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Surface Characteristics and Adhesive Properties of *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 for Preparation of Probiotics

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Abstract Probiotics are generally excreted within a few days if their ingestion in feces at the same rate as or even more quickly than a transit marker (meaning not clear). Ability of probiotics to adhere to intestine prolongs their persistence in gastrointestinal tract, allowing them to exert healthful effects longer. Hydrophobicities, zeta potentials, Alcian blue-binding capacities, and sedimentation profiles of *Pichia farinosa* SKM-1, *P. anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 were determined to evaluate characteristic properties of cell surfaces responsible for adhesion. Results of intestinal Caco-2 cell line *in vitro* and murine intestine *in vivo* studies revealed these strains exhibit adhesive properties regardless of their cell surface hydrophobicity.

Keywords: *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, *Galactomyces geotrichum* SJM-59, adhesion, hydrophobicity

Introduction

Probiotics are live microbial food ingredients that are beneficial to human health. The most widely used probiotic microorganisms are *Lactobacillus* and *Bifidobacteria*, and extensive studies on their beneficial effects have been reported (1-4). Effective probiotic strains should be able to survive under various environmental conditions, i.e., they should have the ability to survive, but not necessarily grow, in the gastrointestinal tract and not transfer antibiotic-resistant gene to other microbes (5). At present, several reports have been made on the side effects of lactic acid bacteria consumption. *Bifidobacterium* species showed resistance against vancomycin, and some lactic acid bacteria exhibited translocation in the host when consumed at high quantity by increasing the gut permeability (5-8). Consequently, attentions are focused on probiotic strains without side effects.

The potential probiotic strains *Pichia farinosa* SKM-1, *P. anomala* SKM-T, and *Galactomyces geotrichum* SJM-59 have shown abilities to resist digestion processes in the gastrointestinal tract and were bile-resistant. Furthermore, *in vitro* study has shown that *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 could assimilate cholesterol, and these strains lowered serum cholesterol *in vivo* (9).

The aim of this study was to investigate the surface characterization and adhesive properties of *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 for the preparation of novel probiotics.

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Materials and Methods

Yeast strains and culture condition *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 were cultured for 24 hr at 30°C in potato dextrose broth (PDB, Difco, Detroit, MI, USA), pH 5.5. In all cases, yeasts were harvested at the logarithmic phase. Cells were collected by centrifugation at 3,000 rpm for 10 min, washed twice in 10 mM phosphate buffer (pH 5.8), and resuspended (OD_{600} = 0.5 ± 0.05) in the same buffer.

Determination of cell surface hydrophobicity Three different solvents were tested in this study: xylene, chloroform, and ethyl acetate. Two milliliters of yeast suspension was put in contact with 0.5 mL solvent by vortexing for 2 min, and the phases were allowed to separate by decantation for 20 min. The aqueous phase was removed, and the A_{600} was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (CSH, %), which was calculated based on the following equation: CSH (%) = $[(A_i-A_f) / A_i] \times 100$, where A_i and A_f are the absorbances measured during the initial and final extractions with the solvent.

Determination of zeta potential and Alcian blue binding The ζ potentials of the yeast resuspension were measured at 25°C using a model ELS-8000 analyzer (Otasuka Electronics, Osaka, Japan). The data are given as mean values of six measurements. Alcian blue binding to yeast cells were measured using a method based on that of Odani et al. (10).

Determination of autoaggregation time (AT) Yeast was grown, and the optical density was adjusted as indicated previously. The suspensions were dispensed in

cuvettes, and the time course of turbidity at timed intervals was evaluated until no change was observed. AT was determined using a method based on that of Bibiloni et al. (11).

Adhesion to Caco-2 cells Enterocyte-like Caco-2 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea), and the cells were grown in DMEM/F12 medium (Gibco BRL, Grand Island, NY, USA) and 20% inactivated fetal bovine serum (Gibco BRL). Monolayers were prepared on glass coverslips, which were placed in 24-well Nunc tissue plates (PolyLabo, Strasbourg, France). Cells were seeded at 1.4×10^4 cell/well and incubated at 37°C in a 5% CO_2 incubator. The Caco-2 monolayers at late postconfluence were prepared on coverslips and washed twice with phosphate buffered saline (PBS; NaCl 0.8%, K_2HPO_4 0.121%, KH_2PO_4 0.034%, pH 7.2). Cells of yeast strains (2 mL; 1×10^5~10^8 cfu/mL in PBS) were mixed with 2 mL culture medium for Caco-2 cells, and the mixture was added to each well of the monolayer Caco-2 cells. The plate was incubate at 37°C in 5% CO_2 - 95% air. After 1 hr of incubation, the monolayers were washed five times with sterile PBS, and fixed with 4% glutaraldehyde for 2 hr at room temperature. The coverslips were washed five times and dehydrated in a graded series of ethanol. Yeast adhesion was evaluated through light microscopy.

Experimental animals and in vivo adhesion assay Male ICR mice (6 weeks old) were purchased from Daihan Bio Link Co. (Eumsung, Korea). The mice were divided into three groups, each group comprising six mice, in stainless steel cages with wire mesh bottoms and maintained on a 12-hr light-dark cycle. Temperature and humidity were controlled at 20±2°C and 60±5%, respectively. The composition of the diet is shown in Table 1. All animals were given food and water *ad libitum* for 4 weeks. All animal procedures described conformed to the principles of *Guide for the Care and Use of Laboratory Animals* (12). At the end of the experimental period, the mice were anesthetized with diethyl ether (Sigma, St. Louis, MO, USA) following a 12-hr fast. After abdominal section, the colon was removed, opened along the antimesenteric line, rinsed with 0.9% saline, and cut into 30-40 pieces. Specimens were fixed with 4% glutaraldehyde for 2 hr, washed two times in PBS, post-fixed with 2.5% OsO_4 (Sigma, St. Louis, MO, USA) for 30 min, and washed again five times in PBS at room temperature. The specimens were dehydrated in a graded series of ethanol, dried in a critical-point dryer (BAL-TEC, CPD 030, City, State, USA), and coated with gold in an ionsputter (Hitachi E101, Tyoko, Japan). *In vivo* adhesion was examined using a scanning electron microscope (Hitachi S-2350, Tokyo, Japan).

Table 1. Composition of experimental diets

Ingredients	Control diet, g%	Experimental diet, g%
Casein	20.0	20.0
Corn starch	15.0	15.0
Corn oil	5.0	5.0
Cellulose	5.0	5.0
Mineral*	3.5	3.5
Vitamin**	1.0	1.0
DL-Methionine	0.3	0.3
Choline bitartarate	0.2	0.2
Lyophilized yeast	-	0.3***
Sucrose	To make 100	To make 100

*AIN mineral mix (g/Kg mix); $CaHPO_4$ 500, NaCl 74, K citrate monohydrate 220, K_2SO_4 53, MgO 24, Mn carbohydrate 3.5, Fe citrate 6.0, Zn carbonate 1.6, Cu carbonate 0.3, KIO_3 0.01, $Na_2SeO_3 \cdot 5H_2O$ 0.01, $CrK(SO_4)_2 \cdot 12H_2O$ 0.55, sucrose 118 (14).
**AIN vitamin mix (g/Kg mix); thiamin°PHCl 0.6, riboflavin 0.6, pyridoxine°PHCl 0.7, nicotinic acid 3, D-calcium pantothenate 1.6, folic acid 0.2, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 0.8 (250,000 IU/g) 0.005, menaquinone 0.005, sucrose 972.9 [14]
***Yeast concentration was 1×10^6 cfu/mL.

Statistical analysis Group data were expressed as means ±S.E. Analyses among three groups were performed using one-way ANOVA with 95% confidence intervals. Post hoc ANOVA analyses were determined by fisher's protected least-significant differences using SPSS program (ver. 10.0).

Results and Discussion

Cell surface characterization Microbial adhesion to chloroform and ethyl acetate was tested to assess the Lewis acid-base characteristics of the yeast cell surface. *P. farinosa* SKM-1 and *P. anomala* SKM-T showed slightly stronger affinity for chloroform (electron acceptor) than for ethyl acetate (electron donor). *G. geotrichum* SJM-59 showed stronger affinity for xylene. The results of solvent partitioning method indicated that *G. geotrcihum* SJM-59 was hydrophobic, whereas *P. farinosa* SKM-1 and *P. anomala* SKM-T were fully hydrophilic (Table 2).

Zeta potential is a measure of the electrical potential resulting from the net charges distributed on the microbial surface. Based on the results from zeta potential measurements, *P. farinosa* SKM-1 and *P. anomala* SKM-T showed negative charge, and *G. geotrichum* SJM-59 showed an intermediate value in PBS.

Alcian blue is a type of phthalocyanine complex that has four positively charged sites in the molecule and is adsorbed by negatively charged yeast cell surfaces, particularly the mannosylphosphate moiety. *P. farinosa* SKM-1 showed low Alcian blue level, while *P. anomala* SKM-T and *G. geotrichum* SJM-59 showed high values. Therefore, it was considered that the mannosylphosphate moiety is distributed abundantly on the surfaces of *P. anomala* SKM-T and *G. geotrichum* SJM-59. Considering the cell surface charge is influenced by the ratio of nitrogen-rich to phosphorus-rich cell wall peptides in yeast, its intermediate zeta value indicates *G. geotrichum* SJM-59 could have abundant nitrogen-rich peptides on its surface (13).

Figure 1 shows the changes in the turbidity of the microbial suspension in which sediments can be spontaneously monitored as a function of time. Note that, over a particular dilution, all curves overlapped. At this point, we graphically obtained the autoaggregation time (AT). Regardless of dilution, the obtained AT values were 2 hr, 4 hr, and 10 min in *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59, respectively,

Table 2. Surface characterization and adhesive properties of *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59

		P. farinosa SKM-1	*P. anomala* SKM-T	*G. geotrichum* SJM-59
CSH, %	Xylene	14.11±0.87 [a]	32.62±0.28 [b]	76.61±0.55 [C]
	Ethyl acetate	72.14±4.69 [a]	73.91±2.64 [a]	45.19±1.85 [b]
	Chloroform	79.27±0.94 [a]	88.72±0.73 [b]	31.83±3.47 [C]
Zeta potential, mV		-78.0±1.9 [a]	-53.32±1.8 [b]	0.27±0.06 [C]
ABC, μg/mg		14.51±7.09 [a]	53.60±2.09 [b]	47.57±9.46 [b]
Adhesion	Caco-2, %	6.86±0.44 [a]	9.20±0.41 [b]	10.19±0.38 [b]
	Murine intestine	+	+	+

CSH; cell surface hydrophobicity, ABC; alcian blue binding capacity.

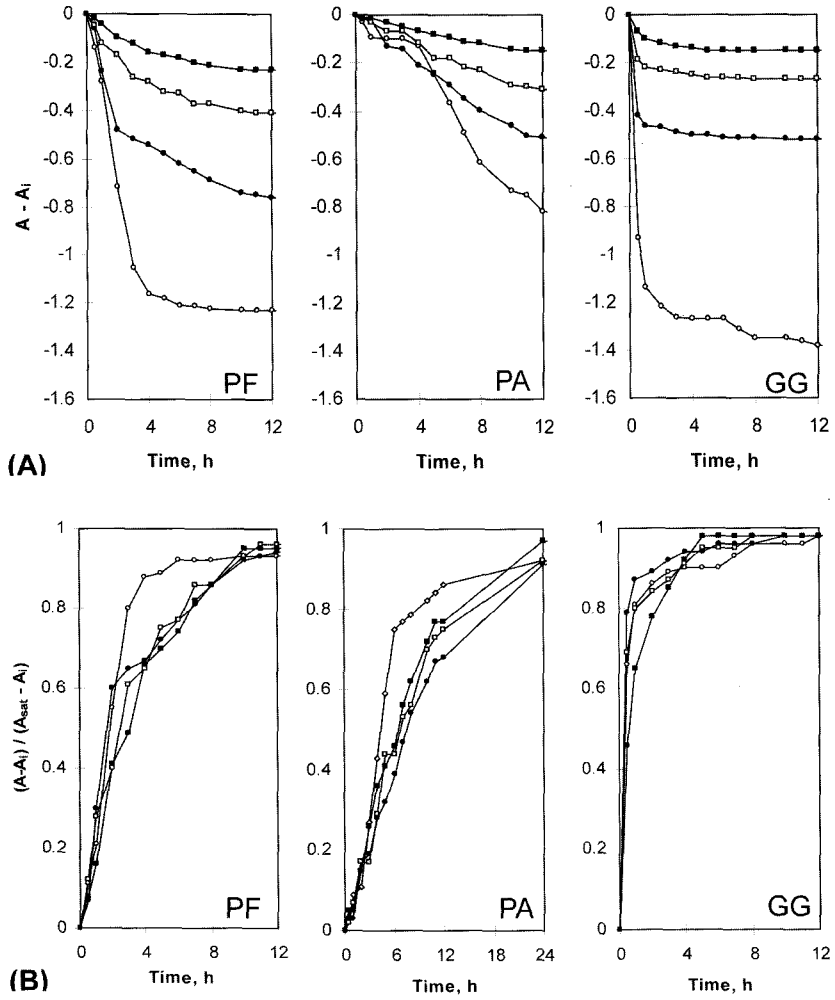

Fig. 1. (A) Sedimentation profile monitored at A_{600} for strain *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 grown in PDB. The assay was performed at four serial dilutions of single stock suspension. (B) Analysis of sedimentation curves. PF; *P. farinosa* SKM-1, PA; *P. anomala* SKM-T, GG; *G. geotrichum* SJM-59. -○-; stock solution, -●-; 1/2 dilution, -□-; 1/4 dilution, -■-; dilution.

suggesting that *P. farinosa* SKM-1 and *P. anomala* SKM-T were not autoaggregating strains, while *G. geotrichum* SJM-59 was. These results indicated that the rate of sedimentation depends on the initial density of yeast and the microbial surface electrostatic state. The decrease in electric repulsion could result in autoaggregation by allowing the approach to the hydrophobic surfaces, thus the hydrophobicity appears to be necessary for autoaggregation.

Adhesion to Caco-2 cell and murine intestinal surface *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 were examined for their abilities to adhere to polarized human intestinal epithelial Caco-2 cells. The number of yeast bound to Caco-2 cell cultures was directly related to the number of yeast added; however, the number of yeast bound did not increase linearly above 4×10^7 added cfu/well, an indication that the saturation level was reached (data not shown). All tested yeast strains showed adhesiveness regardless of

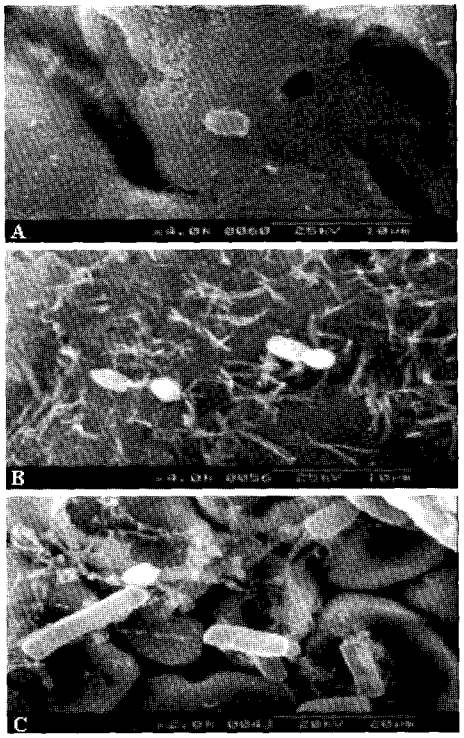

Fig. 2. Presumptive results of adhesion assays performed with strains *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 on murine intestine. A; *P. farinosa* SKM-1, B; *P. anomala* SKM-T, C; *G. geotrichum* SJM-59.

their surface hydrophobicity (Table 2), with *G. geotrichum* SJM-59 being the most adhesive strain; approximately 10% of the added yeast bound to the Caco-2 cell cultures. Adherence of *G. geotrichum* SJM-59 could not be reliably evaluated, because *G. geotrichum* SJM-59 formed clusters like biofilm (data not shown). On the average, approximately 1,000 yeasts per randomized microscopic fields were observed. *P. anomala* SKM-T adhered significantly better than *P. farinosa* SKM-1. No statistically significant differences were observed for the adhesion between *P. anomala* SKM-T and *G. geotrichum* SJM-59, although *G. geotrichum* SJM-59 showed higher hydrophobic value than that of *P. anomala* SKM-T. Cell surface hydrophobicity may therefore not be an appro-priate marker for potential adhering microorganisms.

Three different lyophilized yeasts were administered to mice for 4 weeks. Side effects, such as weight loss, fur and/or skin modification, activity change, and food intake change, were not inspected during the experimental period (Tables 3-4). The adhesions on to murine intestines of *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 were observed using scanning electron micrograph (Fig. 2). Examination of the adhered *P. farinosa* SKM-1 was difficult due to its poor adhesion, while *P. anomala* SKM-T and *G. geotrichum* SJM-59 showed good adhesion. The results of *in vivo* adhesion showed a tendency similar to that of Caco-2 cell adhesion.

Results of this study indicate that *P. farinosa* SKM-1, *P. anomala* SKM-T, and *G. geotrichum* SJM-59 are good candidates as new probiotic strains with the ability to adhere to Caco-2 cells and murine intestine regardless of their surface characteristics. The adhesive mechanisms and other probiotic properties of the tested strains, such as immune stimulation, antimicrobial production, and safety, are now under investigation.

Table 3. Effect of yeast consumption on body weight, food intake, and food efficiency

	Weight gain, g/day	Food intake, g/day	FER*
Control	0.28±0.001	3.65±0.260	7.63±1.95
P. fainosa SKM-1	0.22±0.101	3.62±0.370	6.14±2.80
P. anomala SKM-T	0.22±0.006	3.39±0.350	6.14±2.80
G. geotrichum SJM-59	0.21±0.004	3.49±0.340	5.92±1.32
p value	0.226	0.267	0.543

*; Food efficiency ratio = body weight / food intake

References

1. Fuller R. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378 (1989)
2. Lee YK, Salminen S. The coming age of probiotics. Trends Food Sci. Technol. 6: 241-245 (1995)
3. Moon JH, Om AS, Kwon HS, Kim YJ, Ji KE. Fermented product development of *Cyananchum wilfordii hemsley* by lactic acid bacteria and Bifidobacteria. Food Sci. Biotechnol. 13: 285-288 (2004)
4. Lee NK, Kim TH, Choi SY, Lee SK, Paik HD. Identification and probiotic properties of *Lactococcus lactis* NK 24 isolated from Jeot-gal, a Korean fermented food. Food Sci. Biotechnol. 13: 411-416 (2004)
5. Salminen S, Wright A, Morelli L, Marteau P, Brassaert D, Vos WM, Fond NR, Saxelin M, Collins K, Mogensen S, Birkeland E, Sandholm TM. Demonstration of safety of probiotics. Int. J. Food Microbiol. 44: 93-106 (1998)
6. Adams MR. Safety of industrial lactic acid bacteria. J. Biotechnol. 68: 171-178 (1999)
7. Isolaurie E, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilomi H. Lactobacillus casei GG reverse increased intestinal permeability induced cow milk in suckling rats. Gastroenterol. 105: 1643-1650 (1993)
8. Saalera M, Mogensen G, Fonden R, Matto J, Mattila-Sandholm T. Probitic bacteria: safety, functional and technological properties. J. Biotechnol. 84: 197-215 (2000)

Table 4. The weight of major organ from mice fed yeasts for 4 weeks

	Liver	Heart	Lung	Renal	Spleen
Control	1.98±.24	0.163±.001	0.21±.003	0.56±.001	0.093±.001
P. farinosa SKM-1	2.05±.39	0.150±.001	0.22±.001	0.55±.001	0.097±.002
P. anomala SKM-T	2.35±.54	0.153±.002	0.21±.001	0.52±.001	0.105±.025
G. geotrichum SJM-59	2.14±.39	0.160±.001	0.21±.001	0.53±.001	0.099±.001
p value	0.618	0.519	0.155	0.671	0.144

The organs were removed quickly, washed with cold saline, blotted dry on filter paper and then weighed (g).

9. Mo EK. Characterization and application of three probiotic yeasts form human feces. Ph.D. thesis, Chungnam Natl. Univ., Daejon, Korea (2003)
10. Odani T, Shimma Y, Wang XH, Jigami Y. Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the *MNN4* gene in *Saccharomyces cerevisiae*. FEBS Lett. 420: 186-190 (1997)
11. Bibiloni R, Perez PF, Garrote GL, Disalvo EA, De Antoni GL. Surface characterization and adhesive properties of Bifidobacteria. Methods Enzymol. 336: 411-427 (2001)
12. Institute of laboratory Animal Resources. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, D.C. (1996)
13. Hazen KC, Hazen BW. Surface hydrophobic and hydrophilic protein alterations in *Candida albicans*. FEMS Microbiol. Lett. 107: 83-88 (1993)
14. Reeves PG, Nielson FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A rodent diet. J. Nutr. 123, 1939-1951 (1993)