

Stimulatory effect of *Shoyu* polysaccharides from soy sauce on the intestinal immune system

HIROAKI MATSUSHITA¹, MAKIO KOBAYASHI¹, RYO-ICHI TSUKIYAMA¹,
MAYUMI FUJIMOTO², MAKOTO SUZUKI², KEISUKE TSUJI² and KENJI YAMAMOTO³

¹Research Laboratory, Higashimaru Shoyu Co. Ltd., Tatsuno, Hyogo 679-4167;

²School of Human Science and Environment, University of Hyogo, Shinzaike-honcho, Himeji, Hyogo 670-0092;

³Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Received March 24, 2008; Accepted May 5, 2008

DOI: 10.3892/ijmm_00000015

Abstract. Soy sauce (*Shoyu*) is a traditional Japanese fermented seasoning and is available worldwide. We investigated the effect of *Shoyu* polysaccharides (SPS) prepared from soy sauce on the intestinal immune system of mice. SPS enhanced the production of immunoglobulin A (IgA) from Peyer's patch cells *in vitro*, and its oral administration to 7-week-old male BALB/c mice for 2 weeks at a dose of 1.5 mg per day significantly ($p < 0.01$) increased the concentration of IgA in the intestine as compared to control mice. Furthermore, experiments on the intestinal transport of SPS *in vitro* using the human intestinal epithelial cell line Caco-2 confirmed the permeation of uronic acid to be time-dependent. In conclusion, SPS of soy sauce enhanced the production of IgA *in vitro* and *in vivo*, and the digested SPS might cross the enterocytic monolayer. Thus, soy sauce is a potentially promising food for enhancing host defenses.

Introduction

Soy sauce (*Shoyu*) is a traditional Japanese fermented seasoning available worldwide (1). In Japanese *shoyu*, soybeans and wheat are the two main raw materials used in almost the same quantity. Proteins of the raw materials are completely degraded into peptides and amino acids by microbial proteolytic enzymes after fermentation, and no allergens of the raw materials are present in soy sauce (2). In contrast, polysaccharides originating from the cell wall of soybeans are resistant to enzymatic hydrolysis. These polysaccharides are present in soy sauce even after fermentation and are termed *shoyu* polysaccharides (SPS) (3). Recently,

we reported that SPS exhibited potent anti-allergic effects (3), enhancing both macrophage and lymphocyte functions (4) and also iron absorption (5) *in vitro* and *in vivo*. Furthermore, oral supplementation of SPS was an effective intervention for patients with allergic rhinitis (6,7) and enhanced iron absorption in healthy women (5) in two double-blind placebo-controlled clinical studies.

The intestinal mucosa is the first line of host defense. Orally administered antigens interact with the gut-associated lymphoid tissue (GALT), which is a well-developed immune network that is involved not only in protection of the host from pathogens but also in preventing unusual reactions to ingested proteins. This immune response is mainly a humoral immune response mediated by immunoglobulin A (IgA)-producing cells and results in the secretion of IgA which constitutes almost 80% of all the antibodies produced in mucosal-associated tissue (8,9). This antibody inhibits microbial adherence and also prevents the absorption of antigens into mucosal surfaces (10).

Antigen uptake occurs through a specialized system represented by the M cells overlying Peyer's patch (PP) (11). PP is the major site in the intestine for inducing an active immune response between macrophages and dendritic cells and T and B lymphocytes. The activation of the oral immune system is clearly associated with activation of T cells in PP. Recently, the oral administration of certain lactic acid bacteria (12,13) and water-soluble dietary fibers such as glucomannan and pectin (14,15) has also been reported to enhance the mucosal IgA response.

The human intestinal epithelial cell line Caco-2 has been known to exhibit characteristics of both the small and large intestines and to express several carrier-mediated transport processes for amino acids, sugars, dipeptides, bile acids and nucleosides (16-18). This cell line forms confluent monolayers having the functional properties of transporting epithelia and has been widely used for studying drug transport mechanisms (19,20). Recently, we elucidated the biological activities of SPS after their oral administration (3-7), but the absorption characteristics of SPS are still unknown.

Therefore, in the present study, we examined the effect of SPS on the promotion of IgA production in PP cells and the intestinal transport of SPS using the Caco-2 human intestinal

Correspondence to: Dr Makio Kobayashi, Research Laboratory, Higashimaru Shoyu Co. Ltd., 100-3 Tominaga, Tatsuno-cho, Tatsuno, Hyogo 679-4167, Japan
E-mail: mkobayashi@higashimaru.co.jp

Key words: immunoglobulin A, Peyer's patch, Caco-2, *shoyu* polysaccharides, soy sauce

epithelial cell line in order to clarify the immunological function of SPS as a functional dietary component from soy sauce.

Materials and methods

Preparation of Shoyu polysaccharides. Soy sauce (*Shoyu*) was fermented by Higashimaru Shoyu Co., Ltd. (Tatsuno, Hyogo, Japan) as described previously (3). *Shoyu* polysaccharides (SPS) were prepared from the dialysate of soy sauce according to the method of Kikuchi and Sugimoto (21) as follows: 10 ml of soy sauce in seamless cellulose tubing (small size 18, pore size 25Å, molecular weight cut-off 12,000; Wako Pure Chemical Industries Ltd., Osaka, Japan) was dialyzed overnight in water at 4°C and then freeze-dried.

Animals. Male BALB/c mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and bred in our laboratories. They were housed, 5 per cage, and fed a basal diet, CE-2 (Clea Japan, Inc.) and water *ad libitum*, in an animal room under a 12 h light/dark cycle at a temperature of 24±1°C and a humidity of 60±5%. After a week of acclimation, they were used for the experiment.

Preparation of Peyer's patch (PP) cells. PP cells from male 8-week-old mice were prepared as described by Lim *et al.* (22). Mice were sacrificed by cervical dislocation, and the intestine from each mouse was carefully removed and trimmed free of PP cells. PP cells were placed in 5 ml of RPMI-1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) in a plastic dish. After being gently dispersed using a 5-ml sterile syringe plunger, the cell suspensions were passed through a 150-mesh nylon sieve and centrifuged at 600 x g for 5 min. The cell pellet obtained was washed with 10 ml of RPMI-1640 medium and then resuspended in 2 ml of RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 100 U/ml of penicillin (Sigma Chemical Co., St. Louis, MO, USA) and 100 µg/ml of streptomycin (Sigma Chemical Co.) which was termed RPMI-10% FBS. The viability of PP cells was >98% as determined with the dye exclusion test using trypan blue (Gibco BRL). The cells were cultured at 1x10⁶ cells/100 µl in 96-well culture plates (Nalge Nunc International, Roskilde, Denmark). Next, 100 µl of the test sample dissolved in RPMI-10% FBS was added to the cell suspension, and the mixture was incubated for 72 h in a humidified 5% CO₂ incubator (BNA-11, Tabai Espec Co., Osaka, Japan) at 37°C. The culture supernatant was then harvested to evaluate IgA levels. Lipopolysaccharide (LPS) from *Salmonella typhimurium* (IL7261, Sigma Chemical Co.) was used as a PP activator.

Measurement of total IgA. A sandwich ELISA was principally conducted using a modified version of the method of Williams *et al.* (23). First, 96-well culture plates were coated with 100 µl of goat anti-mouse IgA (α-chain-specific, 5 µg/ml, Southern Biotechnology Associates Inc., Birmingham, CA, USA) in 0.01 M carbonate-bicarbonate buffer (Sigma Chemical Co.), pH 9.6, for 1 h at 37°C. Then, after three

washes with TPBS [phosphate-buffered saline (PBS) containing 0.05% Tween-20], the plates were coated with 300 µl of bovine serum albumin in PBS for 1 h at 37°C and washed again with TPBS three times. Next, 50 µl of culture supernatant was added to each well, and the plates were incubated for 1 h at 37°C. After the plates were washed as above, 100 µl of horseradish peroxidase-conjugated goat anti-mouse IgA (α-chain-specific, 1 µg/ml) was added to each well. The plates were incubated for 1 h at 37°C and washed again. Finally, 100 µl of azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (0.6 mg/ml, Sigma Chemical Co.) in 0.2 M citrate buffer containing 0.006% hydrogen peroxide (pH 4.0) was added. After incubation at room temperature for 30 min, color development was halted by adding 100 µl of 1.5% oxalic acid dihydrate (Wako Pure Chemical Industries Ltd.). The absorbance was measured with a microplate reader (Fluostar Galaxy; BMG Labtechnologies Pty. Ltd., Victoria, Australia) at 492 nm. The results were expressed as micrograms of IgA/milliliter of supernatant, as calculated from a standard curve.

Feeding procedure. A total of 15 male BALB/c mice, 6 weeks old (weighing 20–23 g), were used for this experiment. After preliminary breeding for 4 days, the mice were divided into 3 groups of 5. Mice of the test group were fed *ad libitum* with a basal diet, CE-2, containing 0.01 or 0.05% (w/w) SPS for 2 weeks. It was estimated that the oral dose of SPS was approximately 0.3 or 1.5 mg per day per mouse. The control group was fed with the basal diet only. Mice were sacrificed on day 14, and the intestines were removed from each mouse.

Collection of intestinal fluid. The intestinal fluid was obtained at the end of the feeding period. The procedure for its collection was previously described by Lim *et al.* (22) with modifications. The intestine was carefully removed between the stomach-duodenum junction and the ileum ascending-colon junction. The intestinal contents were washed out using 1 ml of cold PBS (pH 7.2) and centrifuged at 2,000 x g for 30 min. The supernatant was collected for determination of IgA.

Measurement of the permeation of SPS through Caco-2 cell monolayers. The procedure used to measure the permeation of SPS through the Caco-2 cells was a modified version of that described by Hidalgo *et al.* (20). The Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% FBS, 1% non-essential amino acids (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin (10% FBS-DMEM) in a humidified 5% CO₂ incubator at 37°C. The Caco-2 cells were seeded at a density of 1.25x10⁶ cells/0.2 ml/well in a 6.5-mm-diameter membrane (0.4-mm pore size, 24-well plate; Transwell, Corning, NY, USA). The cells were used at 21 days after seeding. Transepithelial resistance was measured using Millicell ERS (Millipore, Eschborn, Germany), and a value >800 ohm x cm² was considered sufficient to study permeability. After washing with preheated 10% FBS-DMEM, 0.1 mg of SPS dissolved in 200 µl of 10% FBS-DMEM was added to the apical chamber (0.1 mg of SPS/well), and 700 µl of 10% FBS-DMEM was added to the

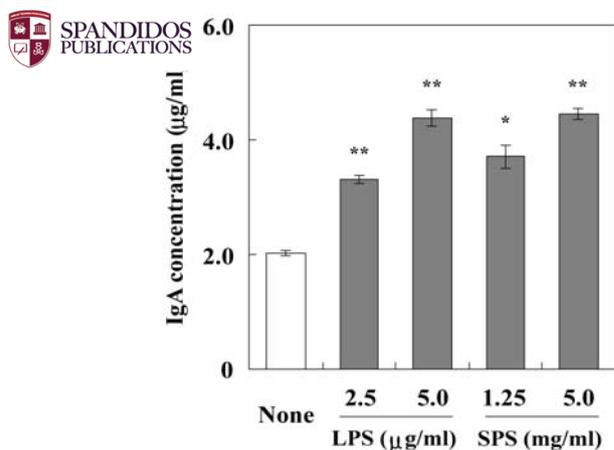


Figure 1. *In vitro* effect of SPS on IgA production in PP cells. For details concerning these experiments, see Materials and methods. LPS from the microbial cell wall was used as a positive control. The IgA concentrations in the culture supernatant were determined using the ELISA system. Each value is the average of triplicate cultures, and each bar indicates the mean \pm SE (n=5). Significantly different from the control (none) at * $p < 0.05$ or ** $p < 0.01$ (Dunnett's test). LPS, lipopolysaccharide from *Salmonella typhimurium*.

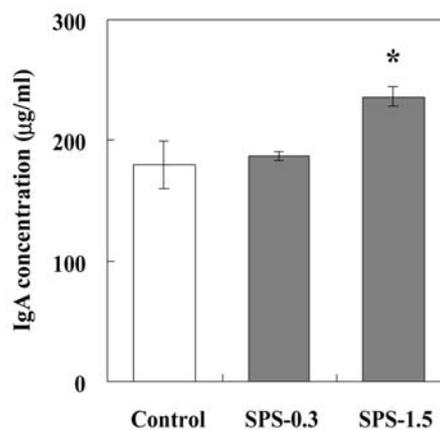


Figure 2. *In vivo* effect of SPS on production of intestinal secretory IgA. For details concerning these experiments see Materials and methods. The oral dose of SPS was approximately 0.3 (SPS-0.3) or 1.5 (SPS-1.5) mg per day per mouse. The IgA concentration in the intestinal contents was determined using the ELISA system. Each value is the average of triplicate cultures, and each bar indicates the mean \pm SE (n=5). Significantly different from the control (none) at * $p < 0.01$ (Dunnett's test).

basolateral chamber. The monolayers were incubated at 37°C for a period of 1, 3, or 6 h. At each fixed time, the solution on the apical side was harvested to determine the permeability to SPS. Permeation rates of SPS were calculated based on the decreased amount of uronic acid.

Data analysis. Permeation rates were calculated according to the following equation: Permeation rate (%) = 1-A/B, where A is the uronic acid concentration in the apical chamber, and B is the initial uronic acid concentration in the chamber.

Assay of uronic acids. The procedure used to determine concentrations of uronic acids was a modification of that described by Blumenkrantz and Asboe-Hansen (24). To 0.2 ml of the sample containing from 0.5 to 20 µg of uronic acid, 1.2 ml of sulfuric acid containing 0.0125 M tetraborate was added. The mixture was heated in a water bath at 100°C for 5 min. After cooling in ice water, 20 µl of 0.5% NaOH containing 0.15% of m-hydroxydiphenyl was added. The absorbance values were measured at 520 nm. The uronic acid content was determined using galacturonic acid (Nacalai Tesque Inc., Kyoto, Japan) as a standard.

Statistical analysis. Statistical analysis was performed using the Dunnett's test. In all cases, a p-value <0.05 was considered to be statistically significant.

Results

Activation of PP cells by the addition of SPS. Fig. 1 shows the effect of SPS on the production of IgA by PP cells of mice. LPS of the microbial cell wall, which is known to activate PP cells and to produce IgA, was used as a positive control sample in this experiment. According to the results, production increased significantly ($p < 0.05-0.01$) upon the addition of SPS, as it did in response to LPS. The increase seemed to be dependent on the concentration of SPS.

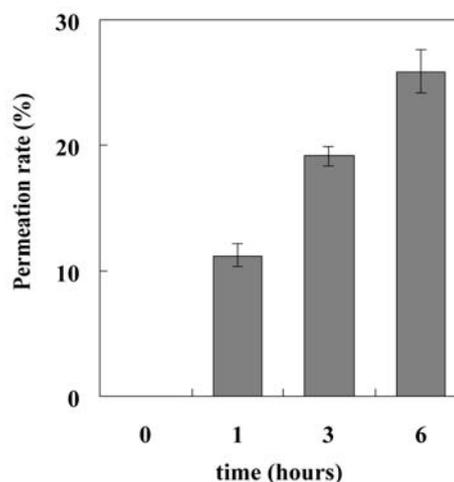


Figure 3. The permeability to SPS of Caco-2 cell monolayers. For details concerning these experiments, see Materials and methods. SPS was applied to the apical chamber. The samples were taken at 1, 3, and 6 h. At each fixed time, the solution on the apical side was harvested to determine permeability to SPS. Permeation rates were calculated according to the following equation: Permeation rate (%) = 1-A/B, where A is the uronic acid concentration in the apical chamber, and B is the initial uronic acid concentration in the chamber. Each bar indicates the mean \pm SE (n=4).

The effect of an oral dose of SPS on secretion of IgA in mouse intestines. The effect on the secretion of IgA in the intestines in mice fed SPS was estimated. The oral dose of SPS was approximately 0.3 or 1.5 mg per day per mouse, as the animals ingested about 3 g of the diet per day per mouse throughout the feeding period. There were no significant differences in body weight among the groups (data not shown). As shown in Fig. 2, the production of IgA was significantly ($p < 0.01$) greater in the SPS-1.5 group than in the control group.

The permeability to SPS of Caco-2 cell monolayers. Fig. 3 shows the time course of the apical to basolateral transport of

SPS in Caco-2 cell monolayers. The amount of uronic acid moving across Caco-2 cell monolayers increased with time for up to 6 h.

Discussion

The gastrointestinal tract is the most common site of entry for infectious agents at the mucosal surfaces. Secretory immunity is the basis for specific protection at the mucosal level, where IgA is the main immunoglobulin. Mucosal IgA prevents the adhesion of bacteria or viruses, and reduces the absorption of food antigens in the intestine (25-27). Yasui *et al* (28) reported that certain lactic acid bacteria which generate large quantities of IgA could prevent infections of the rotavirus. In the present study, we demonstrated that SPS of soy sauce enhanced IgA production in PP cells *in vitro*. In fact, compared to that of the control group of mice, the IgA concentration in the intestine of the mice fed SPS was significantly higher. These results suggest that SPS enhances IgA production through stimulation of the intestinal immune system and that SPS could be expected to prevent allergic reactions by interfering with the absorption of allergens and microbial infections. We previously reported that SPS exhibit strong anti-allergic activities (3), and this effect was exerted through the Th1/Th2 balance (4). In short, the results obtained in this study, together with our previous findings, confirm that SPS effectively enhances not only the systemic immune system but also the mucosal one, and provide new insight into the physiological role of SPS.

Next, we evaluated the absorption of SPS to epithelial cells using a Caco-2 cell model. It has been reported that Caco-2 cells undergo spontaneous differentiation in culture and exhibit the characteristics of mature enterocytes by forming polarized monolayers with a brush border and functionally competent tight junctions (20). Caco-2 cells can serve as a screen since compounds with high permeability in this model are typically well absorbed *in vivo* (29). Using this model, we showed that SPS might be transported across cell monolayers. This finding confirmed that intestinal absorption of uronic acid occurred and digested SPS was distributed within the body.

In conclusion, we demonstrated that SPS effectively enhanced mucosal IgA production. The results obtained here may provide new insight into the immunomodulating functions of SPS. Thus, soy sauce is a potentially promising food for enhancing host defenses.

Acknowledgements

The authors are grateful to Mr. Yoshimichi Hiramitsu, Dr Jun Wada and Mr. Ryuji Yamamoto (all from Kyoto University) for the technical suggestions and discussions. The authors are also grateful to Dr Kohei Ushio (Research Laboratory, Higashimaru Shoyu Co. Ltd.) for the critical reading of the manuscript and for helpful advice. This work was supported in part by a subsidy from the Ministry of Agriculture, Forestry and Fisheries of Japan (Program for new technology development to activate agriculture, forestry, fisheries and food industry by cooperating industry, academia and the government).

References

1. Yokotsuka T: Soy sauce biochemistry. *Adv Food Res* 30: 195-329, 1986.
2. Kobayashi M, Hashimoto Y, Taniuchi S and Tanabe S: Degradation of wheat allergen in Japanese soy sauce. *Int J Mol Med* 13: 821-827, 2004.
3. Kobayashi M, Matsushita H, Yoshida K, Tsukiyama R, Sugimura T and Yamamoto K: *In vitro* and *in vivo* anti-allergic activity of soy sauce. *Int J Mol Med* 14: 879-884, 2004.
4. Matsushita H, Kobayashi M, Tsukiyama R and Yamamoto K: *In vitro* and *in vivo* immunomodulating activities of *Shoyu* polysaccharides from soy sauce. *Int J Mol Med* 17: 905-909, 2006.
5. Kobayashi M, Nagatani Y, Magishi N, Tokuriki N, Nakata Y, Tsukiyama R, Imai H, Suzuki M, Saito M and Tsuji K: Promotive effect of *Shoyu* polysaccharides from soy sauce on iron absorption in animals and humans. *Int J Mol Med* 18: 1159-1163, 2006.
6. Kobayashi M, Matsushita H, Shioya I, Nagai M, Tsukiyama R, Saito M, Sugita T, Sugimura T and Yamamoto K: Quality of life improvement with soy sauce ingredients, *Shoyu* polysaccharides, in perennial allergic rhinitis: a double-blind placebo-controlled clinical study. *Int J Mol Med* 14: 885-889, 2004.
7. Kobayashi M, Matsushita H, Tsukiyama R, Saito M and Sugita T: *Shoyu* polysaccharides from soy sauce improve quality of life for patients with seasonal allergic rhinitis: a double-blind placebo-controlled clinical study. *Int J Mol Med* 15: 463-467, 2005.
8. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H and Zinkernagel RM: A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288: 2222-2226, 2000.
9. McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M and Kiyono H: The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* 10: 75-88, 1992.
10. Lamm ME: Interaction of antigens and antibodies at mucosal surfaces. *Annu Rev Microbiol* 51: 311-340, 1997.
11. Kraehenbuhl JP and Neutra MR: Epithelial M cells: differentiation and function. *Annu Rev Cell Dev Biol* 16: 301-332, 2000.
12. Perdigon G, Vintini E, Alvarez S, Medina M and Medici M: Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *J Dairy Sci* 82: 1108-1114, 1999.
13. Takahashi T, Nakagawa E, Nara T, Yajima T and Kuwata T: Effects of orally ingested *Bifidobacterium longum* on the mucosal IgA response of mice to dietary antigens. *Biosci Biotechnol Biochem* 62: 10-15, 1998.
14. Yamada K, Tokunaga Y, Ikeda A, Ohkura K, Kaku-Ohkura S, Mamiya S, Lim BO and Tachibana H: Effect of dietary fiber on the lipid metabolism and immune function of aged Sprague-Dawley rats. *Biosci Biotechnol Biochem* 67: 429-433, 2003.
15. Lim BO, Lee SH, Park DK and Choue RW: Effect of dietary pectin on the production of immunoglobulins and cytokines by mesenteric lymph node lymphocytes in mouse colitis induced with dextran sulfate sodium. *Biosci Biotechnol Biochem* 67: 1706-1712, 2003.
16. Blais A, Bissonnette P and Berteloot A: Common characteristics for Na⁺-dependent sugar transport in Caco-2 cells and human fetal colon. *J Membr Biol* 99: 113-125, 1987.
17. Chen J, Zhu Y and Hu M: Mechanisms and kinetics of uptake and efflux of L-methionine in an intestinal epithelial model (Caco-2). *J Nutr* 124: 1907-1916, 1994.
18. Hidalgo IJ and Borchardt RT: Transport of bile acids in a human intestinal epithelial cell line, Caco-2. *Biochem Biophys Acta* 1035: 97-103, 1990.
19. Pinto M, Robine-Leon S, Appay MD, Keding M, Triadou N, Dussaulex E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J and Zweibaum A: Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47: 323-330, 1983.
20. Hidalgo IJ, Raub TJ and Borchardt RT: Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96: 736-749, 1989.
21. Kikuchi T and Sugimoto H: Detailed structure of an acidic polysaccharide in soy sauce, confirmed by use of two kinds of purified pectinases. *Agric Biol Chem* 40: 87-92, 1976.
22. Lim TS, Messiha N and Watson RR: Immune components of the intestinal mucosae of ageing and protein deficient mice. *Immunology* 43: 401-407, 1981.



SPANDIDOS DJL, Newson J and Naessens J: Quantitation of bovine immunoglobulin isotypes and allotypes using monoclonal antibodies. *Vet Immunol Immunopathol* 24: 267-283, 1990.

24. Blumenkrantz N and Asboe-Hansen G: New method for quantitative determination of uronic acids. *Anal Biochem* 54: 484-489, 1973.
25. Andre C, Lambert R, Bazin H and Heremans JF: Interference of oral immunization with the intestinal absorption of heterologous albumin. *Eur J Immunol* 4: 701-704, 1974.
26. Silbart LK and Keren DF: Reduction of intestinal carcinogen absorption by carcinogen-specific secretory immunity. *Science* 243: 1462-1464, 1989.
27. Stokes CR, Soothill JF and Turner MW: Immune exclusion is a function of IgA. *Nature* 255: 745-746, 1975.
28. Yasui H, Kiyoshima J and Ushijima H: Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed *Bifidobacterium brebe* YIT4064. *J Infect Dis* 172: 403-409, 1995.
29. Balimane PV, Chong S and Morrison RA: Current methodologies used for evaluation of intestinal permeability and absorption. *J Pharmacol Toxicol Methods* 44: 301-312, 2000.