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NOTE

Comparison of Bifidobacteria Selective Media for the Detection of Bifidobacteria in Korean Commercial Fermented Milk Products

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Abstract

This study was carried out to compare the efficacy and selectivity of TOS and BS media for enumeration of bifidobacteria in commercial fermented milk products. First, bifidobacteria was isolated from 20 fermented milk products, and all isolated bifidobacteria were identified by genomic technology as *Bifidobacterium lactis*. The two media significantly differed from each other with regard to the recovery of *B. lactis*, that is, the recovery of this organism was as much as 6 logs lower on BS medium than on TOS. When the concentration of BS solution (mixture of paromomycin sulfate, neomycin, sodium propionate, and lithium chloride) used in BS medium was reduced to 50% (BS50), a relatively high percentage recovery of bifidobacteria from pure cultures was achieved. Susceptibility tests to antibiotics and tests for selective agents for the isolated bifidobacteria and lactic acid bacteria were conducted. The BS solution inhibited some lactic acid bacteria and *Bifidobacterium* species, while mupirocin (MU) suppressed the growth of all tested lactic acid bacteria but not *Bifidobacterium*. As compared with BS50 medium, TOS with or without MU showed good bifidobacteria recovery and readily distinguishable colonies; in particular, TOS supplemented with MU had a high selectivity for bifidobacteria. In conclusion, all results suggested that TOS medium with or without MU was found to be suitable for selective enumeration of bifidobacteria from mixed cultures in fermented milk, and better in that capacity than BS medium.

Key words: TOS medium, bifidobacteria, fermented milk, BS medium, selective enumeration

Introduction

The increasing interest of health care and the desire for improving quality of life are driving factors for research and development of functional foods. Among a number of functional compounds so far, bioactive compounds from fermented foods and probiotics still take the center stage due to their long tradition of safe use, and established and postulated beneficial effects (Vasiljevic and Shah, 2008).

A number of probiotic cultures have been exploited extensively by the dairy industry as a tool for the development of novel functional products, but the main species believed to have probiotic characteristics are *L. acidophilus, L. casei* and *Bifidobacterium* species (Shah, 2007; Vasiljevic and Shah, 2008).

It is claimed that a high number of bifidobacteria in the colon is positive for human health. A high number of bifidobacteria may prevent colonization of pathogens, and may have positive effects on intestinal peristalsis, the immune system, cancer prevention, cholesterol metabolism and carbohydrate metabolism in the colon (Hartemink *et al*., 1996).

Fermented dairy products are generally considered to be one of the most suitable vehicles for the administration of an adequate number of probiotics including bifidobacteria to the consumer (Van de Casteele *et al*., 2006). As there is growing interest in using bifidobacteria as probiotics as well as in the use of bifidogenic factors, bifidobacteria are usually used in combination with other lactic acid bacteria, such as, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for health benefits and ease of manufactures.

Since the products are prepared from many different strains, it was required a selective enumeration of different microbial species (Camaschella *et al*., 1998). In practice, the differential enumeration of bifidobacteria is difficult to achieve due to the presence of multiple species of lactic acid bacteria in products (Van de Casteele *et al*., 2006). Therefore, it is necessary to have a medium

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that selectively promotes the growth of bifidobacteria, whereas other bacteria growth is suppressed (Hartemink *et al*., 1996). To increase the selectivity and enumeration of bifidobacteria, antibiotics such as neomycin, kanamycin or nalidixic acid and other factors (lithium chloride, sodium propionate and propionic acid) inhibiting the growth of other lactic acid bacteria, as well as component which improve the growth of bifidobacteria, for example riboflavin, nitrogenous base and carbohydrates such as raffinose, lactose and oligosaccharides are required (Biavati and Matarelli, 2005; Lim *et al*., 1995; Tanak and Mutai, 1980). Several culture media have been developed and evaluated for the selective enumeration of bifidobacteria (Hartemink *et al*., 1996; Ji *et al*., 1994; Lee *et al*., 1994; Lim *et al*., 1995; Martineau, 1999; Nebra and Blanch, 1999; Roy, 2001; Shin *et al*., 1994; Silvi *et al*., 1996; Tanaka and Mutai, 1980; Van de Casteele *et al*., 2006). Of them, BS medium is most commonly used medium for detection of bifidobacteria in Korea. However, the problems of BS media, as many researchers investigated (Ji *et al*., 1994; Lee *et al*., 1994; Tanaka and Mutai, 1980), are either not sufficiently selective for bifidobacteria or inhibit bifidobacteria growth resulting in inaccurate low counts. Recently the use of TOS agar has been increased in Europe and Japan. TOS agar, originally developed by the Japanese Association of Fermented Milk Drinks, is designed for use with dairy products and is easy to prepare and stable, and uses galactooilgosaccharides as a bifidobacteria carbon source (IDF, 2007). Oligosaccharides are used as bifidogenic factors to stimulate the growth of bifidobacteria. It is assumed that these carbohydrates are selectively fermented by bifidobacteria and not by lactic acid bacteria (Roy, 2001). International Dairy Federation (IDF) confirmed that TOS agar was highly suited for the enumeration of *Bifidobacterium*.

The aim of this study was to evaluate the selectivity, efficacy and performance of TOS medium for complete recovery of viable bifidobacteria in comparison to BS medium. Also the sensitivity to antibiotics and selective agents used in TOS and BS agar of isolated bifidobacteria and lactic acid bacteria from commercial fermented milk products was investigated.

Materials and Methods

Sample collection

Before collecting samples, fermented milk products, which were claimed to contain bifidobacteria, were investigated. A total of 20 yogurt products (12 drinkable and 8 spoonable) were selected and purchased from markets in Seoul from March to April 2009, and stored at 5°C prior to examination. The list of products is shown in Table 1. None of them had exceeded their expiry date.

Table 1. The starter cultures and probiotics used in Korean commercial fermented milk products

Brand Code	Company Code	*Str. thermophilus*	*Lb. bulgaricus*	*Lb. acidophilus*	*Lc. lactis*	*Lb. casei*	*Bifidobacterium*
DY-01	A	O		O		O	O
DY-02	A	O	O	O			O
DY-03	A	O	O	O			O
DY-04	A					O	O
DY-05	B	O	O				O
DY-06	C	O		O			O
DY-07	D	O		O			O
DY-08	E	O	O	O			O
DY-09	F	O	O				O
DY-10	G	O	O				O
DY-11	G					O	O
DY-12	H	O		O			O
SY-01	A	O		O			O
SY-02	H	O	O	O			O
SY-03	G	O	O				O
SY-04	D	O	O				O
SY-05	E	O		O			O
SY-06	E	O		O			O
SY-07	I	O	O				O
SY-08	J	O	O		O		O

Table 2. The list of type strains and commercial strains

Species	Strain	Source	Species	Strain	Source
Lb. acidophilus	NCFM	KCTC	*Str. thermophilus*	Commercial	Products
Lb. acidophilus	ATCC4356	KCTC	*Str. thermophilus*	Commercial	Products
Lb. acidophilus	Commercial	Products	*Bif. adolescentis*	ATCC15703	KCTC
Lb. casei	Commercial	Products	*Bif. angulactum*	ATCC27535	KCTC
Lb. rhamnous	Commercial	Products	*Bif. breve*	ATCC15700	KCTC
Lb. bulgaricus	Commercial	Products	*Bif. infantis*	ATCC15697	KCTC
Lb. plantarum	KCTC3099	KCTC	*Bif. longum*	Commercial	Products
Lc. lactis	ATCC7962	KCTC	*Bif. longum*	ATCC15707	KCTC
Lc. lactis	Commercial	Products	*Bif. lactis*	Commercial	Products
Str. thermophilus	Commercial	Products	*Bif. lactis*	Commercial	Products

KCTC: Korea Collection for Type Culture
ATCC: America Type Culture Collection

Bacterial strains

Type and commercial strains used in this study and their origins were listed in Table 2. Nine type strains have been stored in Danone Korea R&I Center after obtaining from Korea Collection for Type Culture (Seoul, Korea), and 9 commercial strains were isolated from commercial fermented milk products. Two type strains, *Bif. Lactis*, BB-12 and DN-173010 were supplied by Christian Hansen (Hoersholm, Denmark).

Media preparation

All products were examined using selective media under standardized cultivation conditions. Each medium was prepared by following the methods provided by the manufacturer or authors (Silvi *et al*., 1996; Temmerman *et al*., 2003). For the preparation of BS agar, blood glucose-liver (BL) agar (Difco, Sparks, MD, USA) was used as a basal medium and BS solution was added to the basal medium. BS solution was composed of sodium propionate (Junsei Chemical Co., Tokyo, Japan), paromomycin sulfate (Sigma-Aldrich Co., Steinheim, Germany), neomycin (Sigma-Aldrich Co.) and lithium chloride (Oriental Chemical Ind., Seoul, Korea). TOS agar (Yakult Pharmaceutical Co., Tokyo, Japan) was used without antibiotics or modified by supplementation with mupirocin (MU)-lithium (USP, Rockville, MD, USA) which was labeled as TOS-MU. Table 3 shows the composition of the media.

Table 3. Composition of the three selective media

	g/L
BS agar	
BL agar	58
BS solution	
Sodium propionate	15
Paromomycin sulfate	0.05
Neomycin	0.1
Lithium chloride	3
TOS agar	
Peptone	10
Yeast extract	1
KH_2PO_4	3
K_2HPO_4	4.8
$(NH_4)_2SO_4$	3
$MgSO_4$	0.2
L-cysteine	0.5
Sodium propionate	15
Galacto-oligosaccharide	10
Agar	15
TOS-MU agar	
TOS agar	62.5
Mupirocin lithium	0.05

Enumeration

We made a series of 10-fold dilution (10^{-1}-10^{-7}) of each yogurt products with the 0.85% saline solution and, 0.1 mL samples of the 10^{-4} to 10^{-7} was spread onto a bifidobacteria selective plate media, TOS agar, TOS-MU agar and BS agar with the aid of sterile T-shaped plastic rods (SPL Life Science, Seoul, Korea). After inoculation, the plates were anaerobically incubated at 37ºC for 3 days by using anaerobic jars and AnaeroPack® (Anaero-3.5L, Mitsubishi Gas Chemical Co., Tokyo, Japan).

Isolation and identification of bifidobacteria from commercial yogurt products

Bifidobacteria from commercial fermented milk products was isolated from the colonies grown on the surface of TOS-MU agar, Gram stain reaction, cell morphology, carbohydrate fermentation patterns and genus-specific 16S rRNA gene sequencing analysis.

Cell morphology was observed using a microscope

(Olympus BX 51TF, Tokyo, Japan) after Gram staining. Carbohydrate fermentation patterns were determined using API 50 CHL test (BioMerieux, Marcy L Etoile, France) and we followed the manufacturer's instructions.

Identification of bifidobacteria using 16S rRNA gene analysis

Colonies were picked up with a sterilized toothpick, suspended in 0.5 mL of sterilizes saline in a 1.5 mL centrifuge tube and then centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 mL of InstaGene Matrix (Bio-Rad, Hercules, CA, USA). Incubated at 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant can be used for PCR.

For PCR, added 1 μL of template DNA in 20 μL of PCR reaction solution. Used 27F/1492R primers (Table 4) for bacteria, and then performed 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments were amplified in the case of bacteria, including a positive control (*E. coli* genomic DNA) and a negative control in the PCR. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore, Billerica, MA, USA).

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described in Table 4. Sequencing was performed by using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, Foster City, CA, USA). Sequencing products were resolved on an Applied Biosystems Model 3730XL Automated DNA Sequencing System (Applied BioSystems, Foster City, CA, USA) at the Macrogen, Inc. (Korea).

Susceptibility against antibiotics and selective agents of isolated bifidobacteria

At least one isolate per identified species recovered from a given product was tested for resistance against the selected antibiotics and selective agents including sodium propionate, 50 mL solution (mixture of 50 mg paromomycin sulfate, 100 mg neomycin, 3 g lithium chloride and 15 g sodium propionate), MU-lithium and sodium propionate with addition of 0.05% MU-lithium. The inoculums were prepared by suspending cultures grown in TPY broth. Cell density after collecting from TPY was checked using UV spectrophotometer (UV mini 1240, Shimadzu, Tokyo, Japan). The suspension (0.1 mL) was spread evenly on the BL agar. An 8 mm paper disc (Toyo Roshi Kaisha Ltd., Tokyo, Japan) was placed on the agar surface, and then 50 μL of each antibiotics or selective agents was inoculated. The plates for bifidobacteria and lactic acid bacteria were incubated under anaerobic conditions and aerobic conditions, respectively at 37°C for 72 h. The susceptibility to each antibiotics or selective agent was determined by measuring the diameter of clear zone formed around 8 mm paper.

Table 4. Primers for amplification and sequencing of 16S rRNA gene

		amplification	sequencing
27F	AGA GTT TGA TCM TGG CTC AG	●	
1492R	TAC GGY TAC CTT GTT ACG ACT T	●	
518F	CCA GCA GCC GCG GTA ATA CG		●
800R	TAC CAG GGT ATC TAA TCC		●

Results and Discussion

Isolation and identification of bifidobacteria from commercial yogurt products

All commercial fermented milk products tested in this study claimed to contain bifidobacteria. As shown in Table 5, no colonies on TOS medium were detected in DY-11 when commercial yogurt products were inoculated on TOS medium. The colonies isolated from TOS medium was identified by observation of its morphology on the plates, and then confirmed by observation of cell morphology via optical microscopy after Gram staining; large and round colonies, and Gram positive polymorphic cells. All colonies isolated from commercial fermented milk products showed same carbohydrate fermentation patterns by the commercial kit API 50 CHL. The results of Gram staining, cell morphology and carbohydrate patterns confirmed the colonies isolated on TOS medium as bifidobacteria species. All presumptive bifidobacteria strains were identified as *Bif. lactis* by genomic identification using 16s rRNA gene analysis. It was reported that *Bif. lactis* has been recently applied in fermented dairy products instead of other bifidobacteria species due to its better technological characteristics (Collado *et al*., 2005).

Comparison of viable cell counts of bifidobacteria isolated from commercial yogurt products

Several media have been developed for the differential enumeration of the bifidobacteria in the fermented dairy products. BS medium, originally developed by Mitsuoka, is widely used for the recovery and enumeration of bifidobacteria by researchers and quality control laboratories in Korea. BS medium is also an official agar for enumer-

Table 5. Identification of bifidobacteria isolated from Korean commercial fermented milk products

Brand Code	Company Code	TOS-Mu selection	Morphology	API 50CHL	Homology
DY-01	A	O	O	O	100%, *Bif. lactis* DSM10140
DY-02	A	O	O	O	100%, *Bif. lactis* DSM10140
DY-03	A	O	O	O	100%, *Bif. lactis* DSM10140
DY-04	A	O	O	O	99%, *Bif. lactis* DSM10140
DY-05	B	O	O	O	99%, *Bif. lactis* DSM10140
DY-06	C	O	O	O	100%, *Bif. lactis* DSM10140
DY-07	D	O	O	O	99%, *Bif. lactis* DSM10140
DY-08	E	O	O	O	100%, *Bif. lactis* DSM10140
DY-09	F	O	O	O	99%, *Bif. lactis* DSM10140
DY-10	G	O	O	O	100%, *Bif. lactis* DSM10140
DY-11	G	Not detected	-	-	-
DY-12	H	O	O	O	100%, *Bif. lactis* DSM10140
SY-01	A	O	O	O	100%, *Bif. lactis* DSM10140
SY-02	H	O	O	O	100%, *Bif. lactis* DSM10140
SY-03	G	O	O	O	100%, *Bif. lactis* DSM10140
SY-04	D	O	O	O	99%, *Bif. lactis* DSM10140
SY-05	E	O	O	O	100%, *Bif. lactis* DSM10140
SY-06	E	O	O	O	100%, *Bif. lactis* DSM10140
SY-07	I	O	O	O	99%, *Bif. lactis* DSM10140
SY-08	J	O	O	O	100%, *Bif. lactis* DSM10140

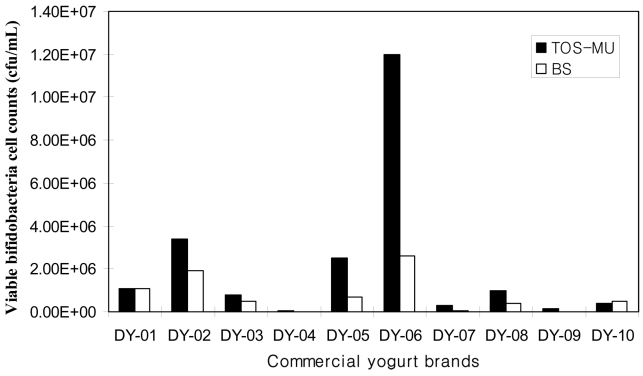

Fig. 1. Comparison of bifidobacteria recovery on BS and TOS-MU media.

ation of bifidobacteria under Korean Food Code. However, we found poor recovery of bifidobacteria on BS medium. As comparing with TOS-MU medium, the bifidobacteria recovery on BS medium was 13-100% depending on the fermented milk product brands (Fig. 1). As antibiotics are used as selective agents in BS medium, they might have affected the growth of bifidobacteria. Several researches (Ji *et al*., 1994; Lee *et al*., 1994; Silvi *et al*., 1996; Tanaka and Mutai, 1980) also have reported that BS media was not fully selective and showed lower recovery of bifidobacteria than other selective media, that is, BS medium inhibited the growth not only of other lactic acid bacteria but also of bifidobacteria. Therefore, the counts obtained on BS agar may not be representative of the bifidobacteria present in the commercial products, which consequently suggests a need to check the antibiotics concentration of BS solution and efficacy of BS medium with pure cultures before the medium is adopted for enumeration purposes.

Re-establishment of the antibiotic concentration of BS medium

For the right selection of optimal concentration of BS solution, the effect of antibiotic (BS solution) concentration on the bifidobacteria recovery on BS agar was determined using pure cultures of two *Bifidobacterium lactis* strains, BB-12 and DN-173010. BS solution with various concentrations (10-100%) was added into BS medium.

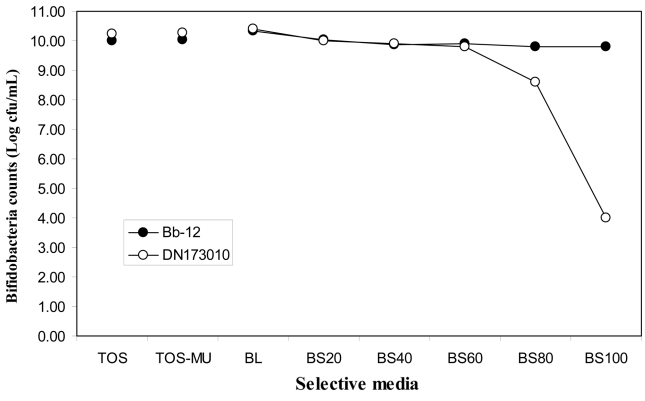

Fig. 2. Effect of antibiotic (BS solution) concentrations in BS medium on bifidobacteria recovery as compared with bifidobacteria recovery on other selective (TOS and TOS-MU) and non-selective (BL) media.

The recovery of bifidobacteria on BS medium was compared with other selective media (TOS, TOS-MU) and non-selective medium (BL agar). As show in Fig. 2, TOS, TOS-MU and BL media showed good recovery of both BB-12 and DN-17010 strains. However, these two strains showed completely different results on BS agar. BB-12 strain was hardly influenced by the BS solution concentration whereas DN-173010 strain was highly affected by the high concentration of BS solution added into BS medium. Therefore, it could be said that the susceptibility to specific antibiotics was strain-specific not species-specific. When 100% BS solution was used, the viable cell count of DN-173010 strain was only 4 log cycle. However, bifidobacteria recovery on BS medium with lower concentrations of BS solution (<60%) was comparable with those on other media. For this reason, the concentration of BS solution was reduced to 50% in BS medium, which was labeled as BS 50 in the following study.

Comparison of susceptibility against antibiotics and selective agents of isolated bifidobacteria

Most fermented milk products contain bifidobacteria in combination with other lactic acid bacteria. The appearance of colonies of other lactic acid bacteria may influence the bifidobacteria counting, which consequently may cause under-estimation of the counts. In order to reach selectivity and efficacy of selective medium for bifidobacteria when mixed cultures are present, several antibiotics and ingredients are used to suppress the growth of other lactic acid bacteria as well as to support the bifidobacteria growth. A test on susceptibility of bifidobacteria strains and other lactic acid bacteria strains to antibiotics and selective agents used in BS and TOS media was conducted in this study. Several antibiotics and selective agents are also used in BS and TOS media: paromomycin, neomycin, lithium chloride, propionic acid and MU-lithium. As bifidobacteria may be stimulated by short-chain fatty acids, like propionate and butyrate, several bifidobacterial selective media have used propionate alone or in combination with lithium chloride (Hartemink *et al.*, 1996). Lithium chloride is commonly used for the selective isolation of bifidobacteria (Roy, 2001). Paromomycin, neomycin and MU are antibiotics to suppress other bacteria growth. Neomycin sulphate is included as growth inhibitors of Gram-positive rods (Van de Casteels *et al.*, 2006).

As it was indicated that some of these agents may affect the bifidobacteria growth in the previous study, susceptibility of bifidobacteria and lactic acid bacteria to antibiotics and selective agents were investigated (Table 6). It

Table 6. The susceptibility of type strains and commercial strains of bifidobacteria and lactic acid bacteria to the tested antibiotics and selective agents.

Species	Strain code	Clear zone (mm)									
		BS solution (mg/L)				Mu-lithium (mg/L)			Mu-lithium + sodium propionate (mg/L)		
		145,200	72,600	36,300	18,150	50	25	12.5	15,050	7,525	3,763
Lb. acidophilus	NCFM	28	18	10	-	28	24	21	26	25	15
Lb. acidophilus	ATCC4356	31	26	-	-	34	32	28	30	28	15
Lb. acidophilus	Commercial	28	16	-	-	24	22	16	30	26	15
Lb. casei	Commercial	26	20	18	16	30	24	18	28	24	8
Lb. rhamnosus	Commercial	14	14	12	10	16	10	-	21	18	10
Lb. bulgaricus	Commercial	22	16	14	-	10	8	8	15	8	8
Lb. plantarum	KCTC3099	20	16	13	12	-	-	-	10	8	-
Lc. lactis	ATCC7962	23	18	16	15	20	18	14	21	14	13
Lc. lactis	Commercial	28	26	22	-	14	14	12	18	16	12
Str. thermophilus	Commercial	15	12	-	-	30	24	18	26	20	16
Str. thermophilus	Commercial	16	13	-	-	26	24	22	32	24	16
Str. thermophilus	Commercial	22	18	14	-	28	24	18	26	22	18
Bif. adolescentis	ATCC15703	12	10	-	-	-	-	-	-	-	-
Bif. angulactum	ATCC27535	-	-	-	-	-	-	-	-	-	-
Bif. breve	ATCC15700	-	-	-	-	-	-	-	-	-	-
Bif. infantis	ATCC15697	16	13	10	-	-	-	-	-	-	-
Bif. longum	Commercial	-	-	-	-	-	-	-	-	-	-
Bif. longum	ATCC15707	25	16	12	10	-	-	-	-	-	-
Bif. lactis	Commercial	12	10	-	-	-	-	-	-	-	-
Bif. lactis	Commercial	-	-	-	-	-	-	-	-	-	-

was reported that low propionate concentration enhanced the growth of bifidobacteria and lactic acid bacteria but high concentration of propionate (20-25 g/L) had inhibitory effects on *Lactobacillus* species (Hartemink *et al*., 1996). However, there were no marked inhibitory effects of propionate on all tested lactic acid bacteria except *Str. thermophilus* isolated from a commercial fermented milk product (data not shown).

High concentration of BS solution had inhibitory effects on not only lactic acid bacteria but also on some bifidobacteria species. However, *Lb. acidophilus, Lb. bulgaricus* and *Str. thermophilus*, the most commonly used starter cultures or probiotics in fermented milk products, had relatively high resistance on standard concentration of BS solution (18,150 mg/L). The inhibitory effect of BS solution on bifidobateria may be mainly due to antibiotics added in the selective media. Some researches indicated that propionic acid did not inhibit the bifidobacteria growth, but neomycin and paromomycin suppressed the growth of some bifidobacteria species, even though bifidobacteria strains showed different antibiotic susceptibility (Ji *et al*., 1994; Shin *et al*., 1994).

As MU was discovered as a selective agent suppressing the growth of commonly used lactic acid bacteria but did not affect bificobacterial growth, it was considered as a relevant compound for a selective medium (IDF, 2007). In this study, MU-lithium also showed highly inhibitory effects on all tested strains of *Lb.acidophilus, Lb. casei, Lc. lactis*, and *Str. thermophilus*, but the level of resistance varied. On the other hand, bifidobacteria species and *Lb. plantarum* had high resistance to MU-lithium. It was reported that the addition of MU at levels as high as 100 μg/mL was found to suppress the growth of lactic acid bacteria (Thitaram *et al*., 2005). However, reducing the concentration of MU to12.5 μg/mL resulted in highly inhibitory effect to lactic acid bacteria. When MU-lithium was used in combination with propionic acid, it had an inhibitory effect to *Lb. plantarum* which had high resistance to sole use of MU-lithium. With these results, it was indicated that MU was an effective antibiotic for using in a bifidobacteria selective medium and its concentration could be reduced than standard dosage.

Comparison of efficacy and selectivity of selective media

It has been suggested that bifidobacteria and other lactic acid bacteria should reach the intestine alive and in sufficient numbers to adhere, implant or multiply in the intestinal tract (Collado *et al*., 2005). Therefore, it is important for manufacturers and retailers to be able to confirm the viable counts of bifidobacteria and probiotics in fermented milk products until the consumption of products. Recommended lower limit of IDF for bifidobacteria counts in dairy product is 10^6 CFU/mL at the time of consumption of strain added to the product (Payne *et al*., 1999; Roy, 2001; Vlkova *et al*., 2004).

As shown in Fig. 3, the bifidobacteria counts in products tested varied from 10^4 to 10^7 CFU/mL, and SY-08 showed outstanding bifidobacteria count, 10^8 CFU/mL which is about 10 times higher than bifidobacteria counts in other samples. Bifidobacteria was not detected in DY-11 and SY-01 even though these products claimed to contain bifidobacteria. The low number of bifidobacteria cells contained in some products (DY-01, DY-04, DY-09, DY-10, DY12 and SY-02) could limit their probiotic effect because the viable bifidobacteria was lower than 10^6 CFU/mL. Generally bifidobacteria showed poor viability in fermented dairy products and various studies had indicated that not all probiotic products contain the recommended levels of viable microorganisms (Vlkova *et al*., 2004).

As three selective media were compared in this study, BS50 showed up to one log reduction in bifidobacteria colony count. Other researchers (Ji *et al*., 1994; Lee *et al*., 1994) also observed poor growth of bifidobacteria on BS medium. However, the recovery of bifidobacteria on BS50 medium was much improved in comparison with BS medium supplemented with 100% BS solution.

The size and appearance of the colonies may influence the counting. In particular, pin point colonies usually may cause under-estimation of the counts (IDF, 2007). Bifidobacteria formed tiny colonies on BS50 medium on which large number of pin points were also observed. Therefore, it was difficult to count bifidobacteria, as some colonies of bifidobacteria were not distinguishable with the colonies of other lactic acid bacteria. Silvy *et al*. (1996) also showed that BS medium allowed the growth of other bacteria species. With these results, it was indicated that the efficacy for bifidobacteria enumeration increased but selectivity decreased when the concentration of BS solution was reduced to 50% in BS medium.

On the other hand, bifidobacteira formed well-developed colonies on TOS and TOS-MU media. According to Thitaram *et al*. (2005), the colony size of bifidobacteria observed from TOS medium supplemented with MU was clearly larger than that of TOS medium without MU supplementation. But in this study, the colonies of bifidobacteria from TOS medium regardless of MU supplementation

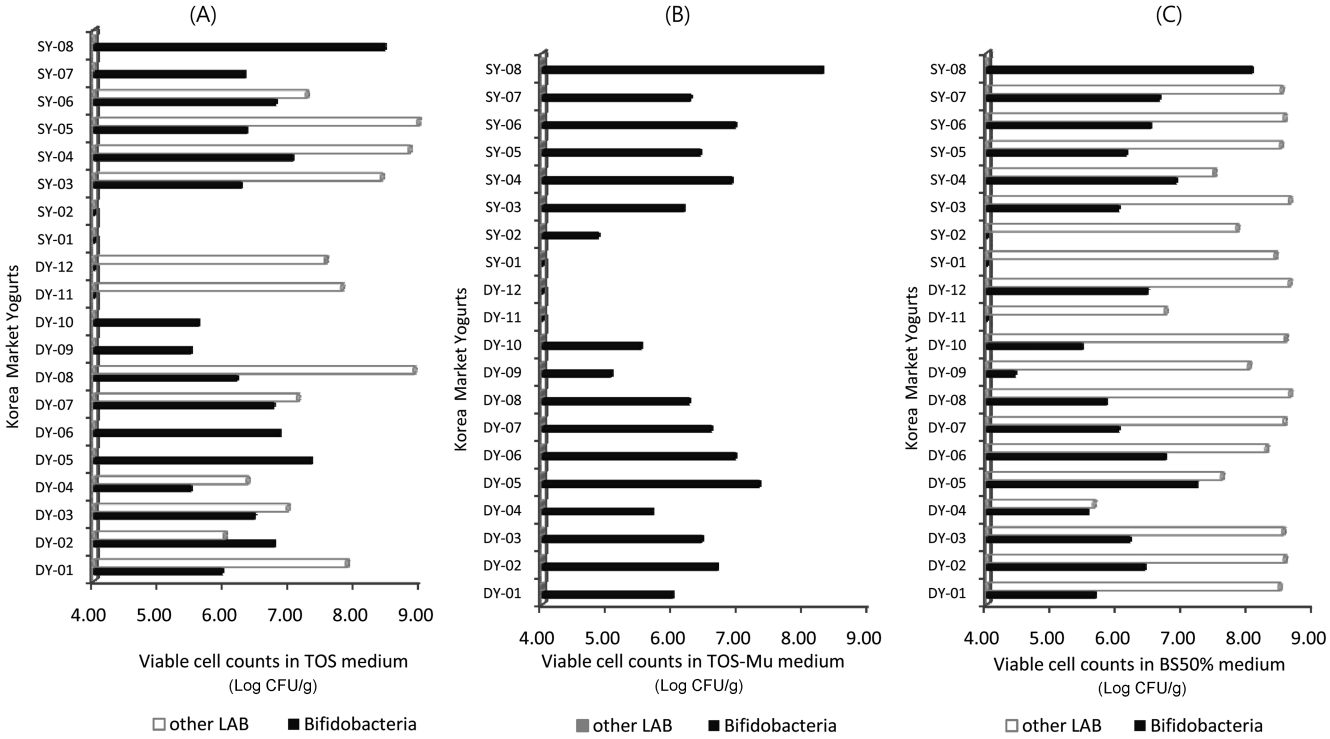

Fig. 3. Comparison of the recovery of bifidobacteria and other lactic acid bacteria on three selective media. A: TOS medium, B: TOS-MU medium, C: BS medium.

had large size and were distinguishable with those of other lactic acid bacteria. The selectivity of MU was also confirmed in a series of tests carried out by eight laboratories participated in the IDF working group in 2003, in which faster individual growth, bigger bifidobacterial colonies and higher colony counts were resulted from using TOS-MU than counter medium (IDF, 2007).

Without MU, pin points on the TOS agar plates in some products were observed, whereas no other lactic acid bacteria were grown on TOS-MU. It was reported that TOS medium supplemented with low MU concentration resulted in inconsistent selectivity against non-bifidobacteria species, whereas TOS supplemented with 50 μg/mL MU showed a high selectivity and readily distinguishable presumptive colony morphology of bifidobacteria species from fermented milk (Thitaram *et al*., 2005). Nonetheless, TOS medium without MU can be used for enumeration of bifidobacteria depending on the MU susceptibility of other lactic acid bacteria used in conjunction with bifidobacteria in the fermented milk, because not all the products tested showed pin points on the TOS medium.

In conclusion, a TOS-MU medium was found to be highly selective and suitable for isolation and enumeration of bifidobacteria in dairy products which contain other lactic acid bacteria. TOS without MU also showed high efficiency on bifidobacteria recovery and relatively high selectivity depending on the MU sensitivity of lactic acid bacteria strains. BS medium has been considered as suitable media for the selection of bifidobacteria. However, our data on selective enumeration and susceptibility to antibiotics indicated that BS50 medium gave better recovery of bifidobacteria than BS100 medium, but did not suppress the growth of other lactic acid bacteria used in the commercial fermented milk products. Therefore, TOS with or without MU can be strongly recommended for selective enumeration of bifidobacteria in fermented milk products as proved in this study and supported by other research results.

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