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Mixed cultures of Kimchi lactic acid bacteria show increased cell density and lactate productivity

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This study was carried out to determine the characteristics of cell growth, lactate production and amino acid secretion among four kimchi lactic acid bacteria (*Leuconostoc mesenteroides* JEI, *Leuconostoc kimchi* 132, *Lactobacillus sakei* 171, and *Weissella koreensis* 521) alone and in selected mixtures. In solo culture, *L. sakei* 171 was superior in cell growth, lactate production and the release of amino acids to the extracellular medium. In contrast, *W. koreensis* 521 showed the least cell growth, lactate production and amino acid release among the tested bacteria. *W. koreensis* 521 consumed essential amino acids for growth, whereas *L. sakei* 171 released several of the essential amino acids important for the growth of *W. koreensis* 521. When we mixed *L. sakei* 171 and *W. koreensis* 521 at optimal concentrations, the obtained cell growth and lactic acid production were higher than those seen with either strain alone, presumably reflecting mutual effects between the two strains. Mixed culture of two kimchi lactobacilli on batch fermentation increased the cell density and lactic acid production with low nutrients consumption. These results suggest that mixed culturing of kimchi lactobacilli may be more effective than single culturing of kimchi lactic acid bacteria for improving lactic acid production.

Key words: Kimchi lactic acid bacteria, amino acid utilization, nutrients consumption.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of related bacteria that produce lactic acid through carbohydrate fermentation. These microbes are broadly used in the production of fermented food products, such as yogurt, cheese, sauerkraut and sausage. Live LAB is also probiotics, in that they can confer a health benefit to the host by improving its intestinal balance (Fuller, 1989). In general, probiotics can reduce serum cholesterol levels, improve gastrointestinal function, enhance the immune system, and lower the risk of colon cancer (Berner and O'Donnell, 1998; McNaught and MacFie, 2001; Rafter, 2003; Saarela et al., 2002). Recently, the focus of scientific investigation has shifted from the primary role of food as the source of energy and body-forming substances, to the more subtle action of biologically active food components on human health. As a result, a new term functional food has been proposed (Ozen et al., 2012). The functional foods include probiotics, which may exert positive effects on the composition of gut microbiota and overall health, and the market for probiotics is on the rise. Kimchi, a traditional Korean food, is gaining popularity as a functional food. As a lactic

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Abbreviations: 3G-Bio, Green Bioresource, Green Biotechnology, Global Bioproduct for Functional Cosmetics and Food.

acid-fermented vegetable product that is consumed raw, kimchi is considered a good source of potentially beneficial and useful LAB (Lee, 1997). The LAB found in kimchi are psychrophilic or psychrotrophic facultative anaerobic organisms that are highly resistant to salts. Probiotic cultures for use in dairy products need to be particularly resistant to acidic conditions, because the pH of this type of product is typically 4.5 or lower (Vinderola and Reinheimer, 2003). Fresh isolated LAB of human origin is often sensitive to acidic conditions (Park et al., 2002). However, the LAB in kimchi can survive under harsh conditions (nutrient limitation and the presence of natural antimicrobial substances). Furthermore, LAB in kimchi have a superior ability to decompose and utilize nutrients, and show excellent productivity for various bioactive substances. This suggests that *Lactobacilli* isolated from kimchi could potentially be used for functional food. Foods containing probiotics can eliminate constipation symptoms arising from irregular eating habits, stress and excessive dieting (Kapka-Skrzypczak et al., 2012). Mixed cultures of lactic acid bacteria have been recognized to be effective for certain fermentation. Mixed cultures of lactic acid are currently used in the dairy industry for cheeses and fermented milks. The existence of a symbiotic relationship among various bacteria has been demonstrated (Secchi et al., 2012; Moon and Reinbold, 1976).

The aim of this investigation was to produce higher cell growth and lactic acid production in batch cultures by using a mixed culture system containing *Lactobacillus sakei* 171 and *Weissella koreensis* 521. In addition, the production and consumption of free amino acids were examined in order to improve our understanding of the symbiotic interactions between populations. Then, the differences between single *Lactobacillus* cultures and mixed-type lactobacilli culture, by comparing their fermentative abilities, were investigated.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Four Lactobacillus strains were used in this study: *Leuconostoc mesenteroides* JEI, *L. kimchi* 132, *L. sakei* 171, and *W. koreensis* 521, which are hereinafter referred to as strains JEI, 132, 171, and 521, respectively. The strains were isolated from kimchi in South Korea and stored at -80°C in de Man-Rogosa-Sharpe (MRS) medium (De Man et al., 1960) supplemented with glycerol (20%). The bacteria were grown under anaerobic conditions in glucose-supplemented MRS broth for 32 h at 37°C. Pure and mixed culture experiments were conducted in 500-ml Erlenmeyer flasks containing 100 ml of the MRS medium (150 rpm, pH 6.5, 37°C). The final inoculum volume of each bacterial strain was 1 ml ($1x10^4$ cfu/ml); mixed cultures contained 1 ml of strain 521 and different inoculation proportions (0.1, 0.2, 0.3, 0.4, 0.5 or 1 ml of strain 171, as indicated).

Bioreactor fermentation

Fermentation runs were performed anaerobically in a fermentor (Fermentec Inc. Co. Daejeon, Korea) with 3 L working volume, agitation (150 rpm), and temperature (37°C). The bacteria were grown under anaerobic conditions in the MRS broth containing glucose. The medium composition per liter was as follows: (a) 10 g peptone, 10 g beef extract, 5 g yeast extract, 3 g diammonium citrate, 5 g sodium acetate, 1 g Tween, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot 4H_2O$; and (b) 15 g glucose. Components (a) and (b) were autoclaved separately and aseptically mixed together before starting the cultivation. The pH was maintained constant at pH 5.5 by automatic addition of 5 N NaOH solution and the total time of fermentation was approximately 32 h. Unless otherwise stated, the fermentation medium was inoculated with 5% (v/v) of the seed MRS broth culture. Each experiment was repeated three times.

Analysis of amino acid concentration

Free amino acids were quantified from culture supernatants taken at the indicated time points. The culture samples were filtered with a membrane (0.45 μm, GS; Millipore, [Bedford, U.S.A]) and hydrolyzed with 6 M HCl for 24 h at 110°C under a vacuum, and amino acid contents were measured using a Hitachi model L8800A automated amino acid analyzer (Hitachi, Japan).

Other analytical methods

Bacteria growth was monitored by spectrophotometric measurement at 600 nm. The fermentation broth was centrifuged at 20,000 x *g* for 10 min, the supernatant was collected, and lactic acid and residual glucose concentrations were assessed using an HPLC apparatus equipped with a refractive index detector (Agilent, USA). The utilized column was an Aminex HPX-87H (Bio-Rad Co., USA), and chromatography was performed at 40°C using 0.01 N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min. The protein content was estimated by the bicinchoninic acid method (Smith et al., 1985).

RESULTS

Flask fermentation

This study sought to evaluate the possible synergistic effects of mixed cultures for lactic acid production. The first series of fermentations were carried out using pure cultures of each strain alone. As shown in Figure 1A, strain 171 produced the highest culture turbidity ($OD_{600} \approx 1.36$) in the course of cultivation, possibly indicating that strain 171 had the highest proteolytic activity among the tested strains. This is notable because the peptides and amino acids generated from protein hydrolysis may be of great nutritional value for bacterial growth (Kunji et al., 1996). In contrast, strain 132 showed the lowest cell growth. Figure 1B shows the glucose consumption curves observed during the fermentation process. The four strains showed significant glucose consumption up to 8 h, but strains JEI, 171, 521 and 132 still had 2.9 and 5.63 (g/l) residual glucose, respectively, at 32 h. Strain 132 consumed very little glucose after 8 h, whereas strains JEI, 521 and 171 had similar amounts of residual glucose at 32 h. In terms of the lactic acid production over time (Figure 1C), the maximum concentration of lactic acid (12.5 g/l) was obtained from strain 171 at 32 h,

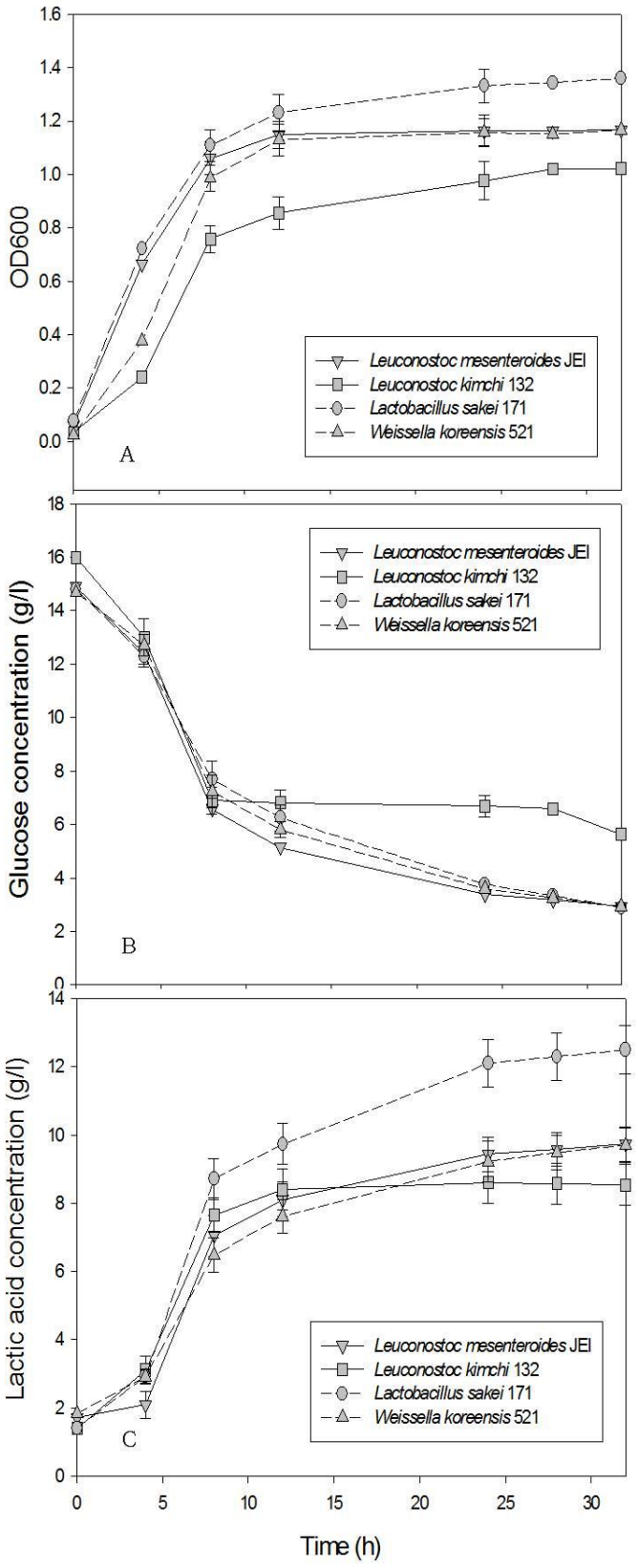

Figure 1. Profiles of cell growth, sugar consumption, and lactic acid production with glucose as carbon source during batch cultivation of four kimchi lactic acid bacteria. Each number represents the mean ± SD of three replicates.

while strains JEI, 521, and 132 yielded maximum lactic acid concentrations of 9.73, 9.7 and 8.54 g/l, respectively, at 32 h.

Amino acid utilization/production of individual Lactobacillus strain

Table 1 shows the changes in individual amino acids in media conditioned by individual cultures of the four strains over time. Strain 171 showed the greatest release of amino acids to the medium, releasing alanine, valine, leucine, methionine, threonine, isoleucine, glutamic acid, arginine, proline, phenylalanine, tyrosine, glycine, glutamine and tryptophan, all of which are non-essential for the growth of this bacterium, while consuming the essential amino acids, cystine and asparagine during the 24 h incubation. Strain 132 released serine, aspartic acid, arginine, phenylalanine and tryptophan, which are non-essential for its growth. In contrast, strains JEI and 521 decreased the amino acid concentration in the medium, showing particular consumption of leucine, cystine, arginine, phenylalanine, asparagine and glutamine, which are essential for the growth of these strains.

Improved lactic acid production by symbiotic mixed culture

We then explored fermentation by mixtures of strains 171 and 521, which showed important differences in cell growth, lactic acid production and amino acid secretion. Basso et al. (2004) have reported that *L. sakei* is a proteolytic strain acting on meat sarcoplasmic proteins. In this work, proteins were hydrolyzed when the extracellular proteinase produced by *L. sakei* 171 accumulated in the medium in pure or mixed culture of this strain. Proteolytic strain 171 showed higher growth parameters and efficiently produced a subset of amino acids that largely corresponded to the amino acids that were consumed by strain 521. We speculated that stimulation of the proteolytic system in the mixed culture would increase the release of amino acids that are essential for the growth of both strains; therefore, co-culturing should enhance the growth yield of both strains (synergism). Changes in cell growth and lactic acid yield during fermentations with single and mixed cultures were assessed. Due to differences in proteolytic rates and the possible dominance of one strain over another, the mixture ratios were adjusted based on the results from preliminary experiments (data not shown). A fixed amount of strain 521 (1 ml inoculum) was mixed with varied amounts of strain 171 (0.1, 0.2, 0.3, 0.4 and 0.5 ml). As shown in Table 2, mixed cultures showed enhanced cell growth and lactic acid yields compared with pure cultures of strain 521. Significant differences in cell growth and lactic acid yields were seen among the mixed cultures,

Table 1. Variation of free amino acids in LAB cultures over time.

Amino acid	Strain			
	171	JEI	521	132
8 h				
Alanine	+	- -	+	
Valine	+ + +	- -	+	
Leucine	+	- -	-	
Serine		- -		+
Cystine	- - -	- - -	- - - -	
Aspartic acid		-	+	+
Methionine	+	- -		
Threonine	+ +	- -		
Isoleucine	+ +	- -		
Lysine		- -		
Glutamic acid	+	- -		
Arginine	+	-	- -	+
Proline	+ +	-		
Phenylalanine	+	- - -	- -	+
Tyrosine	+ +	-		
Glycine	+ +	- -		
Asparagine	- - - -	- - -	- - -	
Glutamine	+	- - - -	- -	- - - -
Tryptophan	+ +	- -		+
24 h				
Alanine	+ +	- -	+ + +	+
Valine	+ +	- -		
Leucine		- -	- - -	
Serine	-	-		
Cystine	- -	- - -	- - -	
Aspartic acid	- - -		+	
Methionine	+ + +	- -	- - - -	
Threonine	+ + +	- -		
Isoleucine	+	- -	-	
Lysine		- -	-	
Glutamic acid	+	- -	- -	
Arginine	+ +	-	- -	+
Proline	+ +	- -	-	
Phenylalanine		- - -	- - - -	
Tyrosine	+	-	-	
Glycine	+ +	- -		
Asparagine	- - - -	- - - -	- - - -	-
Glutamine		- - - -	- -	- - - -
Tryptophan	-	- -	-	

Considering the initial concentration of amino acids in fresh MRS medium as 100%, symbols in Table 1 represent the relative percentage of amino acid concentration in the culture broth with the following definition: No sign, 90-110%; +, 110-130%; + +,130-150%; + + +, 150-200%; + + + +, >200%; -, 90-80%; - -, 80-70%; - - -, 70-50%; - - - -, <50%.

with 0.3 ml of strain 171 showing the highest performance compared to the other inoculation ratios after 32 h of incubation. The higher rates of cell growth, proteolysis and amino acid release in the mixed cultures were associated with increased proteolytic activity. As strain 171 is a proteolytic bacterium and protein hydrolysates were used by both strains in the mixed culture, we conclude that the growth of the non-proteolytic strain 521 was stimulated by the presence of strain 171.

Table 2. Maximum yields of cell growth and lactic acid in mixed cultures.

LAB	Maximum growth Yield*	Lactic acid yield** (%)	Maximum lactic acid concentration (g/l)
Mixed (0.1 ml)	0.099 ± 0.01	69.44± 2.24	10.9 ± 0.35
Mixed (0.2 ml)	0.114 ± 0.01	84.46 ± 2.46	12.1 ± 0.30
Mixed (0.3 ml)	0.125 ± 0.02	88.03 ± 2.46	12.5 ± 0.35
Mixed (0.4 ml)	0.121 ± 0.02	84.39 ± 2.31	12.2 ± 0.23
Mixed (0.5 ml)	0.119 ± 0.02	79.44 ± 2.24	11.9 ± 0.25
LAB 171	0.123 ± 0.02	86.23 ± 2.02	12.2 ± 0.28
LAB 521	0.089 ± 0.01	65.98 ± 2.99	9.7 ± 0.29

Data represent the means and standard deviations from three independent experiments (n = 3). For the mixtures, the numbers in parentheses represent how much of strain 171 was mixed with 1 ml of strain 521.* Maximum growth yield = Δ culture turbidity / Δ glucose; ** Yield = g of lactic acid produced / g of glucose consumed x 100.

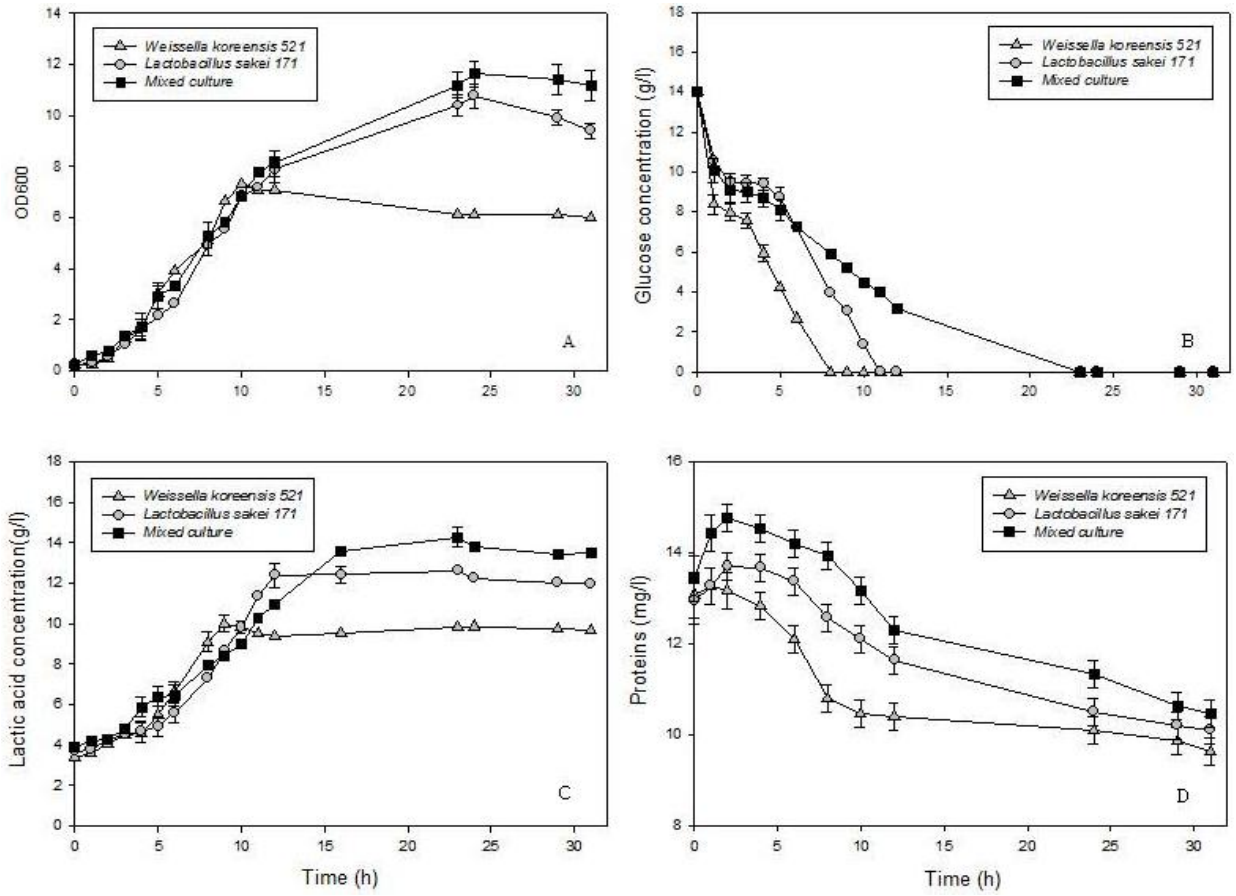

Figure 2. Changes of growth, glucose contents, lactic acid produced and total protein used during fermentation by single and mixed cultures of kimchi lactic acid bacteria. Each number represents the mean ± SD of three replicates.

Comparison of fermentative capacity between single and mixed cultures

The growth characteristics of the single and mixed cultures were conducted in a fermentor. Every strain showed similar exponential growth for the first 10 h and afterwards single cultures of strain 171 and mixed cultures were a large increase in the cell concentration, which was not observed in single cultures of strain 521 (Figure 2A). Mixed cultures showed lower glucose consumption (Figure 2B). Lactic acid concentrations, in the culture of each strain, were estimated and compared (Figure 2C). The benefit of mixed cultures was clearly manifested with lactic acid production. Mixed cultures

produced higher amounts of lactic acid in the culture. The lactic acid yields to glucose of strains 521, 171 and mixed cultures were all comparable: 69.1, 85.5 and 96.2%, respectively, indicating apparent synergistic interactions in co-cultures-helped lactic acid production more than single cultures. Figure 2D shows the changes in proteins in single cultures of strain 521 and strain 171 and in the mixed culture. The protein concentration showed only a marginal increase in the first 2 h but a marked decrease at 32 h fermentation. Initial protein hydrolysis became noticeable after 12 h of incubation (protein consumption was 2.68, 1.3 and 1.12 mg/l, for designated strains 521, 171 and mixed culture, respectively). Then, from 12 to 32 h, no significant variations in protein consumption were detected. Mixed cultures gave lower protein consumption than each single strain. The benefit of mixed cultures was clearly manifested with the nutrient consumption. Proteins were hydrolyzed when the extracellular proteinase produced by proteolytic strain 171 accumulated in the medium in single or mixed culture of this strain. Consequently, growth of the non-proteolytic strain 521 was stimulated by the presence of strain 171.

DISCUSSION

In order to enhance the cell density and lactate productivity from *Lactobacillus* strains, much work has been done in MRS media. High costs of MRS broth and difficulty to scale it up inhibited it to be industrialized. Previous studies found that, mixed culture of *Lactobacillus casei* and *Lactococcus lactis* gave better results regarding lactic acid production and sugar utilization compared to single cells (Nancib et al., 2009). For the first time, *L. sakei* 171 and *W. koreensis* 521 isolated from kimchi were co-cultured to enhance the cell density and lactate productivity. In the present study, co-culturing of both strains enhanced the cell growth and lactic acid production. The reason may be the symbiotic associations in mixed culture that not only overcame but also overcompensated for nutritional limitations in the substrate.

A previous study (Mills and Thomas, 1981) showed that amino acids or proteins with small molecular weight may be a very efficient nitrogen sources for LAB cell growth. Sriphochanart et al. (2011) investigated the effect of amino acid requirements on growth and lactic acid production of *Pediococcus acidilactici* culture. The limitation of phenylalanine, tyrosine, leucine, valine, and histidine significantly affected the production of biomass and lactic acid. Therefore, to enhance the cell density and lactate productivity in the kimchi lactic acid bacteria fermentation, amino acids, as an efficient activator, should be taken into account. In this study, to better understand any synergetic effect between both strains in the high performances in the co-cultured system, MRS broth of free amino acids was identified in individual *Lactobacillus* strain. Our esults show that mixed cultures have a higher fermentative capacity than individual cultures. The synergistic associations between the two strains could overcome the growth repression of the one strain and improve lactic acid production.

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