



New Japanese Yogurts Using Functional Probiotic Lactic Acid Bacteria and Future Strategies to Protect Human Gut

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Abstract: In Japan, milk and food companies have recently developed many functional yogurts using selected probiotic lactic acid bacteria (LAB) and *Bifidobacterium* strains. In 1991, the FOSHU (foods for specified health uses) system began instituted by the Ministry of Health, Labor and Welfare. In 2010, approximately 1,000 commercially available items were labeled as FOSHU products that are specially given brands showing good health effects for humans. Japanese commercial yogurts are classified into three type categories: fermented using selected probiotics, functional components added, and standard types. Many functions are considered in the selection of probiotics including competitive adhesion exclusion of enteric pathogens, cholesterol lowering effects, and positive immuno-modulatory effects. Adhesion to the human intestine is one of the most important characteristics of probiotic LAB and bifidobacteria. Our new screening system for adhesive activity involves a combination of three methods: the rat colonic mucin-micro plate assay, the Carnoy's histochemical staining method, and the carbohydrate probe binding assay. Recently, we developed a new screening assay using the BIACORE biosensor chip coated with human colon mucin (HCM). We found new LAB strains that recognize and specifically bind to the human ABO blood type antigens. We named these "blood type LAB". We identified the molecular LAB binding mechanism occurring in the human intestine through unique adhesin molecules that are expressed on the bacterial cell surface as lectin-like proteins. Bacteria with strong adhesion ability improve gastrointestinal health by continuous proliferation in the intestine; and increase the chance of intake into the Peyer's patches through M-cells that inhibit allergies because of LAB immune-stimulating activity. The future Japanese food market will contain superior functional yogurts containing adhesive effective probiotic LAB and bifidobacteria that are expected to be developed using our proposed mass screening system shown here.

Keywords: probiotics, lactic acid bacteria, blood type lactic acid bacteria, yogurt

Introduction

In Japan, milk and food companies have recently developed and sold many types of functional yogurts (fermented milk) using selected probiotics including beneficial bacteria such as lactic acid bacteria (LAB) and *Bifidobacterium* sp. In 1991, the Ministry of Health, Labor and Welfare developed FOSHU (foods for specified health uses) system, a pioneer in the world for functional foods. First, we would like to introduce briefly the FOSHU system and the representative yogurts. We wish to make choices for probiotics including LAB and bifidobacteria to construct functional yogurts where the me-

thod to select good LAB is being developed. We would like to introduce the history of our development of a mass screening method using a biosensor BIACORE to select probiotics with high binding activity in the human gut to maintain long term health effects. Recently, we proposed a new strategy for the application of our selected human blood type LAB against inflammatory bowel disease (IBD); and I would like to introduce this proposal briefly.

Functional yogurts in the Japanese FOSHU system

The concept of the foods for specified health uses (FOSHU) system developed by the Ministry of Health, Labor and Welfare was introduced in 1991 for the Japanese market. A FOSHU food is permitted to state a beneficial health claim if several strict requirements including human clinical trials are met through government review. The logo for FOSHU is allowed to be shown on the surface of the package (Fig. 1). At present (2011), 983 FOSHU foods are commercially available in Japanese food markets; they are specially labeled brands showing

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Fig. 1. Logo of FOSHU issued by the Japanese Government.

good health effects for humans. Evidence shows FOSHU foods decrease skyrocketing medical bills, preventing diseases such as diabetes, high blood pressure, high blood sugar, and colon cancers.

The FOSHU system is classified into eight categories as foods for: 1) improving intestinal disorders, 2) lowering high cholesterol, 3) lowering high blood pressure, 4) helping mineral adsorption, 5) overall health improvement, 6) increasing dental health (against dental caries), 7) reducing high blood sugar levels and 8) reducing high body fat levels (against obesity). Category 1 FOSHU contains oligosaccharides, yogurts using LAB or bifidobacteria and dietary fibers. In 2008, the FOSHU market size was 7,000 billion yen (93 billion US dollars) per year. Many Japanese personally feel disorder in gut conditions, because more than 50% of the Japanese FOSHU market size is occupied by Category 1 foods.

Japanese functional yogurts are classified into three groups: 1) functional LAB added, 2) functional added components and 3) the others.

Group 1 yogurts contain specially selected *Lactobacillus* (*Lb.*) and/or *Bifidobacterium* (*B.*) sp. as shown in Table 1. For instance, a functional yogurt “Onakani GG! LGG”, the first yogurt permitted as FOSHU, is produced using *Lb. rhamnosus* GG (LGG) by Takanashi Milk Products Co., Ltd.; permitted by a production license from Valio Co., Ltd., Finland. In every FOSHU yogurt, the registered strain number such as *B. longum* BB536, *Lb. gasseri* SP and *Lb. casei* YIT 9029 Shirota is important information as they are proprietary bacteria selected by each food company. The cost for obtaining a FOSHU brand is estimated to be approximately a billion yen (ca. 1.25 million US dollars) primarily because of obtaining costly human clinical data.

The best-selling functional yogurt in Japan is “Probio yogurt LG21” by Meiji Co., Ltd. with sales of more than 200 billion yen (ca. 2.6 billion US dollars) per year continuously for 10 years. The LG21 yogurt may prevent

Table 1. Probiotics used in Japanese functional yogurts

Genus	Species	Strain name
<i>Lactobacillus</i>	<i>acidophilus</i>	<u>CK92</u> , SBT-2062, L92, NCFM
	<i>gasseri</i>	OLL2716(LG21), <u>SP</u>
	<i>casei</i>	<u>NY1301</u> , YIT9029(Shirota)
	<i>fermentum</i>	KLD, RC-14
	<i>helveticus</i>	CK60
	<i>johnsonii</i>	La1
	<i>paracasei</i>	<u>LC1</u> , KW3110, CRL431, F19
	<i>plantarum</i>	299v
	<i>reuteri</i>	DS2112
	<i>rhamnosus</i>	<u>GG(LGG)</u> , 271, LB21
<i>Bifidobacterium</i>	<i>salivarius</i>	UCC118
	<i>breve</i>	Yakult
	<i>longum</i>	BB536, SBT2928
	<i>lactis</i>	<u>Bb-12</u> , <u>BB-12</u> , <u>FK120</u> , HNO19(DR10), <u>LKM512</u>

Underline shows the strain is permitted as FOSHU strain

gastric ulcers or stomach cancer by exclusion of *Helicobacter pylori* from the stomach of adults. The best-selling LG21 yogurt is ironically not FOSHU branded; however, many Japanese consumers are aware of the FOSHU brand when they purchase yogurts in food shops and supermarkets. A popular functional yogurt in the Japanese market with LG21, is “BIO” (“Activia” is the common name used in the US and EU) produced by Danon Co., Ltd. with many variation types. BIO contains *B. animalis* subs. *lactis* BE 80 isolated from animal intestines that may activate the peristaltic movement in the intestine to prevent constipation.

Among the Japanese marketed functional yogurts, the “synbiotic yogurts” such as “Bifiene” type M and S (FOSHU) produced by Yakult Honsha Co., Ltd. are becoming popular. These yogurts contain *B. breve* Yakult (probiotics) as 100 billion (M) and 300 billion (S) cells in a bottle with Galacto-oligosaccharide (GOS), a prebiotics. The big three FOSHU yogurts as plain types in the Japanese market are: “Megumi” by Megmilk Snow Brand Co., Ltd., “Bifidus yogurt” by Morinaga Milk Industry Co., Ltd., and “Bulgaria yogurt” by Meiji; they use probiotics *Lb. gasseri* SP+*Bifidobacterium* SP, *B. longum* BB536, and *Lb. bulgaricus* OLL2038+*Streptococcus thermophilus* OLL1131, respectively.

Group 2 yogurts contain functional food additive components and are popular in the Japanese market. For instance, “Lactoferrin 1000” made by Morinaga Milk Co., Ltd. contains more than 1,000 mg of bovine lactoferrin (bLf) that shows multifunctional bioactivities such as preventing ability of harmful bacteria that require Fe ions and increasing cell proliferation. “Honebuto yogurt” pro-

duced by Megmilk Snow Brand contains “MBP (milk basic proteins)” isolated from bovine milk to prevent osteoporosis. “Glucosamin Power yogurt” by Megmilk Snow Brand is a best-selling product through a home-delivery system especially for elderly persons. The yogurt contains more than 1.3 g of *N*-acetyl glucosamine (GlcNAc) that may relieve joint pain. Recently, “Dr. Piro” produced by Glico Dairy Products Co., Ltd. appeared in the Japanese food market by adding immunoglobulin Y (IgY) as urease (EC 3.5.1.5) antibody from chicken egg yolk that may prevent the adhesion of *H. pylori* to stomach mucosa or mucous membranes. In Korea, this functional yogurt with added anti-urease IgY was introduced in 2000; e.g., “Will” was produced by Korea Yakult Co. Ltd. and “Gut” by Maeil Milk Co., Ltd etc.

Group 3 contains the other products such as B.G.S., gamma aminobutyric acid (GABA), and lactotripeptides produced using LAB fermentation. The concept contained in the products is based on “Biogenetics” by Dr. T. Mitsuoka (Mitsuoka, 1998). “Onaka Katsuryoku (B.G.S.: Bifidogenic Growth Stimulator)” produced by Meiji contains 1,4-dihydroxy-2-naphtoic acid components (DHNA). DHNA is produced by *Propionibacterium* fermentation and stimulates *Bifidobacterium* proliferation as the Bifidus factor. “Pretia” (FOSHU) produced by Yakult contains γ -amino acid produced by *Lactococcus lactis* that may decrease blood pressure through the repression of adrenalin secretion. “Amiel S” (“Evolas” named in EU) produced by Calpis Co., Ltd. contains lactotripeptide including IPP and VPP produced by *Lb. helveticus* and may decrease blood pressure by repressing the activity of angiotensin-I converting enzyme.

There are more than 75 FOSHU yogurts at present and they are expected to become more popular in the future Japanese market. Please confer our review or articles of books (Saito, 2004, 2008).

Development of a new evaluation system for probiotics and discovery of a blood type binding LAB

Probiotics are defined as viable bacteria bringing positive health effects to the host by balancing the intestinal flora. Probiotics show three characteristics: 1) resistant to gastric acid, 2) resistant to bile acid, and 3) binding activity to human intestinal tracts. Among the 20 genera of LAB, lactobacilli isolated from the intestines of animals and humans are used as probiotics in functional fermented milk products; and bacteria belonging to the genera *Enterococcus*, *Streptococcus*, and *Lactococcus* have recently been proposed to be probiotics (Kimoto *et al.*,

1999; Ouwehand *et al.*, 2002).

Almost all harmful bacteria can bind to human intestinal mucosa using special adhesin, lectin-like proteins that are located on the top of pilli and bind the receptor sugar portion in human intestinal mucosa, glycoproteins or glycolipids. Adhesive activity to the human intestine is one of the most important characteristics of probiotic LAB and *Bifidobacterium* that show initial colonization and later proliferation in intestinal tract. To obtain positive immunological activity, incorporation of LAB from M cells, and later stimulation of the entire immune system, much depends on the adhesion ability because of the increased chance of incorporation. The mechanism by which *L. acidophilus* group LAB adheres to the human gastrointestinal tract, has been partially elucidated (Alejunga *et al.*, 1994; Coconnier *et al.*, 1992; Reid *et al.*, 1993). Mukai *et al.*, (1992) and Yamada *et al.* (1994) have revealed, from results of hemagglutination assay (HA), the presence of several lectin-like proteins on the cell surfaces of *L. acidophilus* group LAB.

Such proteinaceous components in the SLP are thought to contribute to cell adhesion through their binding to carbohydrate portions of the colonic mucus layer. Morata de Ambrosini *et al.* (1999) reported that lectin-like surface molecules of *L. casei* CRL 431 stimulated the immune system.

Previously, we developed an evaluation system using rat colon mucosa (RCM) isolated from rat colons (using 400-500 rats) as a replacement for the human colon mucosa (HCM), because their chemical structures resemble HCM (Saito, 2004).

Our initial screening system involved a combination of methods using three steps: 1) the rat colonic mucin-micro plate assay, 2) the Carnoy’s histochemical staining method, and 3) the carbohydrate probe binding assay (Fig. 2).

First step

Eight acidic and neutral *O*-glycosidic (mucin-type) sugar chains that combine with RCM were used (Slominany *et al.*, 1980). The chemical structures of RCM are similar to the structures of those isolated from HCM (Podolsky, 1985). We showed the conventional hemagglutination (HA) test is not suitable for selecting LAB with strong adhesion to the human intestinal tract. We introduced a new screening method using polystyrene beads coated with RCM (Takahashi *et al.*, 1996); and later a modified screening method using RCM-coated microtiter plates without non-specific reaction improving the blocking con-

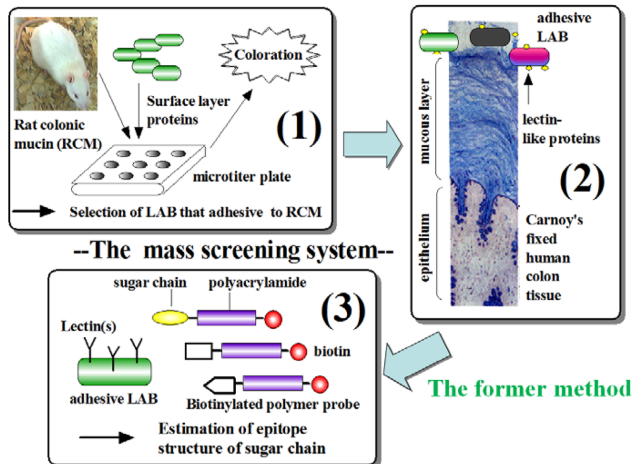


Fig. 2. A proposed mass screening system for probiotic LAB with high adhesion to human intestine. RCM, rat colonic mucin; SLP, surface layer proteins; LAB, lactic acid bacteria (Saito, 2004).

ditions (Matsumura *et al.*, 1999). The method was shown to be useful as a first step in mass screening the adhesiveness of LAB to the human intestinal tract.

Second step

Surface layer proteins (SLP) were prepared from cells of LAB selected by the first mass screening step. The binding of lectin-like protein(s) in SLP to the colonic mucous layer was confirmed using histochemical staining using human colon tissues fixed with Carnoy's fixative (Takahashi *et al.*, 1996).

Third step

We proposed a new method to select LAB using a commercial biotinylated carbohydrate probe that has the representative partial structures of the sugar chains constituting HCM (Saito *et al.*, 2000). High levels of reactivity were detected in 19 strains with a trisaccharide probe that has an A-antigen structure [GalNAc α 1-3(Fuc α 1-2)Gal β 1-]. The adhesion evaluation score proposed was also considered to be useful as an indicator for the final selection of a probiotic LAB.

In the literature, a binding assay using Caco-2 cell line (human epithelial colorectal adenocarcinoma) after co-incubation with testing bacteria was examined (Lebeer *et al.*, 2012; Maccaferri *et al.*, 2012). The method is difficult where control of binding cell numbers by washing and the results tend to be different depending on each researcher. Also, Caco-2 is a cell line that has some sugar chains with abnormal chemical structures. Therefore, we did not use the Caco-2 cell line. Further, the chemical structures

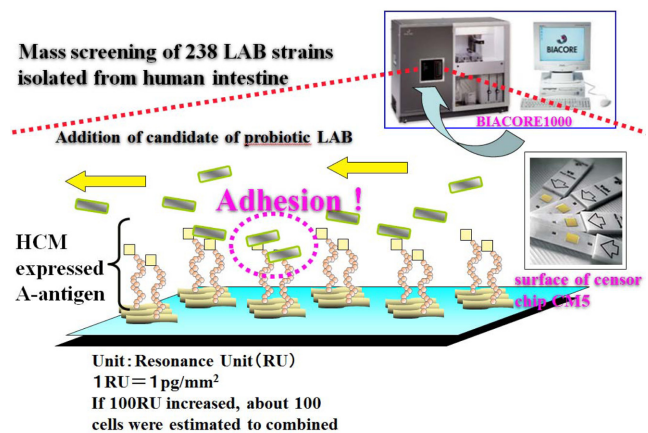


Fig. 3. Sensor chip CM5 coated with human colonic mucin (HCM) for the selection of bacteria with high adhesion activity to human intestine using BIACORE. RU, resonance unit (Uchida *et al.*, 2004).

of sugar chains on colon mucin comparing rat and human are not the same. Later, we used real human mucosa prepared from the normal portion of the human colon obtained during colon cancer surgery through the help of our university's hospital.

Recently, we developed a new screening assay using the biosensor BIACORE 1000 utilizing surface layer plasmon resonance (PSR) with HCM as shown in Fig. 3 (Uchida *et al.*, 2004, 2006a). We performed a mass-screening determining the adhesive activity of 238 LAB strains isolated from the human intestine. We used HCM-A, B and O (BSA-A, B and H; BP-probe A, B and H) individually as ligands to the surface of carboxyl-dextran coated on a sensor chip type CM5. We injected bacterial cells suspended in HBS-EP buffer after lyophilization as the analyte. The sensor chip is considered a para-human intestinal tract and the BIACORE analysis system with the sensor chip also was considered to be a para-colon environment maintained at 37°C under anaerobic conditions. We first developed the ideal evaluation system to select probiotics with strong binding activity to human mucosa using BIACORE. As the system works automatically and the door of the machine is never opened after beginning the experiment, the data cannot be changed unlike the human error that can be introduced in the Caco-2 analysis. From the comparison with the resonance units (RU) on the sensor-gram between human ABO blood type A antigen and B antigen, we found the "blood type LAB" which can recognize the trisaccharide structure and specifically bind to HCM-A stronger than to HCM-B as shown in Fig. 4 (Uchida *et al.*, 2006a, 2006b). We named

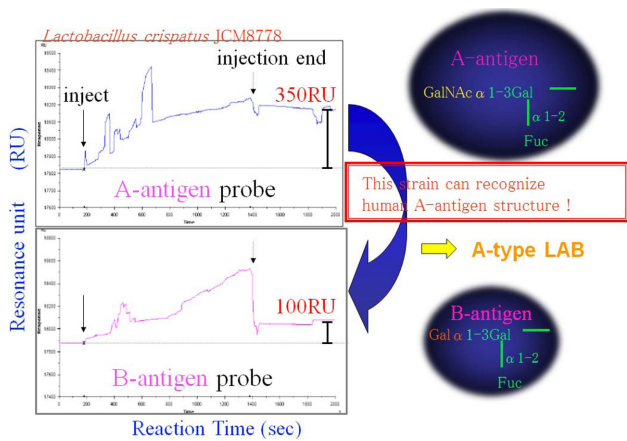


Fig. 4. Probiotic LAB combines to the human A-antigen structure (Uchida *et al.*, 2006).

the bacterium as an “A-type LAB” which can combine through recognition of human A-type antigen structure. Later, we discovered many strains of B-type LAB and O-type LAB strains in our culture collection of probiotics isolated from the human intestine.

Evaluation system for LAB against pollen allergy

The number of patients having pollen allergy has increased in Japan especially among the younger generation. DNA fragments from the genome DNA of strains of LAB were estimated for mitogenic activity using the spleen cells of mouse. In 1995, Krieg *et al.* reported that CpG 1826 (TCCATGACGTCCTGACGTT) prepared from the DNA of *Escherichia coli* showed a strong immune-stimulating activity in spleen cells from mice or rabbits (Table 2). We found many types of CpG and AT motifs as immune-stimulative DNA motifs (ODN) in probiotics such as *L. rhamnosus* GG (LGG), *B. longum* and *L. gasseri*; and dairy LAB such as *L. bulgaricus* and *Str. thermophilus*. We constructed swine TLR9 transfectants using

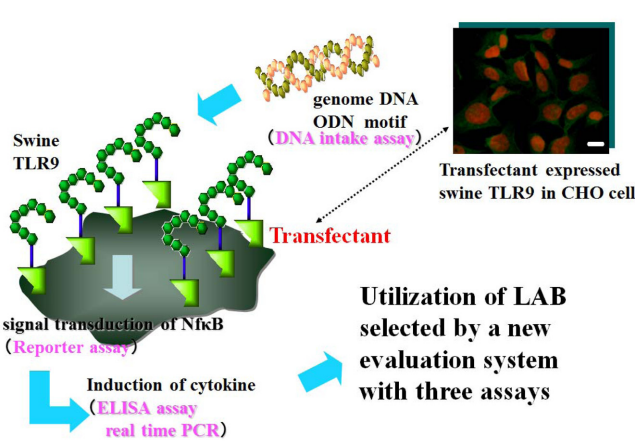


Fig. 5. Establishing a new evaluation system of immune stimulation with transfectant expressing swine TLR9 on the cell surface (Shimosato *et al.*, 2003).

HEK 293 cells and CHO K-1 cells after introducing the expression vector inserting of the structural gene of swine TLR9 (3,093 bp., 1,030 aa., 115.9 kDa) (Shimosato *et al.*, 2003, 2004). The expression of TLR9 on the surface of transfectants was confirmed using anti-swine TLR9 antibody. Finally, we established a new evaluation system with transfectants of swine TLR9 containing three steps: 1) DNA intake assay, 2) reporter assay by determining signal transduction of NF-κB, and 3) ELISA assay or real time PCR to determine induction of cytokines such as IL-12 as shown in Fig. 5.

Adhesins of the blood type LABs and their strategy for survival in the human intestine

We isolated and identified several adhesins and adhesin-like proteins from blood type LABs that specifically combine with HCM. After extraction of the surface layer proteins (SLP) from cells of the A-type LAB with 1-3 M guanidine hydrochloride (GHC1) solution, the adhesin was

Table 2. Immunostimulatory DNA motif (ODN) isolated from genome DNA of several bacteria

Name of ODN motif	Base sequence ('5- -3')	Originated bacteria	Main mammals operated
Probiotics			
ID35	ACT <u>TTTCGTTT</u> TCTGCGTCAA	<i>L. rhamnosus</i> GG (LGG)	human, pig, mouse
BL-7	GCGTCGG <u>TTTCG</u> GTGCTCAC	<i>B. longum</i> BB536	human, mouse
AT5AC-L*	TATA <u>ATTTTT</u> ACCAACTAGC	<i>L. gasseri</i> JCM1131 ^T	human, pig
LGAT243*	TTAACA <u>ATTTTT</u> ACCCAAGA	<i>L. gasseri</i> OLL2716 (LG21)	human, pig
Dairy LAB			
OLLB-7	CGGC <u>ACG</u> CTC <u>ACG</u> ATTCTTG	<i>L. bulgaricus</i> NIAIB6	human, pig, mouse
MsST	CAGGACGTTGTATCACTGAA	<i>Str. thermophilus</i> ATCC19258 lacZ	human, pig
The others			
CpG1826	TCCATG <u>ACG</u> TCCTG <u>ACG</u> TT	<i>Escherichia coli</i>	mouse

* shows AT motif which does not contain C and G in the stimulative sequences. No * shows CpG motif. Underlined letter sequence means the part which confirmed the immunostimulative activity.

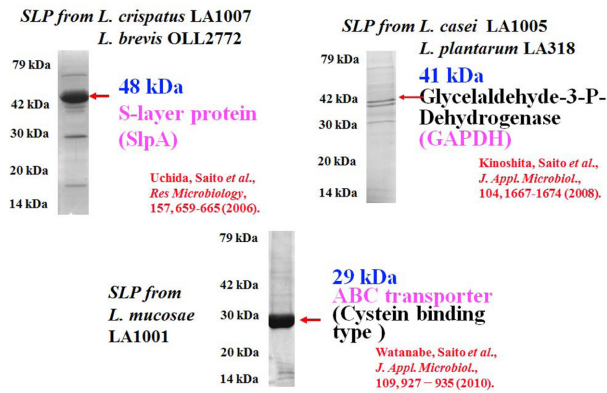


Fig. 6. Three representative adhesins isolated from the cell surface of blood type LAB that specifically combine to blood type antigens in human colonic mucin.

isolated from SLP with the ligand fishing method using PVDF membrane covered with BSA-A (human ABO blood type A antigen on bovine serum albumin). In *L. crispatus* LA1007 and *L. brevis* OLL2772 (A-type LAB), the primary component was identified in the “S-layer protein (SlpA)” after cloning as a lectin-like protein at 48 kDa (Uchida *et al.*, 2006a) as shown in Fig. 6. In *L. casei* LA1005 and *L. plantarum* LA318 (A-type LAB), the primary component was identified as a “glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)” after cloning as a lectin-like protein at 41 kDa (Kinoshita *et al.*, 2008). In *L. mucosae* LA1001 (A-type LAB), the primary component was identified as the “ABC transporter Cys binding type” after cloning as the lectin-like protein at 29 kDa (Watanabe *et al.*, 2010). We also identified other lectin-like proteins, enolase and elongation factor Tu (EF-Tu) from the SLP of A-type LAB (data not shown).

Recently, Katakura *et al.* (2010) reported the SLP prepared from *Lactococcus lactis* IL1403 contained 16 proteins and all were identified as cytoplasmic proteins which recognize yeast mannan. The reason why these proteins including heat shock protein DnaK, GAPDH, 30S ribosomal protein S1, EF-Tu and others exist on the surface is they may be adhesins binding to sugar portions of glycoconjugates and can serve for interaction with another microorganism such as LAB or yeast. In *Lactobacillus* sp. LA1007 (A-type LAB), three lectin-like proteins (SlpA+GAPDH+ABC transporter) were expressed in SLP at the same time as adhesins (data not shown). Probiotic LABs may combine to the sugar portions of HCM using several adhesins and compete with harmful bacteria in human intestines. Although each adhesin of A-type LAB may be weak, the binding ability as the total sum may become very strong and can compete with or overcome harmful

bacteria in the human intestinal environment.

Development of new functional yogurts

In 2009, Turnbaugh *et al.* reported obesity is associated with phylum-level changes in the microbiota and reduced bacterial diversity (Nature, 2009). In 2010, Vijay-Kumar *et al.* reported some intestinal bacteria may cause obesity in rats (Vijay-Kumar *et al.*, 2010). We believe obesity is a disorder caused by a bacterial imbalance between good and harmful bacteria. In 2011, Kadooka *et al.* reported the regulation of abdominal adiposity is possible using probiotic *L. gasseri* SBT2055 in rats with obesity tendencies. A yogurt named “Free yogurt Gasseri SP LAB” using this strain is now commercially available in Japan that may reduce obesity.

In 2011, Nagai *et al.* reported *L. bulgaricus* OLL2073 R-1 producing an extra-cellular polysaccharide (EPS) reduced the infection rate of influenza virus through the activation of NK cells. The yogurt named “R-1 yogurt” using the strain is being sold in Japan that may prevent influenza and respiratory infections.

Recently, we began investigation for the prevention of ulcerative colitis (UC) using probiotics. Although the reason for UC remains unknown, medical researchers estimate that some harmful intestinal bacteria such as *Fusobacterium* (*F.*) *varium* are candidates for causing UC. *F. varium* produces butyric acid inducing apoptosis and triggers the crisis of inflammation through the expression of cytokines such as IL-8 leading to UC (Ohkusa *et al.*, 2003). In the preliminary competitive inhibition experiment, the representative harmful bacteria such as *Staphylococcus aureus* and *Escherichia coli* isolated from human

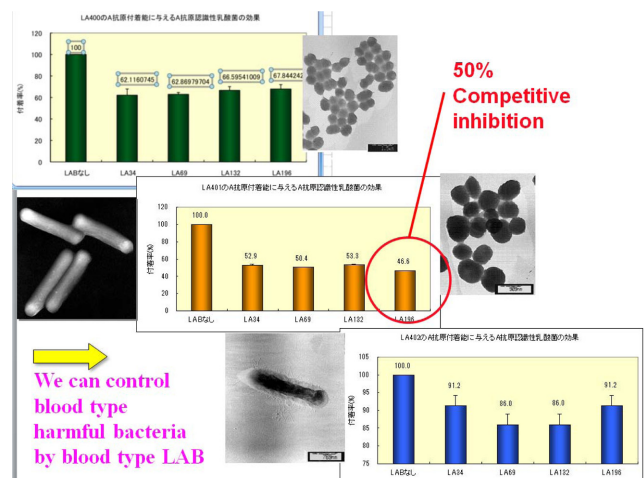


Fig. 7. Competitive inhibition for the adhesion of harmful bacteria by selected blood type LAB on human colonic mucin (HCM).

intestine were inhibited the adhesion to HCM by selected A-type LAB (Fig. 7). We found strains of *F. varium* isolated from the inflammatory part of a UC patient showed high affinity to A/B-type antigens of HCM. As UC usually climaxes with inflammation in the rectum and sigmoid colon, we therefore selected strains of *Bifidobacterium* with strong binding activity to HCM-A from our culture collection using the BIACORE assay. In the preliminary competitive inhibition experiment, two strains of *F. varium* with strong binding activity to HCM-A were inhibited using the A-type *Bifidobacterium* strains with the micro plate assay. The exclusion experiment for *F. varium* using A-type *Bifidobacterium* is getting good results (data not shown). In the very near the future, we will be able to propose a new functional yogurt against UC.

Conclusion

In 2010, some *Bacteroides* sp. with porphyranases were discovered in Japanese gut microbiota that may be transferred from marine bacteria *Porphyra* spp. (Nori) (Hehemann *et al.*, 2010). The reason is speculated to be that Japanese eat seaweeds (NORI) frequently and the gene coding porphyranases must be needed to digest nutritional polysaccharides in Nori. The report suggested to us that some unique and valuable bacteria must exist in human populations of different countries. Although Asians look at the research of the Western countries, we had better pay attention to our countries. Therefore, we hope to collect more information about unique microbiota in Asian environments including animal and human intestines and share the results for each through exchanges with Korean Society for LAB (KSLAB), Japan Society for LAB (JSLAB) and Asian Federation of Society for LAB (AFSLAB).

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