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Effect of Glycine on the Growth of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* in *Kimchi* Fermentation

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Abstract This study was aimed to investigate the effect of glycine supplementation on the growth of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* during *kimchi* fermentation. As preliminary experiment, the effect of supplementation of glycine (0-2.0%, w/v) to MRS medium on the growth of *Leuc. mesenteroides* and *L. plantarum* was evaluated. At 2.0% glycine in the MRS medium, cell growth rate was inhibited by 79% for *L. plantarum* and 27% for *Leuc. mesenteroides*. Subsequently, different concentrations of glycine (0, 0.5, and 2.0%, w/v) were applied for *kimchi* fermentation for 21 days, at 5°C. At day 14, the pH and titratable acidity (TA) of *kimchi* supplemented with 2.0% glycine were 4.83 and 0.38%, respectively, whilst the control *kimchi* had a pH of 4.49 and TA of 0.44%, respectively. The ratio of *Leuconostoc*/*Lactobacillus* in *kimchi* increased as the concentration of glycine increased. The results show that the presence of glycine affected the growth of the 2 lactic acid bacteria, particularly of *L. plantarum*.

Keywords: *kimchi*, fermentation, glycine, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*

Introduction

A number of health benefits have been claimed for lactic acid-producing bacteria (LAB). As a result, many products containing one or more group of LAB are available worldwide (1,2). *Kimchi* is a Korean traditional fermented vegetables similar to sauerkraut, which is a popular fermented vegetable product in the West. *Kimchi* has been developed to give vegetables an extended storage life (3,4). The starter culture for *kimchi* fermentation consists of the normal mixed biota of Chinese cabbage and other vegetables (5). Some important species believed to be responsible for *kimchi* fermentation include *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus lactis, Leuconostoc mesenteroides,* and *Leuconostoc pseudomesenteroides.* Of these, the typical *kimchi* fermentation is dominated by the 2 species of LAB, *Leuc. mesenteroides* and *L. plantarum. Leuc. mesenteroides,* a dominant microbe at the early stage of *kimchi* fermentation, is a heterofermenter producing acetic acid and lactic acid as the major products from fermentation. Homofermentative *L. plantarum* is predominant at a relatively high fermenting temperature and predominates at the later stage of *kimchi* fermentation, making overly acidic *kimchi*, which is unacceptable by consumers. After the fermentation period, these microbial species continue to grow, which results in a sour and bitter taste, off-odor, and softening of the product resulting in deterioration of *kimchi* (6,7). Therefore, a control of the fermentation process is needed to preserve the quality of *kimchi* and to extend its shelf-life.

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Over the last several decades, considerable data have been published on ways of extending shelf-life of *kimchi*, including use of antimicrobial agents (8), ark shell powder (9), heat treatments (10), and a combination of N_2-packaging and heating with irradiation (11). Even though these methods have shown some positive effect in extending the shelf life, chilled storage between 1 to 5°C is generally acceptable to maintain the quality of *kimchi* (12). In our previous studies (13,14) and those of others (15), high dose of glycine showed a reduction in cell growth of microbes, including Gram-positive and Gram-negative bacteria. Hammes *et al.* (16) reported that the structure of peptidoglycan in the Gram-positive cell wall was altered following growth in the medium containing glycine. These authors suggested that glycine substituted for alanine in the peptidoglycan, impairing cross-linking and weakening the peptidoglycan structure. However, there is little information available regarding the growth of 2 LAB, *L. plantarum* and *Leuc. mesenteroides*, in the presence of glycine in the MRS medium. In order to assess viability and survival of *L. plantarum* and *Leuc. mesenteroides*, it is also important to have data for enumeration of these bacteria in the presence of other microbes in *kimchi*. The objective of this study was to investigate the effect of glycine supplementation on the growth of *L. plantarum* and *Leuc. mesenteroides* during *kimchi* fermentation.

Materials and Methods

Bacterial cultures and propagation *Lactobacillus plantarum* KCTC 3928 and *Leuconostoc mesenteroides* (GeneBank Accession No. AY675249) were obtained from the Cell Biotech Co., Ltd. (Gimpo, Gyeonggi, Korea). MRS (Difco, St. Louis, MO, USA) was used for the cultivation of the 2 bacteria. The purity of glycine was above 99% and it was purchased from Samchun Pure

Chemical Co., Ltd. (Pyeongtaek, Gyeonggi, Korea). To test the effect of glycine on the growth of the 2 LAB, cells were inoculated using 1% inoculum into 30 mL quantities of MRS broth supplemented with 0, 0.5, 1.0, 1.5, and 2.0%(w/v) of glycine, with an initial $Abs_{600\,nm}$ of 0.2-0.3, and incubated at 5±3°C without agitation.

Cell growth rates were compared by measuring m value (specific cell growth rate, μ, 1/hr) and the period of lag phase of the growth. When $Abs_{600\,nm}$ exceed 0.6, cultures were diluted in the MRS and $Abs_{600\,nm}$ recorded against MRS. The μ value (μ, 1/hr) of the 2 LAB was expressed as the percentage relative to the growth in the MRS without glycine. For calculation of m value, samples were withdrawn at hourly intervals and optical density was measured at $Abs_{600\,nm}$ using UV-visible spectrophotometer (UV-1650; Shimadzu, Kyoto, Japan). The doubling time was measured during the exponential growth period form the slope of the curve obtained by plotting the logarithm of $Abs_{600\,nm}$ against time, and cell specific growth rate was calculated according to Aiba *et al.* (17) as follows:

Cell specific growth rate (μ, 1/hr)=0.693/doubling time (hr)

***Kimchi* preparation** *Kimchi* was prepared with the following composition; Chinese cabbage 85.7, hot pepper 5.6, minced garlic 2.6, salted shrimps 1.7, green onion 1.7, salted anchovies 1.3, and minced ginger 1.3%. To prepare *kimchi*, Chinese cabbage was trimmed and cut into pieces (4×4 cm) and then brined for 2 hr in 20%(w/v) NaCl solution. After completely removing residual salt by repeated washing using water for 1 min, the Chinese cabbage was drained at room temperature for 2 hr. The sliced hot pepper, salted shrimps, green onion, salted anchovies, and minced garlic were mixed together in a metal bowl. The Chinese cabbage was mixed with the condiment followed by addition of 0.5 and 2.0% of glycine. The control *kimchi* contained no glycine. About 300 g of *kimchi* was packed in plastic jars without any headspace, capped, and stored for 21 days at 5°C. All *kimchi* preparations were carried out in duplicate.

Chemical analysis The tissues and juice in *kimchi* were homogenized together using a mixer (HMF 370; Hanil Electric Co., Seoul, Korea) for 1 min and filtered through a sterile gauze. The pH of the supernatant was determined using a pH meter (Lstek Co., Seoul, Korea). Titratable acidity (TA) was determined by titrating 5 mL of the filtrate with 0.1 N NaOH and was expressed as the quantity of lactic acid produced in *kimchi*. Lactic and acetic acids were determined using high performance liquid chromatography (HPLC). Briefly, the filtrate was passed through C_{18} SEP-PAK filter catridges (Sulpeco Co., Bellafonte, PA, USA) to remove residual proteins, liquids, and chromophores. After prewetting and sample addition, 20 μL of the filtrate eluted from the SEP-PAK catridges was injected directly into the HPLC (Agilent Technologies Inc., Santa Clara, CA, USA). The HPLC was equipped with a Supelcogel C-610H (7.8 mm i.d.×30 cm) column (Sulpeco Co.). The HPLC conditions were as follows: mobile phase, 0.1% phosphoric acid (isocratical elution); flow rate, 0.5 mL/min; detector, UV/VIS $Abs_{210\,nm}$; running time, 30 min.

Enumeration of bacteria Following incubation, 10 fold serial dilutions of cells were made and appropriate dilutions spread onto plates to enumerate viable cells of the 2 LAB. For enumeration of *L. plantarum,* Rogosa SL agar (Difco) was used. For *Leuc. mesenteroides*, phenylethyl alcohol agar (Difco) supplemented with 1%(w/v) sucrose was used (18). Bacterial numbers were counted in triplicate after incubation at 30°C for 36-48 hr. Results of viable cell counts were presented as the average values of colony-forming units (CFU)/g of *kimchi*.

Statistical analysis Data were analyzed using one-way analysis of variance (ANOVA) at 95% level of significance (19). All experiments were replicated twice and all analyses were carried out in triplicates. The results presented are a mean of 6 observations±standard deviations (SD).

Results and Discussion

Effect of glycine on growth of *Leuc. mesenteroides* and *L. plantarum* The effect of glycine on growth of 2 LAB, *Leuc. mesenteroides* and *L. plantarum,* associated with *kimchi* fermentation is shown in Fig. 1 and Table 1. For both strains, the growth rate little changed with glycine concentration between 0 to 0.5%, but it decreased with further increasing glycine concentration. *Leuc. mesenteroides* was more resistant to growth inhibition by glycine (Fig. 1). *L. plantarum* was highly sensitive to glycine and the difference in sensitivity was more significant at higher concentrations; 79% inhibition of specific cell growth rate occurred at 2.0% glycine. For *Leuc. mesenteroides,* the specific cell growth rate decreased by 27% when grown in the presence of 2.0% glycine (Table 1). Generally, cell growth started after a short lag phase as demonstrated by the increase in absorbance when the culture entered into the exponential phase of growth. Glycine did not alter the lag period. At above 1.0% level of glycine, the curve showed a similar pattern, but the values of absorbance at each point were lower than those of the control.

Using the purified UDP-*N*-acetylmuramyl:L-alanine ligase from *Escherichia coli*, which is involved in the biosynthesis of peptidoglycan, Gubler *et al.* (20) calculated that the rates of incorporation of glycine into peptidoglycan precursor metabolites could maximally amount to 0.5% of the rate of L-alanine incorporation. Therefore, it can be postulated that 0.5-1.0% glycine may be enough to impair the peptidoglycan structure of *E. coli*. In contrast, 1.0% or higher concentration of glycine may be required to cause irreversible changes in the cell wall structure of Gram-positive bacteria, since the inhibitory effects of glycine on specific growth rates varied from strain to strain, reflecting the differences in cell wall composition and structure between strains (13). The concentration of glycine required to inhibit the cell growth of 2 LAB was between 1.0-2.0%(w/v) and this range is similar to that reported for Gram-positive bacteria, including *Bacillus subtilis* (21) and *Corynebacterium glutamicum* (22). It has been noted that a part of the barrier to efficient DNA transformation into LAB stemmed from the physical structure of the cell wall, and prior growth of *Lactococcus lactis* subsp. *cremoris* in 3.0-4.0% glycine significantly improved transformation efficiency (23).

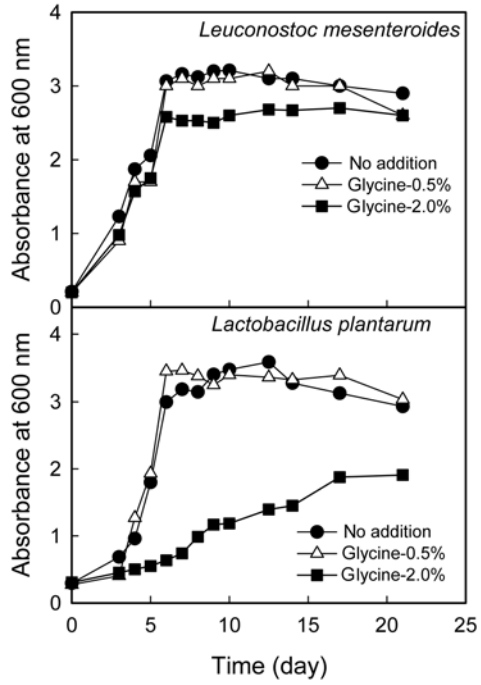

Fig. 1. Effect of glycine on growth of *Leuc. mesenteroides* and *L. plantarum* at 5±3°C.

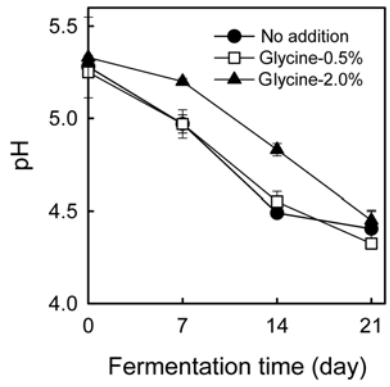

Fig. 2. Changes in pH of *kimchi* at 5°C for 21 days with or without glycine. Data are the mean±SD (n=6).

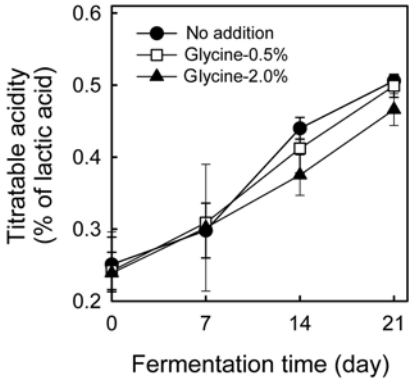

Fig. 3. Changes in titratable acidity of *kimchi* at 5°C for 21 days with or without glycine. Data are the mean±SD (n=6).

Changes in pH, TA, and organic acid content of *kimchi* in the presence of glycine The effect of supplementation of glycine on the *kimchi* fermentation was investigated using 0.5 and 2.0% glycine concentrations (Fig. 2, 3; Table 2, 3). Instead of designing experiments to cover a full possible concentration of glycine, these 2 concentrations were used and the results compared with an appropriate control without any glycine. Since the growth of 2 strains was not affected significantly with glycine concentration between 0 to 0.5%, a glycine concentration of 0.5%, was chosen for further study. A glycine concentration of up to 2.0% was also included for further study since the 2 strains showed significant difference in inhibition with 2.0% glycine. Figure 2 shows the changes in the pH of *kimchi* during fermentation for 21 days.

As fermentation proceeded, pH of the control *kimchi* changed from 5.3 to 4.5 during fermentation for 14 days (Fig. 2). The higher the concentration of glycine in the *kimchi*, the higher the pH remained. For example, at 14 days of fermentation, the *kimchi* supplemented with glycine had a relatively higher pH value than the control *kimchi* (p<0.001). It is interesting to note that, although fermentation in glycine supplemented *kimchi* was retarded for extended periods when *kimchi* contained 0.5 and 2% glycine, the final pH at day 21 remained almost the same as was observed for the control *kimchi*. These observations indicate that glycine was toxic to cells during 14 days of *kimchi* fermentation but, once growth started, cells could cope with this concentration. When we analyzed the glycine concentration in *kimchi* supplemented with 0.5 and 2% glycine using amino acid analyzer, we could not observe the difference in glycine concentration between 0

Table 1. Effects of glycine on cell specific growth rate (μ, 1/hr) and duration of lag phase (hr) of *Leuc. mesenteroides* and *L. plantarum* at 5±3°C

Strain	Parameters[1)]	Glycine concentration (%, w/v)					
		0	0.1	0.5	1.0	1.5	2.0
Leuc. mesenteroides	μ value (1/hr)	0.373	0.321	0.321	0.393	0.393	0.272
	% μ value	100	86.1	86.1	105.3	105.3	72.9
	lag phase (hr)	0.5	1	1	1	1	1
L. plantarum	μ value (1/hr)	0.506	0.506	0.503	0.394	0.321	0.107
	% μ value	100	100	99.4	77.9	63.4	21.2
	lag phase (hr)	2	3	3	3	3	3

[1)]The percentage specific growth rate (% μ value) of the 2 lactic acid bacteria was expressed as a percentage relative to the growth in the MRS without glycine. Duration of lag phase was the time taken before exponential growth started.

Table 2. Concentration of acetic and lactic acids (g/100 g) in *kimchi* supplemented with glycine at 5°C for 21 days[1)]

	Fermentation period			
	Day 0	Day 7	Day 14	Day 21
Lactic acid (LA)				
Control	0.12	0.28	0.54	0.61
0.5% glycine	0.14	0.25	0.43	0.57
2.0% glycine	0.10	0.21	0.33	0.54
Acetic acid (AA)				
Control	0.02	0.07	0.08	0.10
0.5% glycine	0.03	0.06	0.07	0.09
2.0% glycine	0.04	0.06	0.09	0.12
LA/AA[2)]				
Control	6.00	4.00	6.75	6.10
0.5% glycine	4.67	4.17	6.14	6.33
2.0% glycine	2.50	3.50	3.67	4.50

[1)]0.5% glycine, 0.5% glycine supplemented *kimchi*; 2% glycine, 2% glycine supplemented *kimchi*.
[2)]Ratio of lactic acid to acetic acid.

and 21 days *kimchi* samples, showing that majority of glycine supplemented was not metabolized during *kimchi* fermentation (data not shown). However, mechanisms of cell adaptation were not further investigated here. *Kimchi* achieves its best flavor, taste, and texture when properly fermented at pH between 4.1 and 4.5 (24). The pH of the control *kimchi* was 4.5 after 14 days of fermentation ($p<0.001$), while the pH of *kimchi* supplemented with 2.0% glycine was 4.5 after 21 days of fermentation ($p<0.001$) (Fig. 2). The delay in pH drop found in *kimchi* supplemented with 2.0% glycine could be explained by the inhibitory action of glycine on the growth of LAB (Fig. 1).

Changes in the TA of *kimchi* during fermentation for 21 days are shown in Fig. 3. An increase in TA was found as fermentation proceeded but the TA in *kimchi* supplemented with 2.0% glycine increased more slowly than in the control *kimchi*. TA changed significantly after 21 days ($p<0.05$). The differences in TA in *kimchi* supplemented with 0.5% glycine were not statistically significant ($p=0.10$) from that of control *kimchi*. TA and pH are considered as the major quality attributes of *kimchi* because it has the characteristic sour taste (23). Ku *et al.* (24) reported that commercially available *kimchi* showed a TA ranging from 0.28 to 1.00%, which is consistent with the range of 0.30-0.51% at 5°C found in this study (Fig. 3). Kim (25) found that TA during *kimchi* fermentation at 10°C at day 14 was 0.99%.

Acid production, routinely determined by pH measurement, has been used as useful index for evaluating *kimchi* fermentation. The acids found in *kimchi* are lactic, acetic, succinic, malic, and citric. Malic and citric acids are released from the vegetables. However, other acids including acetic, lactic, and succinic are produced during *kimchi* fermentation (9). Lactic acid was the dominant organic acid during the *kimchi* fermentation (Table 2). At the beginning of *kimchi* fermentation, the concentration of acetic acid was less than 0.05%(w/v) (Table 2). With increasing fermentation time, the amount of acetic and lactic acids from *kimchi* supplemented with 2% glycine increased but the rate of their production was much less than for the control *kimchi*. The level of lactic acid formation in control *kimchi* was slightly lower at the later stage of fermentation (Table 2). The production of lactic acid and acetic acid showed a close relationship with the distribution of LAB in *kimchi*. The main LAB in *kimchi* are *Leuc. mesenteroides* and *L. plantarum*. *Leuc. mesenteroides* produces acetic acid, carbon dioxide, and lactic acid, while *L. plantarum* produces solely lactic acid (7,25). Acetic acid in the *kimchi* supplemented with 2.0% glycine, however, was similar to that of the control *kimchi* (Table 2). The ratios of lactic acid to acetic acid were respectively 6.75 in the control *kimchi* and 3.67 in that supplemented with 2.0% glycine at 14 days of fermentation (Table 2). It was reported that the ratio of lactic acid to acetic acid in the *kimchi* during 14-21 days of

Table 3. Changes in the counts of the 2 lactic acid bacteria cells (CFU/g of *kimchi*) in *kimchi* supplemented with glycine during fermentation at 5°C for 21 days

	Fermentation period			
	Day 0	Day 7	Day 14	Day 21
Leuconostoc spp.				
Control	$2.2\times10^{6\,aA1)}$	$7.8\times10^{5\,aA}$	$6.4\times10^{6\,aA}$	$8.0\times10^{7\,aB}$
0.5% glycine	$3.0\times10^{6\,bA}$	$7.0\times10^{5\,aA}$	$3.7\times10^{6\,bA}$	$5.5\times10^{7\,aB}$
2.0% glycine	$2.8\times10^{6\,abA}$	$5.6\times10^{5\,aA}$	$2.2\times10^{6\,bA}$	$6.5\times10^{7\,aB}$
Lactobacillus spp.				
Control	$3.1\times10^{6\,aA}$	$1.7\times10^{7\,aA}$	$7.6\times10^{7\,aB}$	$9.2\times10^{7\,aB}$
0.5% glycine	$3.4\times10^{6\,aA}$	$9.5\times10^{6\,abA}$	$4.2\times10^{7\,bB}$	$5.4\times10^{7\,bB}$
2.0% glycine	$4.4\times10^{6\,aA}$	$6.6\times10^{6\,bA}$	$1.4\times10^{7\,cBA}$	$6.3\times10^{7\,bB}$
Ratio of Leu/Lac[2)] (%)				
Control	71.0	4.6	8.4	87.0
0.5% glycine	88.2	7.4	8.8	101.9
2.0% glycine	63.6	8.5	15.7	103.2

[1)]Values are the means of 6 observations at 95% level of confidence; Means with different alphabets in the same column$^{(abc)}$ and row$^{(ABC)}$ are significantly different, respectively for a particular day of fermentation for each lactic acid bacteria and within a particular treatment for each organism.
[2)]Leu: *Leuconostoc* species, Lac: *Lactobacillus* species.

fermentation ranged between 1.1-11.6 (9,25,26). It appears that there were differences in sensitivity to glycine (Table 1); the production of acetic acid was less affected by the presence of glycine in *kimchi* (Table 2), indicating that the supplementation of glycine (0.5-2.0%) might be more inhibitory to the homofermentative LAB such as *L. plantarum* than heterofermentative LAB such as *Leuc. mesenteroides*.

Viable cell of LAB In order to determine the effect of glycine supplementation on LAB in *kimchi*, enumeration of LAB was carried out using selective media. The growth of *Leuconostoc* species and *Lactobacillus* species in the 3 batches of *kimchi* during fermentation is shown in Table 3. When no glycine was added to the *kimchi*, viable cells of the *Leuconostoc* species reduced slightly from 2.2×10^6 to 7.8×10^5 at 7 days of fermentation, thereafter increased to 8.0×10^7 CFU/ g of *kimchi* at 21 days of fermentation. On day 14, *Leuconostoc* species in control *kimchi* showed 6.4×10^6 CFU/g of *kimchi*, whereas that supplemented with 0.5 and 2.0% glycine showed 58 and 34% of the viable cells in the control *kimchi* ($p<0.05$). *Lactobacillus* species in the control *kimchi* increased markedly until day 14, and then their counts remained similar thereafter. On day 14, *Lactobacillus* species in control *kimchi* showed 7.6×10^7 CFU/g of *kimchi*, whereas that supplemented of glycine 0.5 and 2.0% glycine showed 55 and 18% of the viable cells in the control *kimchi* ($p<0.05$). Supplementation of 0.5% glycine to *kimchi* was found to be suitable for retardation of the growth of *Leuconostoc* species and *Lactobacillus* species. *Kimchi* supplemented with 2.0% glycine showed a more profound effect than that was observed with 0.5% glycine; this is consistent with result in Fig. 1. On the basis of the above results, it can be concluded that glycine can inhibit the growth of *Leuconostoc* species and *Lactobacillus* species in the mixed food system, *kimchi*, as well as in pure culture. Since 2 LAB grew much more slowly in glycine supplemented *kimchi* than in the control *kimchi*, enumeration of cells was expressed as percentage ratio of *Leuconostoc* species/*Lactobacillus* species to enable comparison between different *kimchi* (Table 3). *Lactobacillus* species was more sensitive to glycine than *Leuconostoc* species, as observed from the preliminary experiments (Fig. 1). The ratio of *Leuconostoc* species/*Lactobacillus* species in *kimchi* increased as the concentration of glycine in *kimchi* increased. These observations indicate that glycine was effective in inhibition of both LAB, particularly of *Lactobacillus* species. Another point to be noted is the viable cell numbers of *Lactobacillus* species during fermentation, in particular, in the early stages of the *kimchi* supplemented with 2.0% glycine. For instance, the number of *Lactobacillus* species in *kimchi* supplemented with 2.0% glycine increased slowly by 3 fold at 14 days of fermentation ($p<0.05$), and thereafter further increased by 5 fold at 21 days of fermentation ($p<0.05$). Glycine appeared to be inhibitory, particularly to *Lactobacillus* species during lag phase; however, once growth started, cells were able to cope with these concentrations. Although it is difficult to compare this phenomenon across species, the supplementation of high concentration of glycine into the growth medium often caused increase in the lag phase. For instance, when cells of *Corynebacterium glutamicum* were grown in the presence of 2% glycine and 4 mg/mL of isonicotinic acid hydrazide, the values of $Abs_{600\,nm}$ were often seen to fluctuate; $Abs_{600\,nm}$ increased for several hours before decreasing and, after lag phase, increased continuously until stationary phase (27).

The supplementation of glycine to MRS between 1.0 to 2.0% decreased growth of *Leuc. mesenteroides* and *L. plantarum* associated with *kimchi* fermentation. The organism involved in the latter part of *kimchi* fermentation, *L. plantarum*, was highly sensitive to glycine and the difference in sensitivity was more obvious at higher concentrations of glycine. Based on the results, the fermentation pattern of the *kimchi* supplemented with glycine was superior to that of the control. Supplementation of glycine to *kimchi* had a relatively higher pH value, lower titratable acidity, and lower lactic acid content than the control *kimchi*. In addition, the ratio of *Leuconostoc*/ *Lactobacillus* in *kimchi* increased as the concentration of glycine increased, showing that the presence of glycine inhibited the growth of the 2 LAB, particularly *L. plantarum*. Thus, it appears that supplementation of glycine to *kimchi* may prolong the shelf life of *kimchi*.

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