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Amino acids analysis during lactic acid fermentation by single strain cultures of lactobacilli and mixed culture starter made from them

KiBeom Lee[1]*, Ho-Jin Kim[1] and Sang-Kyu Park[2]

[1]Bio Center Technopark, 7-50 Songdo, Yeonsu-Gu, Incheon 406-840, Republic of Korea.
[2]Nambu University, Chumdan Jungangro 23, Gwangju, Republic of Korea.

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The interactions among *Lactobacillus salivarius*, *Pediococcus acidilactici* and *Lactobacillus plantarum* in utilizing the amino acids of MRS media were investigated. *L. salivarius* alone showed relatively good assimilation of various amino acids that existed at only a little amounts in MRS media (Asn, Asp, Cit, Cys, Glu, His, Lys, Orn, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp and Val), whereas Ala and Gly accumulated in *L. salivarius* cultures. *P. acidilactici*, in contrast, hydrolyzed the proteins found in the medium to liberate various amino acids; it utilized Cit, Cys, and Gly for growth, while promoting the accumulation of Ala, Asn, Glu, His, Lys, Orn, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp, and Val. Similar to *L. salivarius, L. plantarum* showed relatively good assimilation of various amino acids that existed at only trace amounts in MRS medium (Ala, Asn, Asp, Cit, Cys, Glu, Gly, His, Lys, Phe, Pro, Tyr, Arg, Ile, Leu, Met, Ser, Thr, Trp, and Val). Single-strain cultures of *L. salivarius* and *P. acidilactici* exhibited high levels of growth and lactic acid synthesis, whereas single-strain cultures of *L. plantarum* manifested the lowest cell growth and lactic acid synthesis. The unique individual abilities of the three LAB strains to utilize amino acids for growth and lactic acid production were employed to create a mixed culture that showed enhanced cell growth and lactate production. The results from our mixed culture (*L. salivarius* and *P. acidilactici*) experiments suggested that the growth of *L. salivarius* was stimulated by the amino acids produced by *P. acidilactici*, enhancing lactic acid production. Mixed cultures were tested at different inoculation proportions (1:1, 2:1, 5:1 and 9:1; given as OD_{600} ratios of *L. salivarius*: *P. acidilactici*), and the highest cell growth and lactic acid production values were obtained for the 2:1 ratio.

Key words: Amino acids, single and mixed culture, *Lactobacillus salivarius*, *Pediococcus acidilactici*, *Lactobacillus plantarum*

INTRODUCTION

Lactic acid bacteria (LAB), which are used for the fermentation of various foods and beverages, are

*Corresponding author. E-mail: klee02@empal.com. Tel: 82-32-260-0727.

facultative anaerobic bacteria that convert the available sugar into lactic acid as the major end product. Probiotics are live microorganisms that are present in food and are capable of modifying the gut microflora to improve human (or animal) health. The probiotic bacteria are found among the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus* and *Bacillus*. Due to the U.S. Food and Drug Administration's restrictions on the use of antibiotics in animals raised for food, researchers have sought alternative strategies to control infectious diseases in animals such as poultry. One feed supplement that can enhance performance and protect animals from microbial infection is the large-scale application of probiotic bacteria in animal feed (Gaggia et al., 2010). Thus, there is a market for huge LAB biomasses (Schiraldi et al., 2003; Briens et al., 2008; Garvie, 1967), and new production strategies are needed.

LAB of the genus *Lactobacillus* are fastidious bacteria with numerous growth requirements, including the need for rich media containing compounds such as amino acids, peptides, vitamins and nucleic acids (Narayanan et al., 2004; Dumbrepatil et al., 2008; Mills and Thomas, 1981). LAB initiates growth faster and reach higher cell densities in complex media containing fundamental nitrogen components, such as amino acids or low-molecular-weight proteins. The majority of amino acids are either stimulatory or essential for their growth (Garvie, 1967). De Man, Rogosa and Sharpe (MRS) medium is commonly used to grow *Lactobacillus*.

The amino acid requirements and lactic acid production activity of LAB can be optimized by using mixed-strain cultures, wherein strains that produce certain amino acids can compensate for their use by other strains that have higher production activity. Upon noticing that co-cultures of *Lactobacillus acidophilus* plus *Kluyveromyces fragilis* yielded higher lactic acid production rates and values than those seen with either strain alone, Yu et al. (1987) investigated the cross-promoting action of two strains on lactic acid fermentation by examining different sugars (for example sucrose, raffinose and stachyose in soy milk). In this study, to examine the possible synergetic effects between strains of *Lactobacillus* in a co-cultured system, we first monitored the changes in free amino acid levels during single-strain cultures. We assessed the amino acid release, amino acid consumption, cell growth, and lactic acid production in single-strain cultures of LAB and mixed-strain cultures composed of different ratios of *Lactobacillus salivarius* and *Pediococcus acidilactici* starter cultures. This study provides important baseline information for the development of promising and effective means for commercial culturing of LAB.

MATERIALS AND METHODS

Bacterial strain and preparation of inocula

L. salivarius (Ls 21), *P. acidilactici* (Pa 175) and *L. plantarum* (Lp 177), which were previously isolated from a pig farm in Korea (Yun et al., 2008), were used in the present study. Stock cultures of *L. salivarius*, *P. acidilactici*, and *L. plantarum* were maintained at -80°C in MRS medium containing 40% (v/v) glycerol. Precultures were prepared by inoculating 1 ml of frozen stock into 50 ml of MRS medium, followed by incubation at 37°C for 16 h. Cultures (single- or mixed-strain) were prepared by inoculating 1 ml (10^6 CFU/ml) of preculture into 100 ml of MRS medium in a 500 ml Erlenmeyer flask, followed by incubation at 37°C for 16 h with shaking at 150 rpm. The bacteria were grown under anaerobic conditions in the glucose-containing MRS medium. 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 immediately prior to cultivation. For the mixed-strain experiments, cultures were inoculated with 1 ml of precultures mixed at OD ratios ranging from 1:1 to 9:1 (*L. salivarius*: *P. acidilactici*). Each experiment was repeated three times.

Analysis of amino acid concentration

Free amino acids were quantified from culture supernatants taken at 8 h. 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

Bacterial growth was monitored by spectrophotometric measurement at 600 nm. To assess lactic acid production, the fermentation broth was centrifuged at 20,000 x *g* for 10 min, the supernatant was collected, and lactic acid concentrations were assessed using an HPLC apparatus equipped with a refractive index detector (Agilent, U.S.A). 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.

RESULTS AND DISCUSSION

Amino acid utilization/production of individual *Lactobacillus* strain

The free amino acid levels in non-fermented MRS media (0 h) and those subjected to fermentation by the three individual *Lactobacillus* strains at 37°C (8 h, the end of the exponential growth phase) are presented in Table 1. Fermentation of MRS medium by *L. salivarius* decreased the contents of all amino acids except alanine and glycine, all of which are essential growth factors for this bacterium. In contrast, the levels of alanine and glycine increased compared to their levels in unfermented MRS broth. These findings confirm that *L. salivarius* released alanine and glycine, which are non-essential for the growth of this bacterium.

P. acidilactici increased the amino acid content of the medium, releasing numerous (non-essential) amino acids to the medium, including all amino acids except citrulline, cysteine and glycine. Thus, the growth of *P. acidilactici*

Table 1. Change of free amino acid released from single strain culture at the end of exponential growth. 0 h and 8 h.

Amino acids concentration (mg/l)	*L. salivarius*		*P. acidilactici*		*L. plantarum*	
	0 h	8 h	0 h	8 h	0 h	8 h
Alanine	200 ± 4	215 ± 4.3	200 ± 4	254 ± 5.1	200 ± 4	60.8 ± 1.2
Asparagine	55 ± 0.6	25.3 ± 0.3	55.2 ± 0.6	55.8 ± 0.6	55 ± 0.6	7.8 ± 0.1
Asparatic acid	124.3 ± 1	64.7 ± 0.6	124 ± 1.2	124 ± 1.2	124 ± 1.2	25.7 ± 0.3
Citrulline	45.4 ± 0.5	22.2 ± 0.2	46.4 ± 0.5	21.8 ± 0.2	46 ± 0.5	6.08 ± 0.1
Cysteine	7.48	5.23	7.48	5.73	7.48	0.53
Glutamic acid	428 ± 8.6	358 ± 7.2	428 ± 8.6	565 ± 11	428 ± 8.6	102 ± 2
Glycine	115 ± 2.3	140.7 ± 3	114 ± 2.3	40.6 ± 0.8	119 ± 2.4	38 ± 0.76
Histidine	24.5 ± 0.2	18.6 ± 0.2	23.2 ± 0.2	30.4 ± 0.3	27.9 ± 0.3	6.4 ± 0.1
Lysine	149	124	149	219	149	3.3
Ornithine	5.76 ± 0.1	5.5 ± 0.1	5.53 ± 0.1	6.1 ± 0.1	5.9 ± 0.1	21.6 ± 0.2
Phenylalanine	120.4 ± 2	82 ± 1.6	120 ± 2	144.5 ± 3	120 ± 2	11.4 ± 0.3
Proline	89.2 ± 0.9	80.6 ± 0.8	89.2 ± 0.9	121 ± 1.2	89 ± 0.9	26.4 ± 0.3
Tyrosine	59 ± 1.2	40.7 ± 0.8	59 ± 1.2	67.3 ± 1.3	59 ± 1.2	8.14 ± 0.2
Arginine	272 ± 2.7	77 ± 0.8	272 ± 2.7	427 ± 4.3	272 ± 2.7	72.1 ± 0.7
Isoleucine	104.3 ± 2	77 ± 1.5	104 ± 2.1	142 ± 2.8	105 ± 2.1	26.7 ± 0.5
Leucine	224.8 ± 4	143.6 ± 3	225 ± 5	278 ± 5	224 ± 4	52 ± 1
Methionine	49.4 ± 1	18.6 ± 0.4	50 ± 1	64.7 ± 1.3	52 ± 1	17.8 ± 0.4
Serine	109.6 ± 2	91.1 ± 1.8	109 ± 2.2	135 ± 2.7	110 ± 2.2	28.1 ± 0.6
Threonine	87.9 ± 1.8	66.7 ± 1.3	88 ± 1.8	113.5 ± 2	88 ± 1.8	22.2 ± 0.4
Tryptophan	26.8	14.4	27	43	26	6.6
Valine	155 ± 3.1	126.8 ± 3	153 ± 3	217 ± 4	155 ± 3	40.1 ± 0.8

Data represent the mean values from three independent experiments ($n = 3$) and their standard deviation.

produced many amino acids at levels beyond those needed for the organism's metabolism. In contrast, *P. acidilactici* decreased the medium contents of citrulline, cysteine and glycine, which are essential for the growth of this strain. There was no significant difference with respect to the amount of aspartic acid during both 0 and 8 h, which this strain uses as a growth factor, but not much configuration.

Finally, *L. plantarum* decreased the overall amino acid concentration in the medium, showing particular consumption of alanine, asparagine, aspartic acid, citrulline, cysteine, glutamic acid, glycine, histidine, lysine, phenylalanine, proline, tyrosine, arginine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine, which are essential for the growth of this strain. *L. plantarum* released ornithine, which is non-essential for its growth.

Comparisons revealed that *P. acidilactici* showed higher growth parameters and efficiently produced a subset of amino acids that complemented the amino acid requirements of *L. salivarius* and *L. plantarum*. Strain *L. salivarius* was superior in cell growth and lactic acid production, whereas *L. plantarum* showed very poor cell growth and lactic acid production (Figure 1).

Effect of mixed-strain culture of *L. salivarius* and *P. acidilactici* on the metabolism of amino acids in MRS medium

During single- and mixed-strain cultures of *L. salivarius*, *P. acidilactici* and *L. plantarum*, we observed the highest cell growth and lactic acid production from mixed-strain cultures of *L. salivarius* and *P. acidilactici.* A previous study showed that mixed cultures of *Lactobacillus casei* and *Lactococcus lactis* showed better lactic acid production and sugar utilization compared to single-strain cultures (Nancib et al., 2009). However, the present study is the first to examine the co-culture of *L. salivarius* and *P. acidilactici* (two strains isolated from pig feces) for enhancement of cell density and lactate production in MRS medium. We speculate that symbiotic associations in the mixed culture were able to overcome (and possibly overcompensate for) the nutritional limitations of the utilized substrate. In an early study, Mills and Thomas (Mills and Thomas, 1981) showed that amino acids and small-molecular-weight proteins may offer very efficient nitrogen sources for the growth of LAB. More recently, Sriphochanart et al. (2011) investigated the effect of amino acid requirements on growth and lactic acid

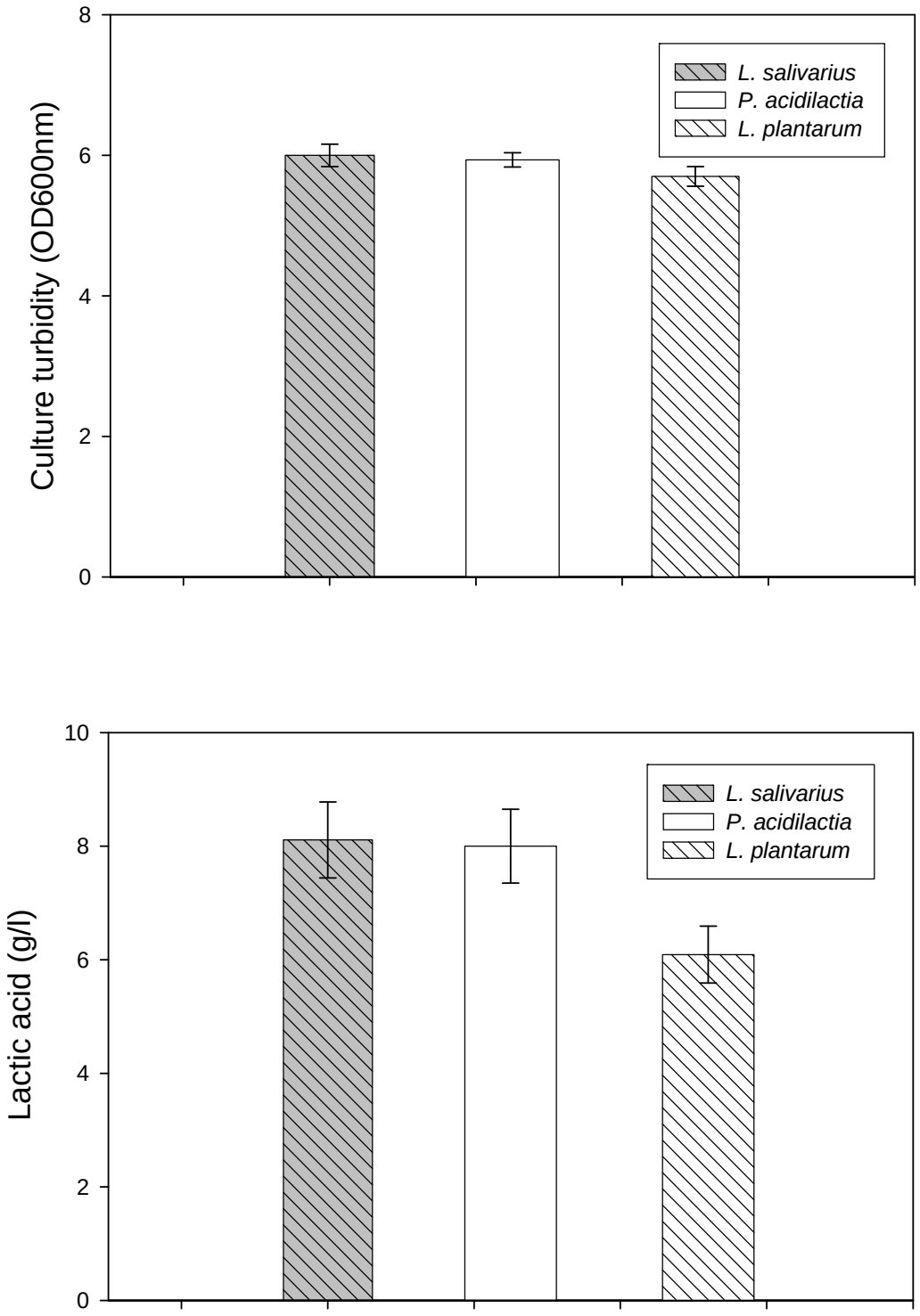

Figure 1. Cell growth and lactic acid production from single-strain cultures. Each number represents the mean ± SD of three replicates.

production in cultures of *P. acidilactici*. In the present study, in an effort to better understand the possible synergetic effects of co-cultured strains, we first examined the free amino acid contents following single-strain cultures of different *Lactobacilli* in MRS medium. We then selected two starter cultures that appeared to overcome each other's nutritional limitations, and showed that the co-culture of *L. salivarius* and *P. acidilactici* increased the

Table 2. Production of free amino acids from mixed cultures grown at optimal inoculation densities at the end of exponential growth (0 h and 8 h).

Amino acids	Ls and Pa (1:1)		Ls and Pa (2:1)		Ls and Pa (5:1)		Ls and Pa (9:1)	
concentration (mg/l)	0 h	8 h	0 h	8 h	0 h	8 h	0 h	8 h
Alanine	200 ± 6	156 ± 5	200 ± 6	232 ± 7	200 ± 6	350 ± 9	200 ± 6	306 ± 9
Asparagine	55 ± 1.7	20.5 ± 1	55 ± 2	29 ± 1	55 ± 2	44.5 ± 1	55 ± 2	33 ± 1
Asparatic acid	124 ± 2	49.8 ± 1	124 ± 2	73 ± 1	124 ± 2	114 ± 2	124 ± 2	95.7 ± 2
Citrulline	45.4	21.2	45.4	18.2	45.4	32.6	45.4	3.12
Cysteine	8 ± 0.1	1.78	8 ± 0.1	4.04	8 ± 0.1	6 ± 0.1	8 ± 0.1	5 ± 0.1
Glutamic acid	428 ± 8	257 ± 7	428 ± 8	388 ± 9	428 ± 8	620 ± 9	428 ± 8	505 ± 9
Glycine	115 ± 2	36.8 ± 1	115 ± 2	173 ± 3	115 ± 2	257 ± 5	115 ± 2	230 ± 4
Histidine	25 ± 0.5	16 ± 0.3	25 ± 0.5	26 ± 1	25 ± 0.5	45 ± 1	25 ± 0.5	33 ± 1
Lysine	149	94	149	129	149	241	149	187.7
Ornithine	5.8 ± 0.1	73 ± 0.7	5.8 ± 0.1	115 ± 1	5.8 ± 0.1	151 ± 2	5.8 ± 0.1	79 ± 0.8
Phenylalanine	120 ± 3	39 ± 1.2	120 ± 3	68 ± 2	120 ± 3	127 ± 4	120 ± 3	113 ± 3
Proline	89 ± 0.9	63 ± 1.3	89 ± 0.9	88 ± 1.8	89 ± 0.9	134 ± 3	89 ± 0.9	120 ± 2
Tyrosine	59 ± 0.6	26 ± 0.3	59 ± 0.6	41 ± 0.4	59 ± 0.6	77 ± 0.8	59 ± 0.6	63 ± 0.6
Arginine	272 ± 3	154 ± 1	272 ± 3	268 ± 2	272 ± 3	193 ± 2	272 ± 3	222 ± 2
Isoleucine	104 ± 1	61.3	104 ± 1	87.3	104 ± 1	143 ± 1	104 ± 1	117 ± 1
Leucine	225 ± 4	112 ± 2	225 ± 4	160 ± 3	225 ± 4	258 ± 5	225 ± 4	217 ± 4
Methionine	49.4 ± 1	20 ± 0.4	49.4 ± 1	21 ± 0.4	49.4 ± 1	17 ± 0.3	49.4 ± 1	668 ± 9
Serine	110 ± 3	65 ± 2	110 ± 3	98 ± 3	110 ± 3	152 ± 4	110 ± 3	131 ± 4
Threonine	88 ± 0.9	53 ± 0.5	88 ± 0.9	76 ± 0.8	88 ± 0.9	141 ± 1	88 ± 0.9	125 ± 1
Tryptophan	27 ± 0.5	8.5 ± 0.2	27 ± 0.5	20 ± 0.4	27 ± 0.5	43 ± 0.9	27 ± 0.5	20 ± 0.4
Valine	155 ± 3	102 ± 2	155 ± 3	135 ± 3	155 ± 3	190 ± 4	155 ± 3	190 ± 4

Data represent the mean values from three independent experiments (*n = 3*) and their standard deviation. Ls, *L. salivarius;* Pa, *P. acidilactici.*

overall cell growth and lactic acid production in our system. In this case, *P. acidilactici* provided essential amino acids (e.g., asparagine, aspartic acid, citrulline, cysteine, glutamic acid, histidine, lysine, ornithine, phenylalanine, proline, tyrosine, arginine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine) required by *L. salivarius*. In particular, the amounts of asparagine, aspartic acid, citrulline, cysteine and arginine had direct effects on lactic acid production.

In an effort to increase the rate and yield of cell growth and lactic acid production in mixed cultures of *L. salivarius* and *P. acidilactici*, in the hope of showing that this system may be suitable for industrial use, we examined four different *P. acidilactici*: *L. salivarius* inoculation ratios: 1:1, 1:2, 1:5, and 1:9 (calculated based on OD_{600}). Due to differences in the proteolytic rates of the two strains and the possible dominance of one strain over another, different inoculation proportions were tested. As shown (Table 2), incubation of mixed cultures for 8 h increased the supernatant levels of alanine, glutamic acid, glycine, histidine, lysine, ornithine, phenylalanine, proline, tyrosine, isoleucine, leucine, methionine, serine, threonine, tryptophan and valine, which were non-essential for the growth of this mixed culture. In contrast, the mixed cultures consumed asparagine, aspartic acid, citrulline, cysteine and arginine, which are essential for the combined growth of these strains. The overall amino acid concentration increased significantly up to ratio 1:5, and decreased thereafter.

In terms of growth and lactic acid production, the 2:1 ratio showed the highest performance compared to the other inoculation ratios (Figure 2), suggesting that this ratio is most appropriate for our mixed culture. The higher tested ratios of the mixed culture (5:1 and 9:1; *L. salivarius* to *P. acidilactici*) inhibited cell growth, whereas a weaker ratio (2:1) was found to be optimal for cell growth and lactic acid production. We speculate that the stronger ratios (5:1 and 9:1) did not provide sufficient essential amino acids for cell growth and lactic acid production, whereas the weaker ratios (1:1 and 2:1) provided sufficient essential amino acids and probably stimulated the metabolism required for cell growth and lactic acid production.

We hypothesized that stimulation of the proteolytic system in a two-strain mixed culture would increase the release of amino acids that are essential for the growth of both strains. Jini et al. (2011) previously reported that *P. acidilactici* exhibited high proteolytic activity and good antagonism toward bacterial pathogens. In the present

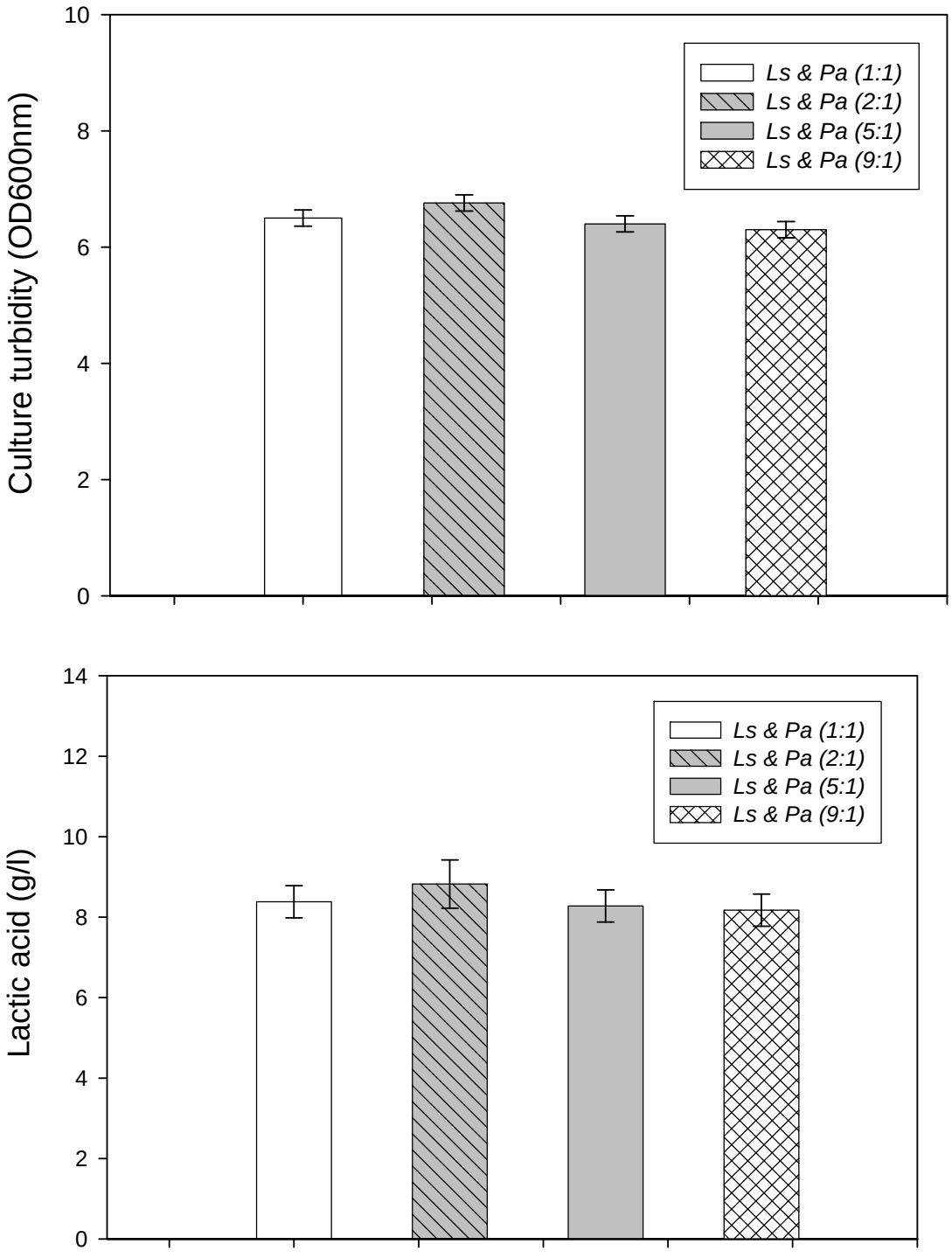

Figure 2. Cell growth and lactic acid production from mixed strain cultures under various inoculation density. Each number represents the mean ± SD of three replicates.

work, proteins were hydrolyzed when the extracellular proteinase produced by *P. acidilactici* accumulated in the medium in pure or mixed cultures of this strain. The proteolytic *P. acidilactici* showed higher growth parameters and efficiently produced a subset of amino acids that largely corresponded to those consumed by *L. salivarius*. We speculate that stimulation of the proteolytic system in a mixed culture of the two would increase the release of amino acids that are essential for the growth of both strains. Hence, we hypothesized that co-culturing

our strains of *L. salivarius* and *P. acidilactici* could enhance the growth of *L. salivarius* and overall lactic acid production.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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