ATCC number	NCBI accession number
<i>Enterococcus faecium</i> IDCC 2102	Breast-fed 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	Breast-fed infant's feces	BAA-2836	EF533991.1 (16S rDNA)
<i>Lactobacillus acidophilus</i> IDCC 3302	Breast-fed infant's feces	BAA-2845	KP325412.1 (16S rDNA)
<i>Lactobacillus paracasei</i> IDCC 3401	Breast-fed infant's feces	BAA-2839	-
<i>Lactobacillus casei</i> IDCC 3451	Breast-fed 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	Breast-fed 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	Breast-fed infant's feces	BAA-2847, 3145	EF58911.1 (16S rDNA)
<i>Bifidobacterium bifidum</i> IDCC 4201	Breast-fed infant's feces	BAA-2850	EF589113.1 (16S rDNA)
<i>Bifidobacterium lactis</i> IDCC 4301	Breast-fed infant's feces	BAA-2848	CP031703.1 (Genome)
<i>Bifidobacterium breve</i> IDCC 4401	Breast-fed infant's feces	BAA-2849	KP325411.1 (16S rDNA)
<i>Lactobacillus johnsonii</i> IDCC 9203	Breast-fed infant's feces	BAA-3147	CP031701.1 (Genome)

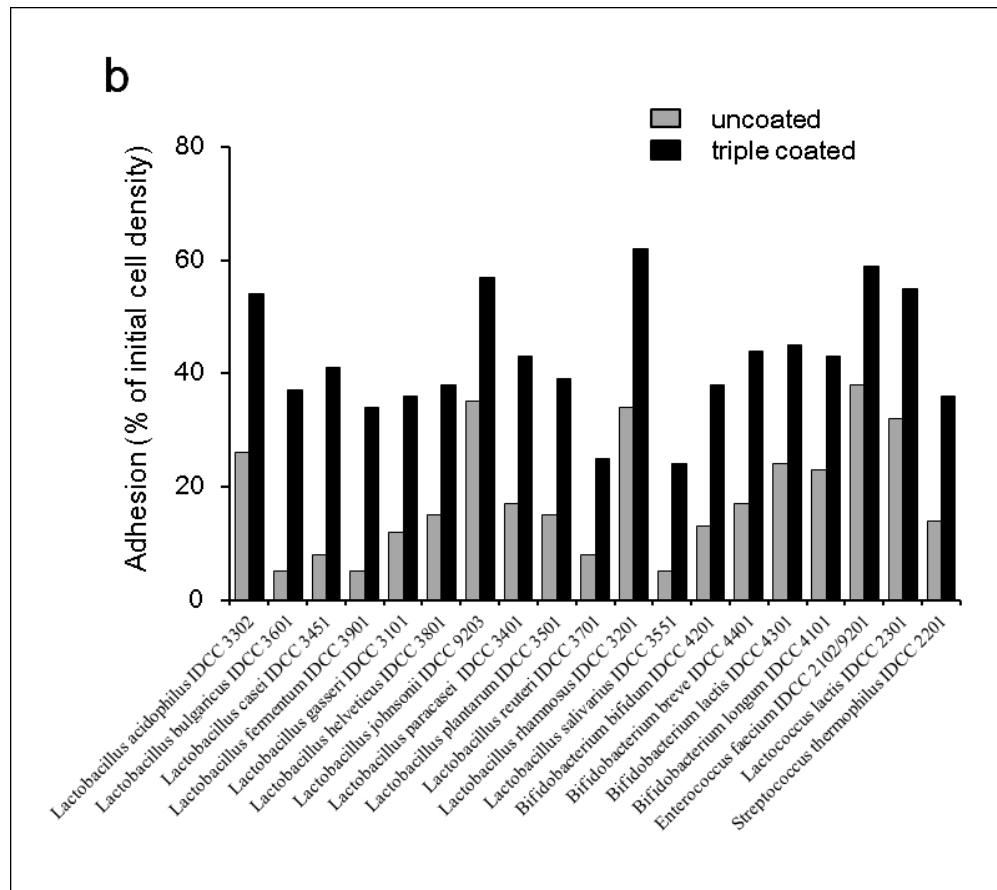
FIGURE CAPTIONS

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.

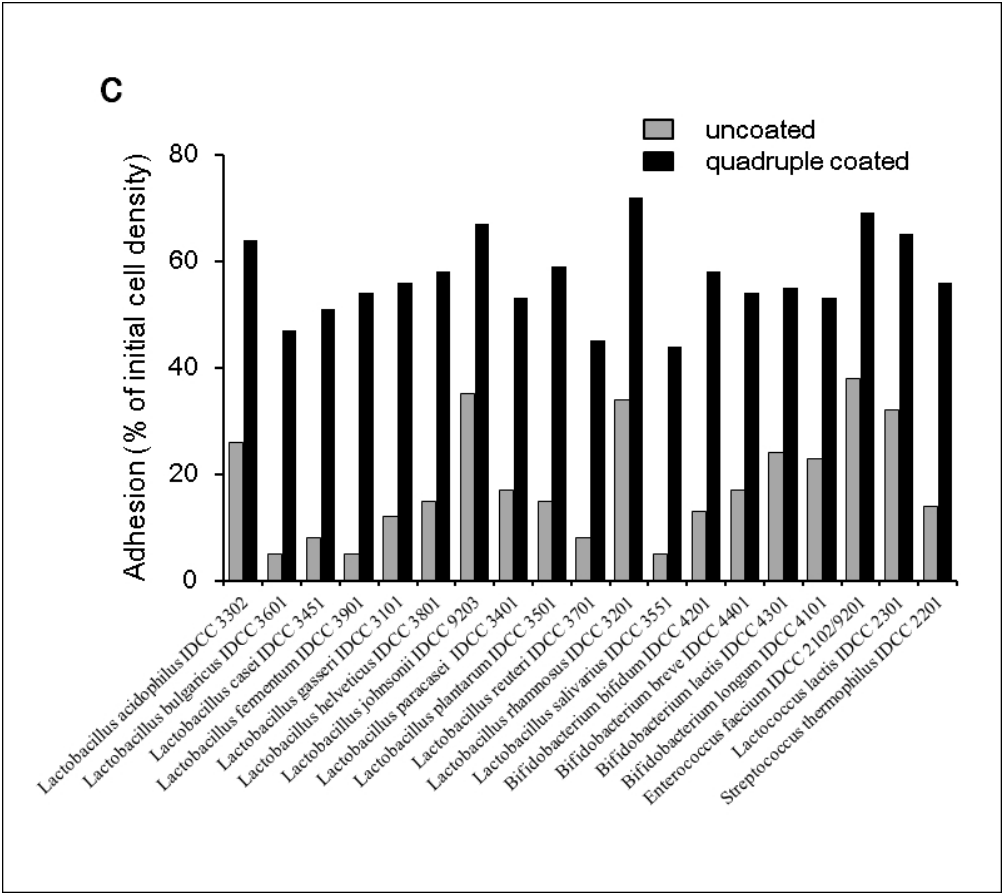
FIG. 2. Effects of coatings on competitive exclusion of *Salmonella* Typhimurium. (a) double coated. (b) triple coated. (c) quadruple coated. (d) customized quadruple coated. The experimental data are expressed as mean from three independent experiments.



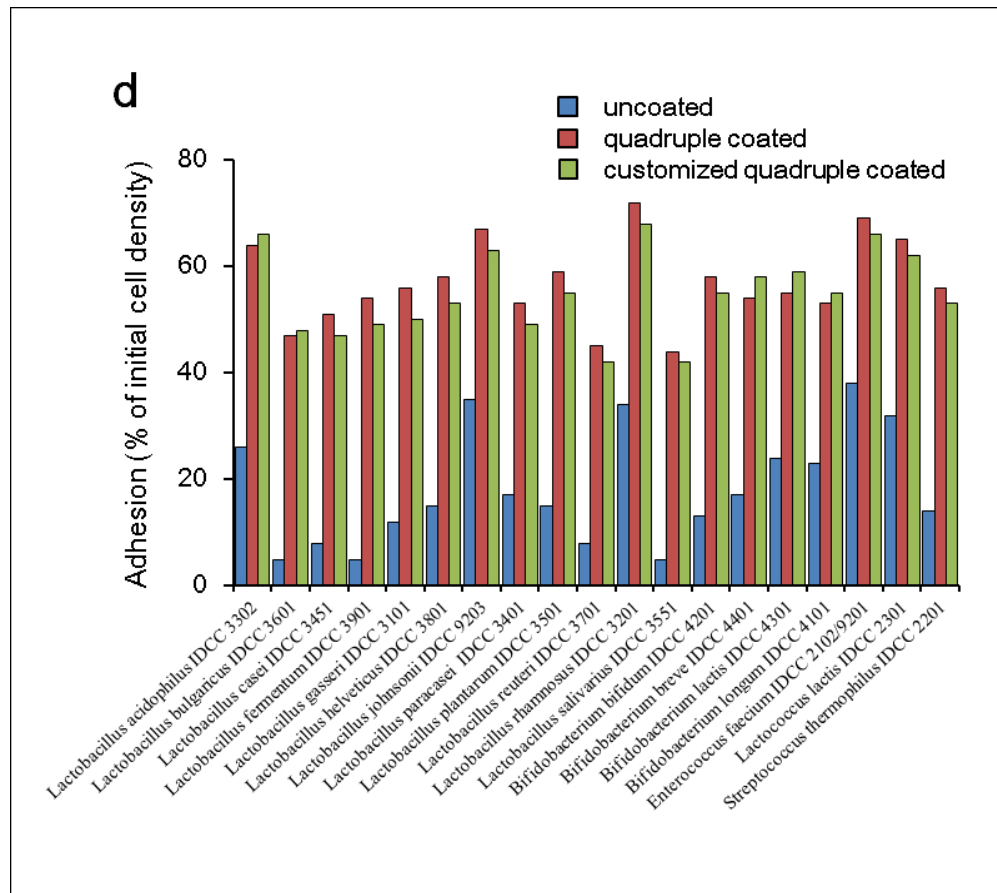
135x120mm (150 x 150 DPI)



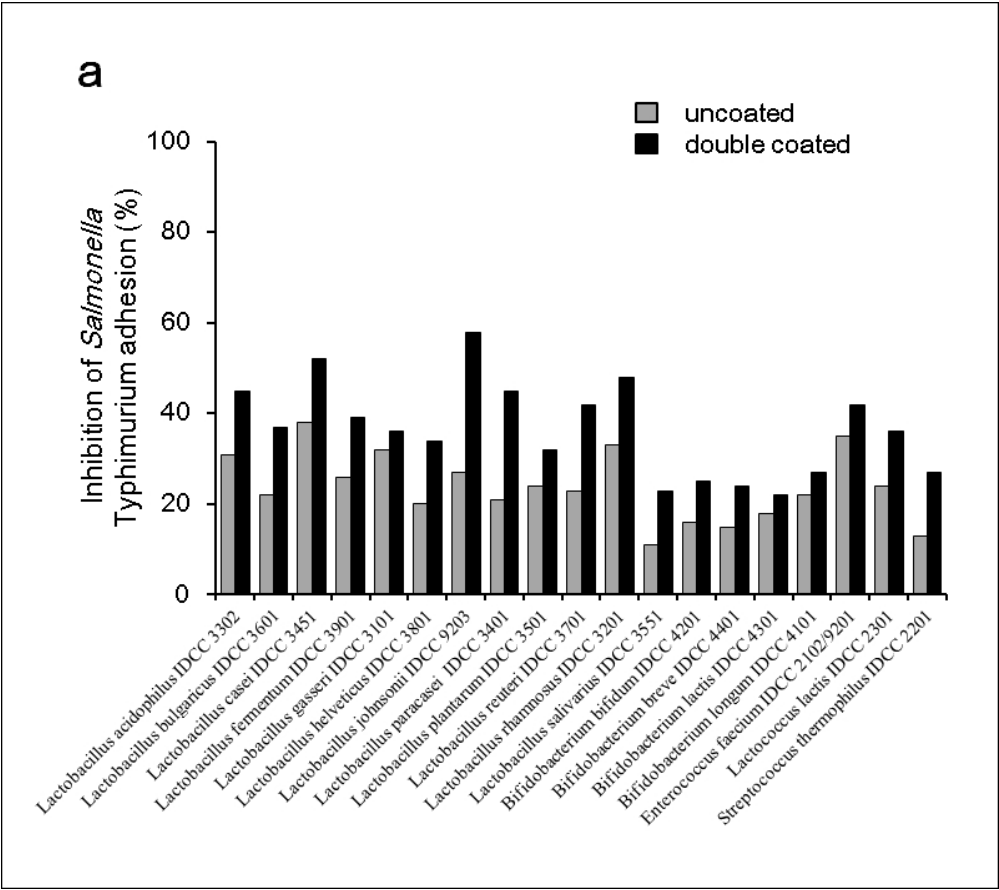
135x120mm (150 x 150 DPI)



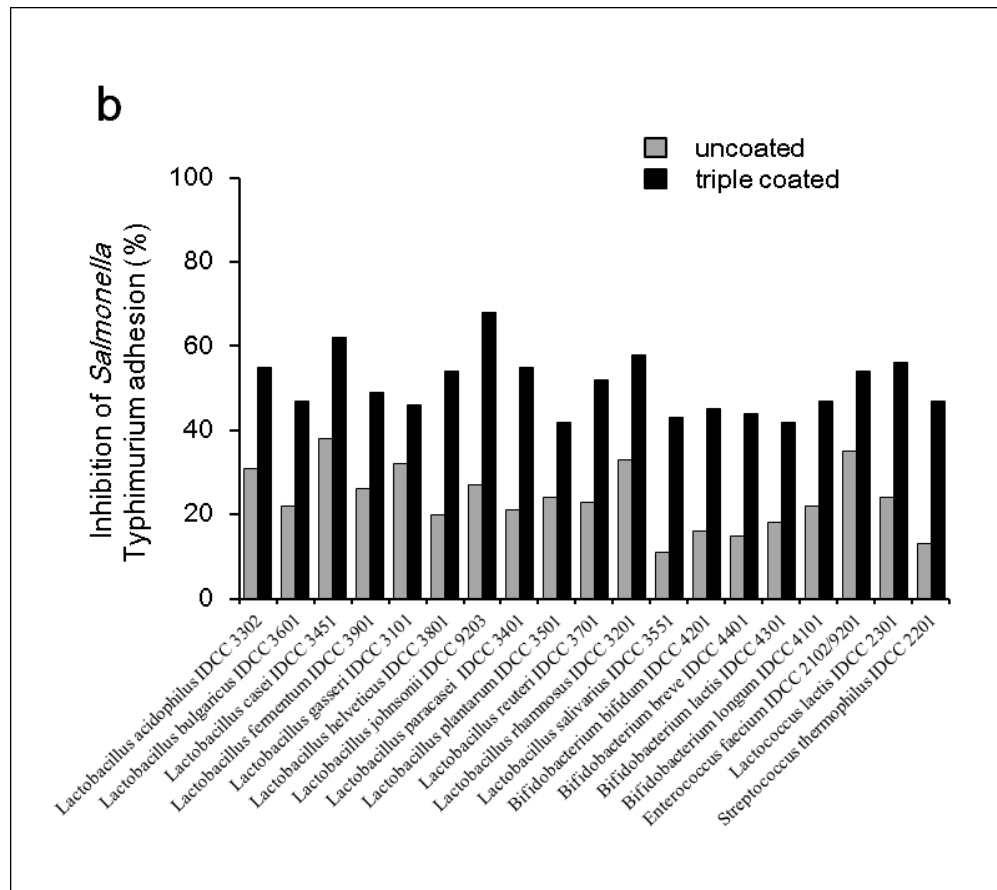
135x120mm (150 x 150 DPI)



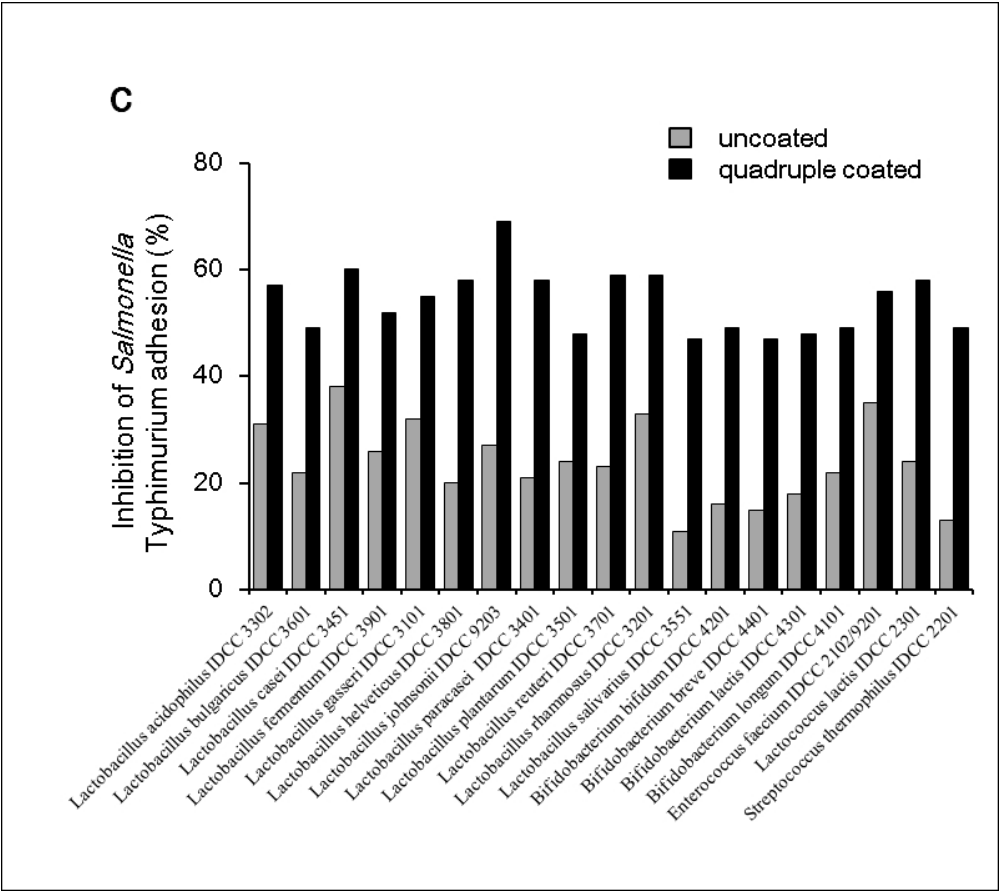
135x120mm (150 x 150 DPI)



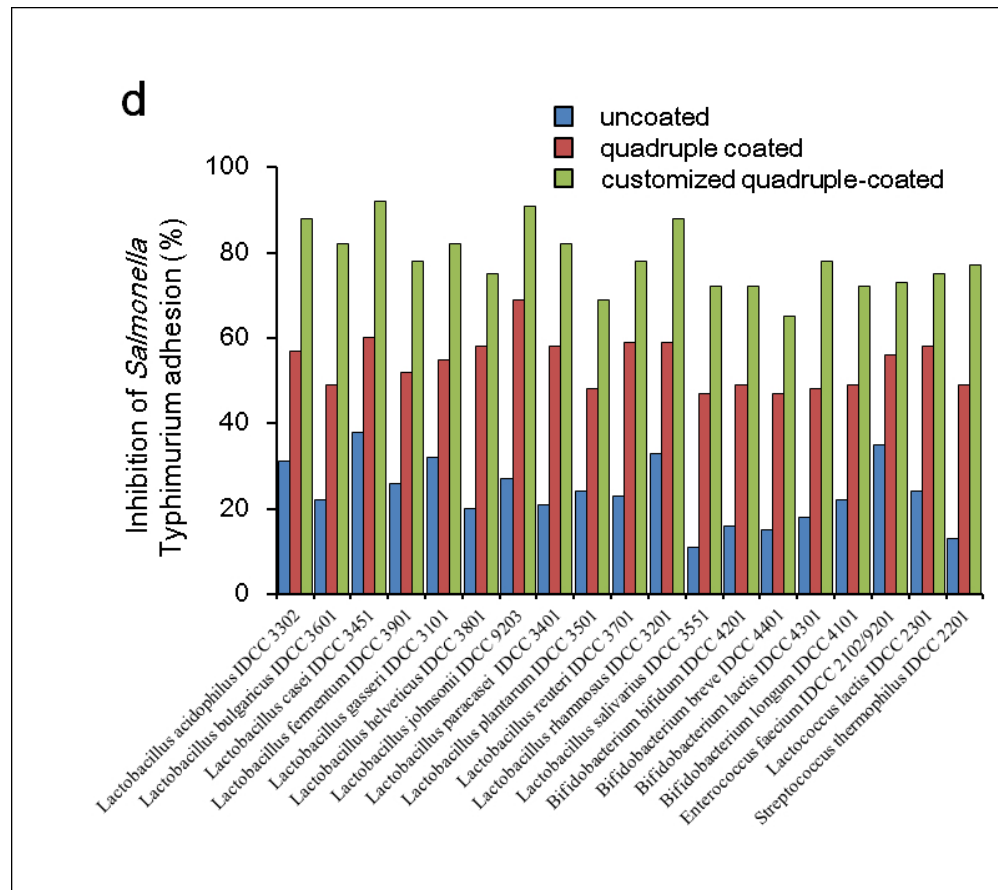
135x120mm (150 x 150 DPI)



135x120mm (150 x 150 DPI)



135x120mm (150 x 150 DPI)



135x120mm (150 x 150 DPI)