주요 개념 : 유산균, 황색포도알균,
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Selection of *Lactobacillus* Species Inhibiting Enteropathogenic Bacteria and Potential Use as Probiotics[†]

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장내세균의 발육억제 효과로 정한 *Lactobacillus* 균주 선정과 Probiotic으로서의 이용가능성

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Abstract

목적 : 본 연구에서는 한국인 소아의 침 및 성인의 질부로부터 분리한 1367개의 유산 생성 균주 중 4가지의 장내 병원성 세균 ; Methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157에 대해 발육억제 효과를 나타내는 유산 균주를 분리하고 분리된 균주의 Probiotic으로서의 가능성을 조사하고자 하였다.

방법 : 네 가지 병원성 세균을 대상으로 agar spot test와 Catalse test를 시행하여 1367개 균주 중 최종적

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인 실험대상 균주를 선정하였다. 최종 선정된 유산균과 네 가지 병원성 세균을 혼합 배양하여 병원성 세균증
식 억제 효과를 보았다. 또한 최종균주의 probiotic으로서의 가능성을 확인하기 위해 균을 동정하고 내산성
등 몇 가지 특성을 관찰하였다.

　　결과 : Agar spot test 결과 전체의 15.7%에 해당하는 215 균주가 MRSA에 대해 억제 효과를 나타내었으며
215 균주 중 9.8%에 해당하는 21 균주가 *L. monocytogenes*에도 동시에 억제효과를 나타냈다. 이들 균주 중
가장 탁월한 효과를 보인 Lb 1250을 실험 대상 균주로 선정하였다. Lb (*Lactobacillus*) 1250과 상술한 병원성
세균을 각각 혼합 배양하면 Lb 1250 존재 하에서 MRSA의 증식은 10^4배 억제되었으며, *L. monocytogenes*는
10^3배의 억제 효과를 보였다. 그러나 *S. typhimurium*, *E. coli* O157에 대해서는 아주 미약한 효과를 나타냈다.
Lb 1250의 몇 가지 특성을 조사한 결과 *Lactobacillus delbrueckii* subsp. *delbrueckii*로 동정되었으며, 이는
H_2O_2를 잘 생산해 내는 균주였다. 또한 이 균주는 우유에서 curd를 약하게 생성하며 이 curd의 향은 달콤
하고 최종 pH가 4.6까지 감소하였으며, pH 2와 같은 강산의 조건하에서 배양한 결과 2시간 후에도 상당한
생존도를 유지하였다.

　　결론 : 이상의 결과에서 Lb 1250은 병원성 세균의 성장을 억제함을 관찰하였고, probiotic으로서의 이용가능
성을 확인하였다.

I. INTRODUCTION

Lactobacilli (Lb) are the predominant normal flora of the human body which play an important physiological role in the maintenance of the ecological balance (Spiegel, 1991). They produce lactic acid responsible for low pH values and also produce many inhibitory substances such as hydrogen peroxide (McGroarty, 1993), various organic acids and bacteriocin-like substances that are active against certain pathogens (Muriana, & Klaenhammer, 1991). The mechanisms proposed for this inhibition are steric hindrance (Chan, Reid, Irvin, Bruce, & Costerton, 1985), competition of nutrients (Freter, Brickner, Botney, Cleven, & Aranki, 1983), stimulation of the immune system (Perdigon, Nader de Macias, Alvarez, Oliver, & Pesce de Ruiz Holgado,

1988) or microbial antagonism (Baker, 1980).

Recently, there has been a growing tendency to use certain products that have proven to be beneficial for both human and animal health. These products, defined as probiotics by Havenaar, Brink, & Veld. (1992) are viable cultures of one or several microorganisms that, when administered to human or animals, produce a beneficial effect on the host, by fulfilling the role of the normal endogenous micro-flora (Chateau, Castellanos, & Deschamps, 1993 ; Nader de Macuias, Apella, Romero, Gonzualez, & Oliver, 1992). Preparations containing Lb have been administered to humans to prevent or cure diarrhea (Gionchetti, Rizzello, Venturi, & Campieri, 2000 ; Pedone, Arnaud, Postaire, Bouley, & Reinert, 2000), as well as bacterial and mycotic urethritis or vaginitis (Redondo Lopez, Cook, & Sobel, 1990). The management of bacteria-associated diarrhea usually ranges

from no treatment to oral rehydration therapy and to antimicrobial drugs. Since there are many problems associated with the routine use of antibiotics, which have potentially serious side effects, the use of probiotics has been suggested as a safer alternative therapy in the management of gastrointestinal disorders caused by infectious agent and one with the potential for preventing such disorders (Drago, Gismondo, Lombarcli, de Haen, & Gozzini, 1997). Several Lb species have been reported to inhibit the growth of pathogenic bacteria (Ocana, Pesce de Ruiz Holgado, & Nader Macias, 1999; Drago et al., 1997). However, thus far, no reports have been published on Lb strains isolated from saliva or vagina in Korean. The present study has been carried out in order to isolate Lb strain inhibiting the growth of enteropathogenic bacteria isolated from infant saliva and adult vagia in Korean and characterize its biologic properties.

II. MATERIALS and METHODS

In this study, we screened for a candidate Lb strain that inhibit the growth of enteropathogenic bacteria among 1367 Lb strains. Catalase test was done to screen for a Lb strain that inhibited the growth of pathogenic bacteria in the broth. Agar spot test determined the inhibitory effect of Lb strains against the pathogenic bacteria on the agar. Since H_2O_2 producing Lb is more effective in inhibiting various enteropathogens, H_2O_2 test was also performed. Finally, potentiality as probiotics was done by the characterization

and identification of the selected candidate.

1. Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (MRSA) was a clinical isolate from a patient who admitted at the Chonnam University Hospital. *S. typhimurium* (LT strain) and *E. coli* O157 were provided from American Type Culture Collection (ATCC). *Listeria monocytogenes* (strain LM10403) was provided from Dr. Bishop in University of Utah. In this study, a total of 1367 Lb strains isolated from infant saliva and adult vagina were used. Lb strains were collected either from saliva (1604 infants) who visited the department of pediatric dentistry or from adult vagina smear (337 woman) who visited the department of obstetrics in Chonnam University Hospital. Samples were collected by the charge doctor. These samples were serially diluted with sterile 0.9% NaCl solution and spread onto MRS (de Man Rogosa Sharpe, Difco, USA) or Rogosa (Difco) agar plates. After 48 hrs of incubation at 37 ℃, the colonies were selected and subcultured on fresh MRS agar plates. A total of 1900 Lb strains were isolated. Each Lb strain was serially numbered. All the stock culture were maintained at -80 ℃ in 20% glycerol.

2. Growth media

The following media were used for individual bacterial culture: medium I, MRS broth or agar for Lb; medium II, BHI (Brain Heart Infusion) broth or agar (Difco) for enteropathogens; medium III, buffered (pH

7.0) BHI and MRS broth or agar for both Lb and enteropathogens.

3. Assays for inhibition of pathogens

Antimicrobial activity was tested by catalase test and agar spot test. The Lb strains and four pathogens were defrosted and cultured for 16 hrs in MRS broth and BHI broth, respectively.

1) Catalase test
The inhibitory effect of Lb against entero-pathogenic bacteria which all produce catalase, catalase production was tested after cocul-turing Lb with the pathogenic bacteria. Re-duced catalase indirectly suggest that the growth of the pathogenic bacteria was inhi-bited. A 1.2×10^7 CFU/ml of each Lb strain and 1.2×10^8 CFU/ml of MRSA were inoculated into 200 $\mu\ell$ of medium III broth (BHI : MRS =

3 : 1, 0.1M MES (2-(N-Morpholino) ethane-sulfonic acid Monohydrate)) in 96well plates, respectively. These plates were incubated in 37 ℃ incubator. After 24 hrs, we added 50 $\mu\ell$ of 35% H_2O_2 in each well and observed the bubble formation.

2) Agar spot test
To further determine the inhibitory effect of Lb against the pathogenic bacteria, agar spot test was done. Each pathogen (100 $\mu\ell$) was inoculated in 3 $m\ell$ of medium III 0.7% agar (BHI : MRS = 1 : 1, 0.1M MES). After vor-tex mixing and pouring on medium III agar, the plates were kept in clean bench until the liquid was absorbed. Individual Lb isolates were spotted on the plate with a toothpick, respectively. These plates were incubated at 37 ℃ for 24 hrs and measured the diameter of the inhibition zone around the spot as shown in Fig. 1.
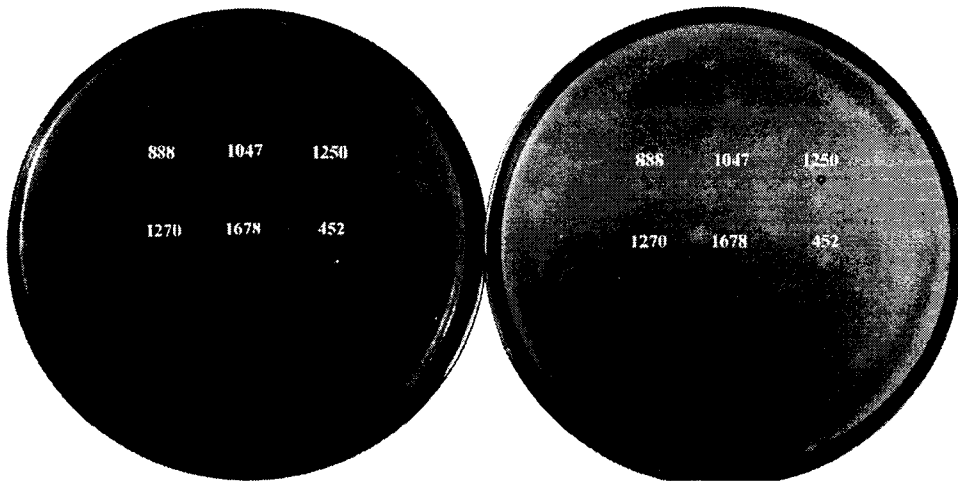
〈Figure 1〉 Formation of inhibitory zone by *Lactobacillus* strains against Methicillin-resistant *Staphylococcus aureus* (left) and *L. monocytogenes* (right). The numbers indicate the serial number of each *Lactobacillus* strain isolated from subjects.

4. H₂O₂ production test

Since H_2O_2 is toxic to many pathogenic bacteria, H_2O_2 producing Lb was further screened. MRS agar with TMB (3, 3, 5, 5-tetramethylbenzidine, 0.25 ㎎/㎖) and peroxidase (0.01 ㎎/㎖) were poured into petri dishes and dried in the clean bench. Lb including Lb 1250 and Lb 245 were spotted on the agar and the plate was incubated at 37 ℃ for 24 hrs in anaerobic condition. Finally, H_2O_2 production was determined by observing the changed colors of the spots.

5. Growth of Lb 1250 and four enteropathogens in mixed cultures

To determine the effect of Lb 1250 on the growth of pathogens, Lb 1250 was grown together with four each pathogens in medium III broth (BHI∶MRS=1∶1, 0.1M MES). As a control, each strain was also grown separately. The inocula were between $10^4 \sim 10^8$ CFU/ml for four pathogens and $10^7 \sim 10^8$ CFU/ml for Lb 1250, respectively. Each pathogen was treated or untreated with catalase (1000 pH

U/ml) and culture fluid was maintained at 6.8~7.2 by adding 5M NaOH every 3 or 4 hrs. At appropriate intervals, the number of viable bacteria was determined by the plate dilution method by using medium I and II agar.

6. Identification and characterization of Lb 1250

Finally, the selected candidate was characterized and identified to determine the potentiality as probiotics. Lb 1250 was identified by morphological and standard biochemical tests including carbohydrate fermentation test and catalase test based on Bergey's manual of systematic bacteriology (Williams & Willkims, 1986). The microorganism was tested for acid tolerance and curd formation.

III. RESULTS

1. Screening of Lb inhibiting enteric bacteria

In catalase test, 982 (71.8%) of 1367 Lb

〈Table 1〉 Screening of *Lactobacillus* strains that inhibit the growth of MRSA by catalase test.

		Agar spot test			Total
		0mm*	0~1mm	1~3mm	
Catalase test	Negative	822	140	20	982
	Positive	330	48	7	385
	Total	1152	188	27	1367

*Inhibition zone size (total diameter-Lb colony diameter) of Methicillin-resistant *Staphylococcus aureus* around *Lactobacillus* strains.
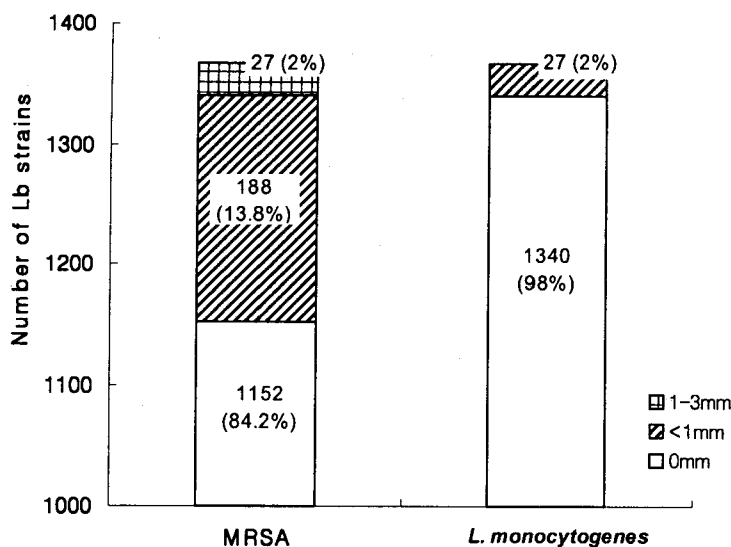
〈Figure 2〉 Number of *Lactobacillus* (Lb) strains that produced inhibition of Methicillin-resistant *Staphylococcus aureus* or *L.* monocytogenes in agar spot test.

strains were negative against MRSA (Table 1).

In a modified agar spot test, 215 (15.7%) of 1367 strains were found to produce inhibition zones against MRSA and 27 (2.0%) strains showed inhibitory activity against *L. monocytogenes*. Only 21 (9.8%) out of 215 strains inhibited the growth of both MRSA and *L. monocytogenes* (Fig. 2). However, none of all the isolates makes clear zone against *S. typhimurium* and *E. coli* O157.

Finally, Lb 1250, isolated from infant saliva, which showed the most strong inhibitory activity through the two tests was selected as a candidate for the following study.

2. H₂O₂ production of Lb 1250

When the spot of cultured Lb 1250 on the modified MRS agar were incubated for 24

hrs in anaerobic condition, the color of spot was changed to blue indicating that Lb 1250 produces H_2O_2 well (Fig. 3).

3. Mixed cultures of Lb 1250 and four enteropathogens

In mixed cultures, the growth patterns of MRSA, *L. monocytogenes*, *S. typhimurium* and *E. coli* O157 in the presence of Lb 1250 were shown in Fig. 4.

Inhibitory effect of Lb 1250 was the most significant against MRSA (10^4 fold) followed by *L. monocytogenes* (10^3 fold) and these effects were reduced about 10^2 fold by adding catalase (1000 U/ml) (Fig. 4-1, Fig. 4-2). But the effects against *S. typhimurium* and *E. coli* O157 were very weak (Fig. 4-3, Fig. 4-4).

The growth patterns of MRSA and *L. monocytogenes* were closely affected by the
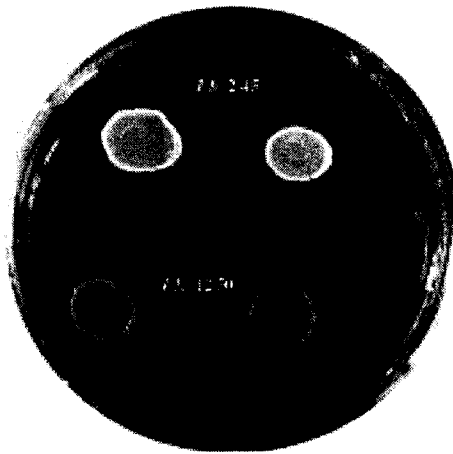
〈Figure 3〉 H_2O_2 production of Lb (*Lactobacillus*) 1250. The plate was cultured at 37 ℃ for 24 hrs in anaerobic condition. Lb 245 was *L. casei* spp. that didn't produce H_2O_2.

initial inoculum sizes of the pathogens (Fig.4-1, Fig. 4-2). The inhibition effects were much higher at $10^4 \sim 10^5$ CFU/ml than $10^6 \sim 10^8$ CFU/ml of pathogen inocula.

4. Identification and biologic properties of Lb 1250

Lb 1250 fermented only 2 carbohydrates (fructose and sucrose) and grew at 45 ℃ and in 4% Nacl. It didn't produce catalase. Based on these characteristics, Lb 1250 was identified as *Lactobacillus delbrueckii* subsp. *delbrueckii* (Table 2).

Even after 2 hrs incubation at pH 2, most of Lb 1250 (10^5 CFU) survived and all the organisms survived at pH 4 (Fig. 5). Lb 1250 produced curd weakly in milk with sweet fragrance and the acidity of the milk was decreased to final pH 4.6. (Table 2)

Taken together, Lb 1250 could serve as a potential probiotic and furter charicterization

are required.

IV. DISCUSSION

Diarrhea caused by *S. aureus*, *S. typhimurium*, *E. coli* O157, and *L. monocytogenes* is a major health problem in many countries. They are frequently indicated pathogens in children and adults with enteric disease ranging from mild diarrhea to severe symptoms (Salyers & whitt, 1993). Especially, MRSA is a main cause of nosocomial infection.

To prevent and treat of diarrheal diseases, lactic acid-forming bacteria are used in several diary products as well as other fermented foods and general medicines.

Foods containing large numbers of Lb and lactic acid-forming bacteria have a long history of safe use. Epidemiological data support the low risk of infection with lactic
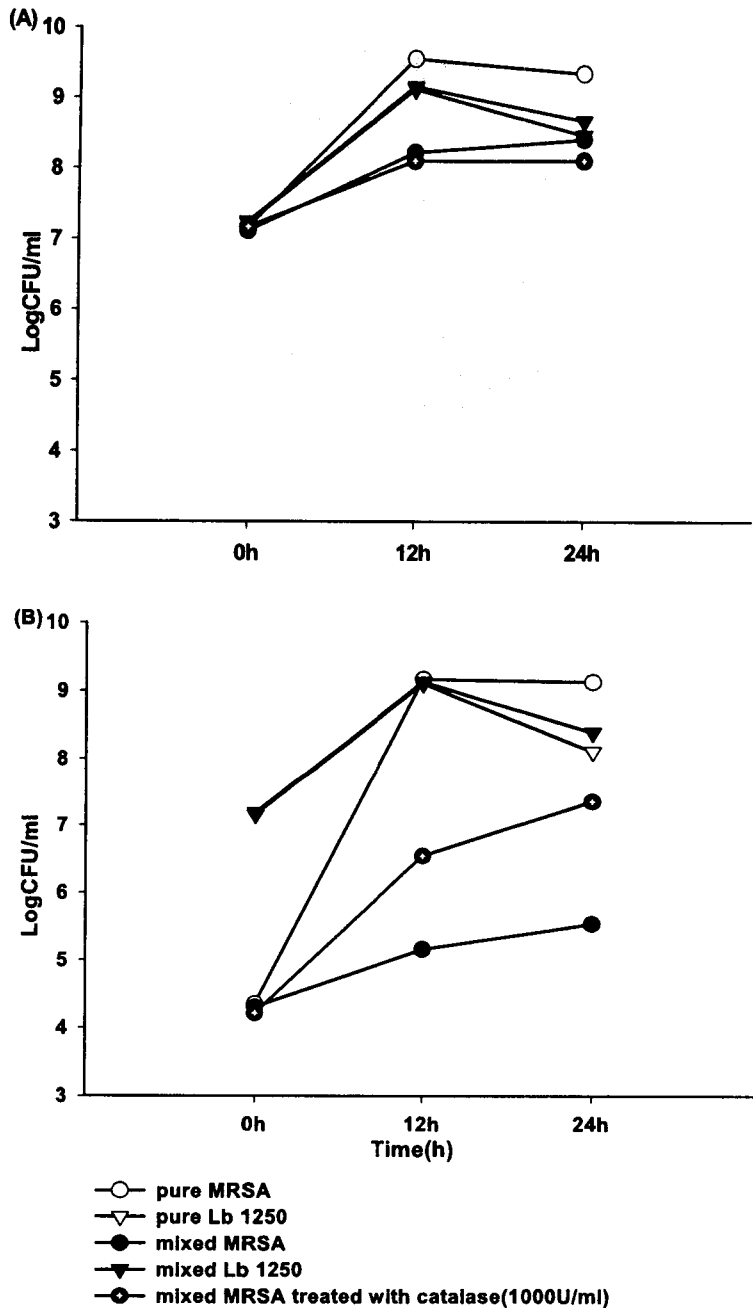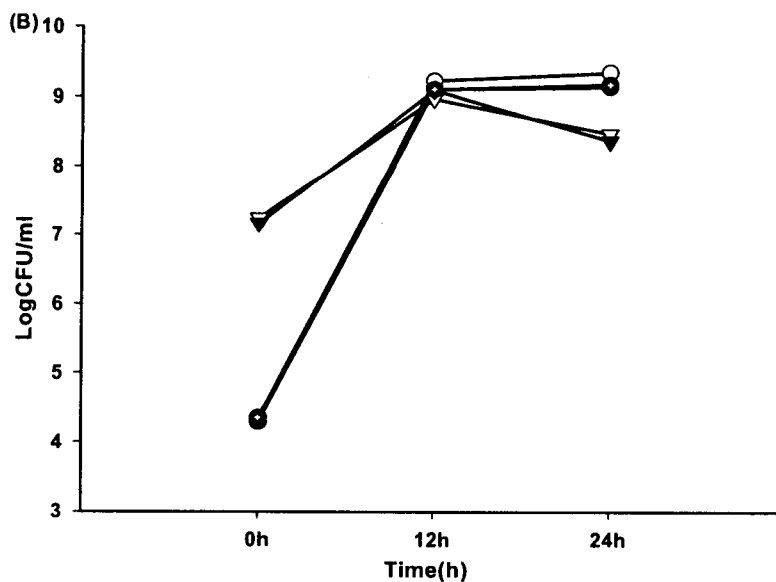
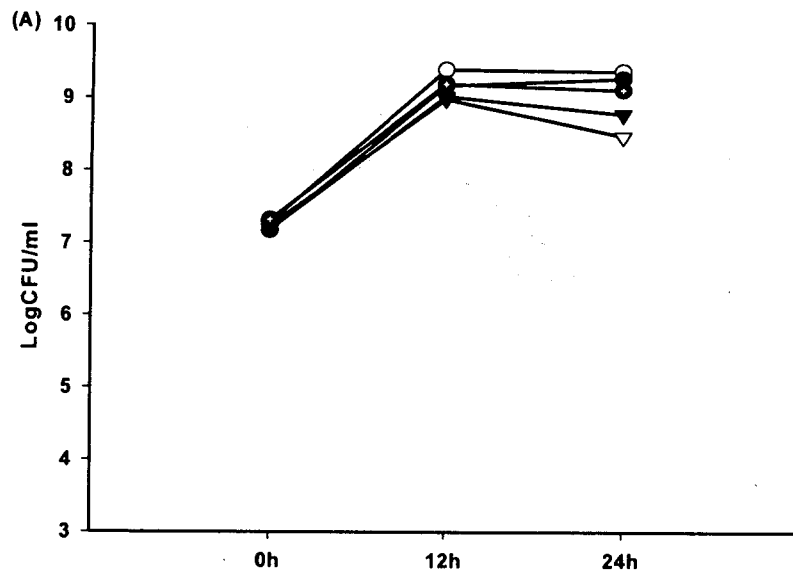⟨Figure 4-1⟩ Growth patterns in the mixed culture of Lb (*Lactobacillus*) 1250 and MRSA (Methicillin-resistant *Staphylococcus aureus*). Different initial concentrations of MRSA. Initial inoculum : (A) 1.5–2.5×10^7 CFU (colony forming unit)/ml, (B) 2.0–3.5×10^4 CFU/ml. Mixed MRSA : colony count of MRSA in mixed culture of MRSA and Lb 1250. Mixed Lb 1250 : colony count of Lb 1250 in mixed culture of MRSA and Lb 1250.
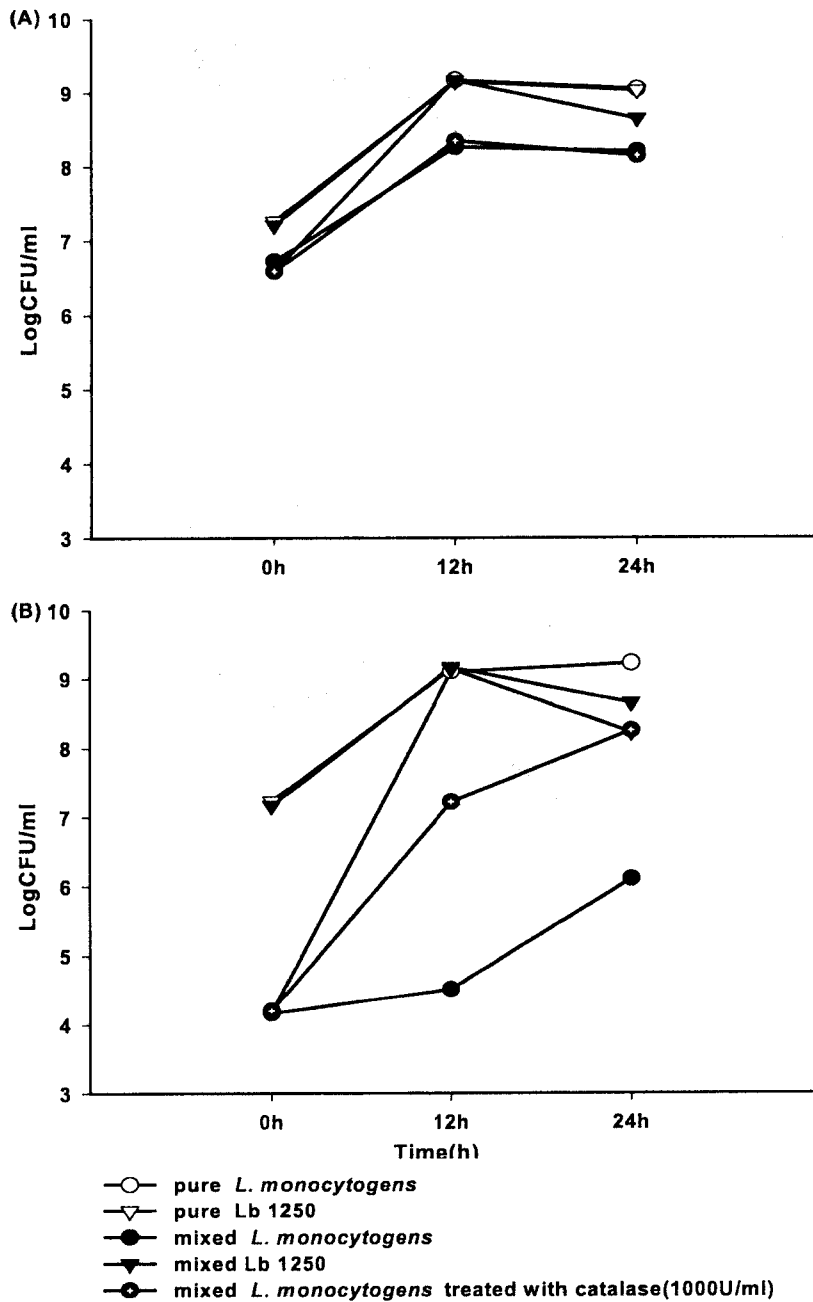
〈Figure 4-2〉 Growth patterns in the mixed culture of Lb (*Lactobacillus*) 1250 and *L. monocy-togenes*. Different initial concentrations of *L. monocytogenes*. Initial inoculum : (A) 5.0–7.0×10⁶ CFU (colony forming unit)/ml, (B) 1.5–2.5×10⁴ CFU/ml. Mixed *L. monocytogenes* : colony count of *L. monocytogenes* in mixed culture of *L. monocytogenes* and Lb 1250. Mixed Lb 1250 : *L. monocytogenes* colony count of Lb 1250 in mixed culture of *L. monocy-togenes* and Lb 1250.

⟨Figure 4-3⟩ Growth patterns in the Mixed culture of Lb (*Lactobacillus*) 1250 and *S. typhimurium*. Different initial concentrations of *S. typhimurium*. Initial inoculum : (A) 2.0–3.0×10^7 CFU (colony forming unit)/ml, (B) 2.5–3.5×10^4 CFU/ml. Mixed *S. typhimurium* : colony count of *S. typhimurium* in mixed culture of *S. typhimurium* and Lb 1250. Mixed Lb 1250: colony count of Lb 1250 in mixed culture of *S. typhimurium* and Lb 1250.
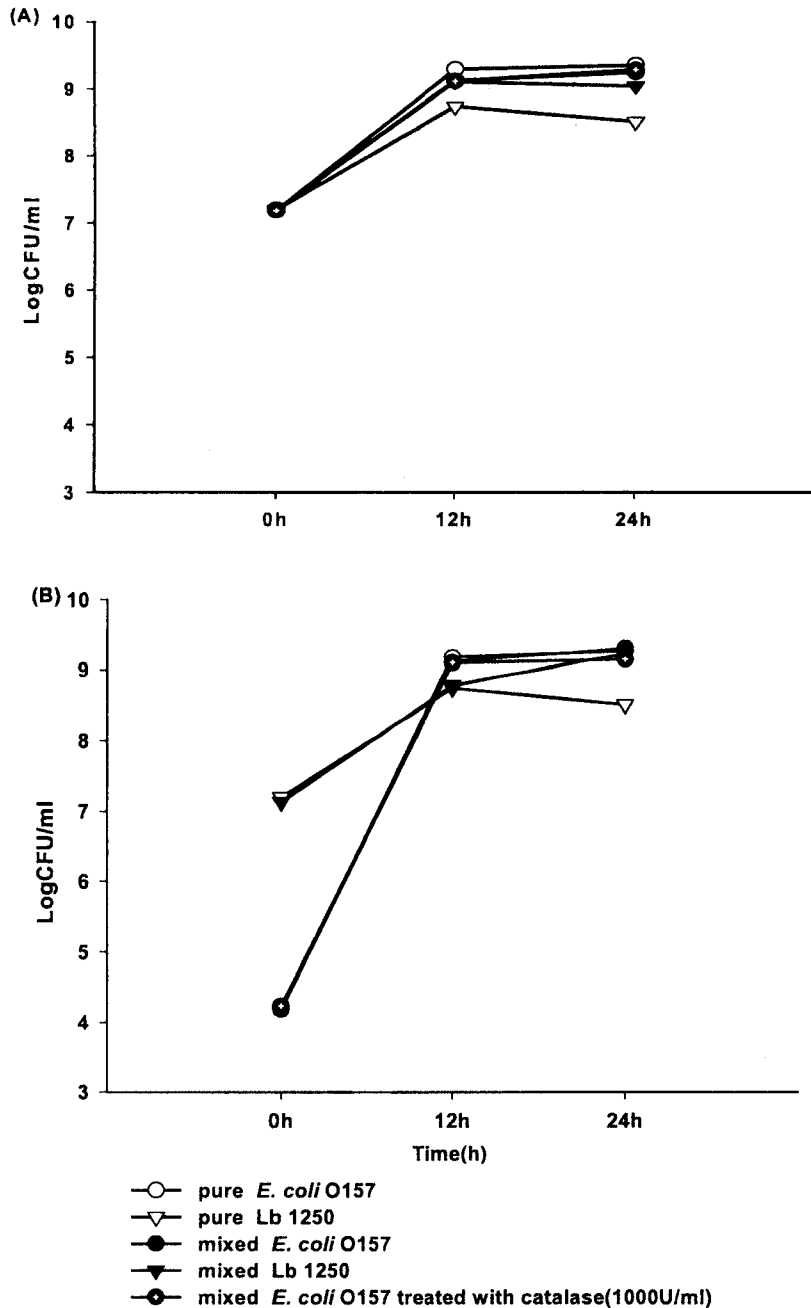
⟨Figure 4-4⟩ Growth patterns in the mixed culture of Lb (*Lactobacillus*) 1250 and *E. coli* O157. Different initial concentrations of *E. coli* O157. Initial inoculum : (A) 1.5–2.0×10^7 CFU (colony forming unit)/ml, (B) 1.5–2.5×10^4 CFU/ml. Mixed *E. coli* O157 : colony count of *E. coli* O157 in mixed culture of *E. coli* O157 and Lb 1250. Mixed Lb 1250 : colony count of Lb 1250 in mixed culture of *E. coli* O157 and Lb 1250.
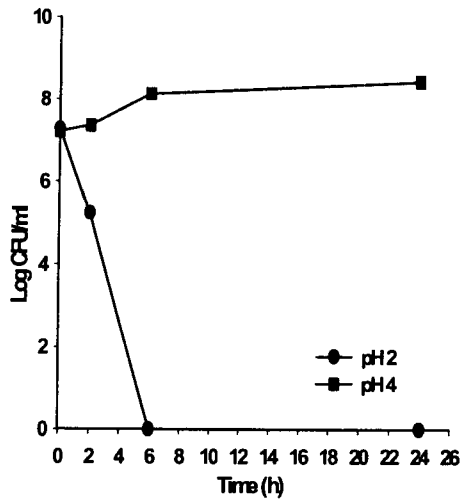
⟨Figure 5⟩ Acid tolerance of Lb (*Lactobacillus*) 1250 during 24 hrs.
CFU : colony forming unit.

acid-forming bacteria (Aguirre & Collins, 1993). More over, no adverse immunologic effects of probiotic bacteria have been reported in healthy persons (Salminen et al., 1998). The incidence of infections caused by or associated with lactic acid-forming bacteria is extremely low considering that they are abundant inhabitants of the healthy microflora of all mucous membranes, and are widely used in fermented food products (Kirjavainen, Apostolou, Salminen, & Isolauri, 1999).

In vitro, many investigators have reported that Lb inhibit various Gram-positive and Gram-negative bacteria (Andersson, Daeschel, & Hassan, 1988; Fuller, 1989; Younts-Dahl et al., 2005; Upreti & Hinsdill, 1973). This inhibition may be related to the production of organic acids, hydrogen peroxidase and bacteriocin like substances.

A number of bacteriocins are produced by lactic acid-forming bacteria including Lb, *Lac-*

tococcus, Pediococcus, Leuconostoc, Entero-coccus and *Carnobacterium* have been reported in recent years (Arihara, Cassens, & Luchansky, 1993; Drago et al., 1997; Piard, Muriana, Desmazeaud, & Klaenhammer, 1992; Ten Brink, Minekus, Van Der Vossen, Leer, & Huis in't Veld, 1994). However, most of these lactic acid-forming bacteria have a narrow spectrum of activity, mainly against other member of lactic acid-forming bacteria. Only a few bacteriocins active against pathogenic bacteria have industrial potential as natural food preservatives or probiotics (Arihara, Ogihara, Mukai, & Kondo, 1996). And Lb species producing H_2O_2 are *L. paracasei, L. delbrueckii, L. agilis, L. crispatus* (Ocana et al., 1999).

In this study, we screened 1367 Lb strains from infant saliva and adult vagina by catalase test and agar spot test. Lb 1250, showing the highest inhibitory activity on the enteric bacteria, was selected as a candidate.

⟨Table 2⟩ Patterns of carbohydrate fermentation of Lb (*Lactobacillus*) 1250 isolated from infant saliva

	characteristics of Lb 1250
Carbohydrate fermentation :	
Amygdalin	−
Cellobiose	−
Fructose	+
Galactose	−
Lactose	−
Maltose	−
Mannitol	−
Mannose	−
Melezitose	−
Melibiose	−
Raffinose	−
Rhamnose	−
Ribose	−
Sorbitol	−
Sucrose	+
Trehalose	−
Morphology	rods, singly and short chain
Gram stain	+
Catalase	−
Growth at 15℃	−
45℃	+
Growth in 4% Nacl	+
Curd formation	+weak(sweet fragrance, final pH : 4.6)
Result	*Lactobacillus delbrueckii* subsp. *delbrueckii*

Symbols: +, 90 % or more of bacteria positive
      −, 90 % or more of bacteria negative

The inhibitory activity of Lb 1250 on the growth of MRSA, *S. typhimurium*, *E. coli* O157 and *L. monocytogenes* was not due to a lowering of pH nor the action of acids normally produced by Lb because the addition of buffer to the culture media and culture fluid was controlled at pH 6.8~7.2 by adding 5M NaOH. It did not impede the normal growth of four enteropatogens and Lb isolates. As another possible reason for growth inhibition, competition for nutritions may be also discarded because Lb 1250 cannot grow well in BHI broth and enteropathogens can not grow in MRS broth.

The inhibition effects of Lb 1250 may be mediated by production of H_2O_2 and bacteriocin. In mixed culture of Lb 1250 with MRSA and *L. monocytogenes*, inhibiting of Lb 1250 was the most significant against MRSA (10^4 fold) followed by *L. monocyto-*

genes (10^3 fold) and there were considerable difference (10^2 fold) of inhibiting effects depending on the culture fluid treated with catalase or not. Additionally, catalase did not impede the normal growth of these organisms. It was shown that the inhibitory substances were products of the metabolic activity of Lb 1250. This products might be H_2O_2 and bacteriocin produced by Lb 1250. Drago *et al.* (1997) reported that two Lb strains inhibited the growth of *E. coli* and *S. enteritidis*. In this study, the inhibitory effects of Lb 1250 against *S. typhimurium* and *E. coli* O157 were very weak.

When MRSA and *L. monocytogenes* were inoculated at $10^6 \sim 10^8$ CFU/ml, lower growth inhibitions were observed compared to at $10^4 \sim 10^5$ CFU/ml. This result was similar to the previously reported result. Ocana, *et al.*

(1999) reported that growth of *S. aureus* was significantly inhibited by *L. casei* and *paracasei* and the inhibitory effects depended on the initial inoculum of *S. aureus*. These may result from the insufficient concentration of H_2O_2 and bacteriocin for the number of pathogens.

In this study, Lb 1250 was identified as *Lactobacillus delbrueckii* subsp. *delbrueckii*. Even after 2 hrs incubation at pH 2, most of It (10^5 CFU) survived. Lb 1250 produced curd weakly in milk with sweet fragrance and the acidity of the milk was decreased to final pH 4.6. This finding suggest that Lb 1250 could be useful as probiotic foods. Further clinical evaluations of Lb 1250 are needed to consider its potential use for probiotics.

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