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Production and Characterization of *Kimchi* with Enhanced Levels of γ-Aminobutyric Acid

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Abstract In the development of a nutrient enhanced functional food, *kimchi* was produced by using high γ-aminobutyric acid (GABA) producing lactic acid bacterium as a starter strain. The strain isolated from *kimchi* was identified by using an API kit and named *Lactobacillus* sp. OPK 2-59. *Kimchi* was produced by 3 methods 1) monosodium glutamate (MSG) added (M group); 2) starter added (S group); 3) MSG+starter added (M&S group). The produced *kimchi* was fermented for 24 hr in an incubator at a temperature of 15°C and stored at 0-1°C to examine its characteristics. The M&S group exhibited a sharper increase in acidity and a steeper fall in pH as well as a higher number of lactobacilli. The M&S group *kimchi* had 18 mg/100 g (fresh weight, f.w.) of GABA, whereas the M and S group each had 6 mg/100 g (f.w.) GABA. According to functional evaluation, the M&S group *kimchi*, which has higher GABA, was not significantly different in taste, color, texture, or smell, but the M&S group was generally superior. In summary, using *Lactobacillus* sp. OPK 2-59 and MSG, a high quality *kimchi* with increased GABA content can be produced as a functional food.

Keywords: *kimchi*, γ-aminobutyric acid, starter, production, characterization

Introduction

Kimchi, a traditional Korean fermented food, is made by salting Oriental radish, Chinese cabbage, and cucumber, mixing them with spices such as red pepper, garlic, green onion, ginger, and pickled fish and then allowing it to mature with lactic acid bacteria at low temperature. In Korean provinces, it is generally called '*ji*' and when performing ancestral rites, it is called '*chimchae*' and at royal palaces, it was called as '*jutkookji*', '*jjanji*', or '*singgunji*'. (1). Making *kimchi* was a way to preserve vegetables for a long period and also to make an excellent fermented food, as it contains various microorganisms and creates organic acid and aroma during storage. *Kimchi* has a proud cultural heritage, and is widely known in other countries as a traditional Korean food. Recently, exports of *kimchi* have been rising and it has been developed as a global cultural product by its registration with CODEX in 2001 (2). Modern research has revealed that *kimchi* contains vitamins such as A, B complex, and C and is full of healthy lactobacilli bacteria (3), so it helps us to digest food and is also known to contain bioactive compounds which prevent growth of cancer cells. So it has been selected as one of the world's five healthiest foods (4).

γ-Aminobutyric acid (GABA) is a non-protein amino acid (5,6) which is known to act in the central nervous system as a postsynaptic inhibitory neurotransmitter in the brain, increase blood flow to the brain in animals and enhances metabolism of brain cells by increasing the oxygen supply. It also participates in the regulation of the secretion of growth hormones (7) and is effective for easing pain and lowering blood pressure (8), so pharmacologically, people are very interested in this material. GABA is produced primarily by the α-decarboxylation of L-glutamic acid (Glu) catalyzed by the enzyme glutamate decarboxylase (GAD) (9). Consumption of GABA-enriched foods such as milk (10), soybean (8), gabaron tea (11), red yeast rice (12), and chlorella (13) have been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs). For example, Hayakawa *et al.* (10) showed that GABA-enriched milk (1 nmol/mL) lowered the blood pressure in spontaneously hypertensive and normotensive Wistar-Koto rats.

Currently, red yeast rice, a kind of yeast, is actively being studied for applications in GABA production by improving the existing strains (14,15). Moreover, strain isolation and its mass culturing were already attempted with the intention of producing GABA using methods compatible with industrial and functional materials (16). However, attempts at producing high-GABA *kimchi* products, using high-GABA generating strains and Glu, have not been done. Recently GABA demand increased upon the easing control over the medicine and food in 2001 in Japan and actively used in chocolate, gum, bean curd, coffee, beverages etc as a healthy food material. Although GABA is widely used as an additive material for food products in Japan, such products with added GABA are still remain prohibited in Korea. If a method for the production of high-GABA *kimchi* products is developed by using GABA producing lactobacilli, it will be a great help for the bioactive *kimchi* production and marketing. Production of such *kimchi* product will enhance a business effectiveness due to its merchandising and effectiveness in protecting

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people's health through the promoted *kimchi* consumption and contribution to the *kimchi* world wide promotion by our effective advancement.

This study examined the characteristics of a functionally favorable *kimchi* with a higher content of GABA, by using monosodium glutamate (MSG) and high-GABA producing strain isolated from *kimchi* to produce *kimchi* with enhanced nutritional functions.

Materials and Methods

Isolation of the ferentation starter strain Samples from homemade *kimchi* were diluted between 10^{-1} and 10^{-8}, using a 1% peptone solution. Then, 1 mL of aliquots was incubated on De Man, Rogosa, and Sharpe (MRS) (Difco, Detroit, MI, USA) agar, with 0.002% bromocresol purple (BCP) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) added, at 25°C for 72 hr in order to observe the colonies. Among the colonies, those which had no ring and a dark blue color were classified as *Leuconostoc* (17). The colonies which had a ring and light blue or white color were classified as *Lactobacillus*. Fermentation characteristics and biochemical features of the isolated strains were tested by using an API 50 CHL kit (BioMerieuxsa, Marcy-I'Etoile, France). The classified yellow colony was inoculated with an MRS medium followed by mixing the cultured strain with glycerol and then stock was made to keep and test. Inoculating the separated strain with the 1% of MSG added MRS and then cultivated 48 hr with 30°C, and then verified the existence or non-existence of GABA productivity using thin layer chromatography (TLC, silica gel 60 F_{254}; Merck, Damstadt, Germany) (18). Standard GABA (Sigma-Aldrich) was prepared with 0.1 M, and spotted over the silica gel 0.5 μL one time. The strain culture solution 0.5 μL was spotted 3 times. In selection strains, spots were selected which showed similar values to the standard retardation factor (R_f) values.

Production of starter The *Lactobacillus* sp. OPK 2-59 (KFCC 11392) strain, which had been grown in the laboratory, was inoculated into *Lactobacillus* MRS broth+ 1% MSG (4%, v/v), and the inoculum incubated at 30°C for 48 hr and centrifuged at 5,000×g for 20 min and cells collected. The cells were then lyophilized and saved for use as a starter for production of *kimchi* (17).

Materials and strains To prepare the *kimchi*, Chinese cabbage, onion, minced ginger, minced garlic, minced green onion, and carrots were purchased at the Noughyup Hanaro market in Jeonju, Korea, and stored in a refrigerator at 5°C. Hot pepper, white sugar, salt (CJ Corp., Seoul, Korea), and MSG (Bedan Enterprise Corp., Taiwan) were purchased from a local food company and stored at room temperature and wrapped in aluminum foil (19).

Salted *kimchi* and dressing materials Cleaned and trimmed Chinese cabbage was cut into pieces, and immersed in a 10%(w/w) NaCl solution for 10 hr. After removing some residual salt with tap water, the cabbage was drained at room temperature for 1 hr (20). The total salt content was 2.7% of the preserved cabbage. Dressing materials are shown in Table 1.

Table 1. The dressing materials of *kimchi*

Materials	Weight (g)
Red pepper	655
Onion	2,415
Ginger	145
Garlic	915
Sugar	70
Salt	80
Green onion	400
Carrot	535
Total	5,215

***Kimchi* production and fermentation** The salted cabbage and dressing materials were mixed in a 7:3 ratio. To the total weight was added 0.1% of MSG and 0.2% of starter. Test samples were; 1) MSG addition sample, 2) starter addition sample, and 3) MSG+starter addition sample (Table 2). The *kimchi* was fermented in the incubator at 15°C for 24 hr and afterward was kept in a *kimchi* refrigerator (0-1°C). Every 3 days the *kimchi* was sampled and evaluated (21).

Measurement of pH The pH of the *kimchi* juice was determined using a pH meter (Microprocessor Bench HI8417; Hanna, Rome, Italy) at a room temperature (22).

Measurement of acidity The 5 mL of the aforementioned *kimchi* juice was mixed with 5 mL 0.1 N NaOH using a vortex mixer and the acidity of *kimchi* was measured using a *kimchi* acidity meter (GMK-885; G-WON Hitech, Seoul, Korea).

Measurement of sugar reduction The reducing sugar was measured according to the dinitrosalicylic acid (DNS) method (23,24). The samples were homogenized in a blender (HMF-392; Hanil, Seoul, Korea) with 50 mL distilled water and filtered through gauze. The filtered juice was then diluted 10-fold and filtered through a 0.45-μm PVDF filter (Millipore Corp., Bedford, IN, USA). One of each filtered sample was added to the 3 mL DNS and vortexed. Then Rochell salt was added and 1 mL was placed in a boiling water bath for 5 min and immersed into cool running water. Optical densities of the samples were measured at 550 nm using an ultra violet (UV)-VIS spectrophotometer (UV1600 PC; Shimadzu, Kyoto, Japan). The reducing sugar was expressed as D-glucose/mL from a standard curve line derived from D-glucose standards.

Lactic acid bacteria analysis The aforementioned gauze filtered juice was diluted with 0.1% peptone water. MRS agar+0.002% BCP was employed for the determination of lactic acid bacteria (25). All plates were triplicated, incubated for 48 hr at 30°C, and the viable cell numbers were determined as yellow colony forming units (CFU)/mL (26).

Measurement of GABA in *kimchi* GABA was extracted essentially as described by Baum *et al.* (27) with minor modifications. Briefly, 800 μL of organic solvent solution

Table 2. The production method of *kimchi* samples[1)]

Group[2)]	Starter (g)	MSG (g)	Salt cabbage (kg)	Material (kg)
M	×	5	3.5	1.5
S	10	×	3.5	1.5
M&S	10	5	3.5	1.5

[1)]The salted cabbage and dressing materials were mixed in a 7:3 ratio. To the total weight was added 0.1% of MSG and 0.2% of starter.
[2)]M, MSG addition sample; S, starter addition sample; M&S, MSG+ starter addition sample.

(methanol:chloroform:water=12:5:3) was added to 200 μL of the *kimchi* suspension sample. The aqueous solution layer containing GABA was obtained by centrifugation (13,000×g, 4°C, 15 min), and the obtained supernatant was recentrifuged to remove the remnant impurities. The supernatant was then freeze-dried, resuspended in water, filtered through a 0.45-μm PVDF membrane and analyzed by high performance liquid chromatography (HPLC, Waters, Milford, MA, USA) after 6-aminoquioly-*N*-hydroxysuccinimidyl carbonate (AQC) derivatization. To separate the derivatives, 3.9×150 mm AccQ · Tag™ (Nova-Pak™ C18; Waters) column with 37°C, mobile phases (AccQ · Tag Eluent A and 60% acetonitrile) with 1.0 mL/min flow rate, and a fluorescence detector (Waters) were used (17). The GABA content was calculated using a commercial GABA standard based on a standard curve.

Sensory evaluation The sensory test was conducted on the M, S, M&S samples incubated at 15°C for 24 hr and then stored at 0-1°C. *Kimchi* aged for 21 days was selected for sensory evaluation after some repeated experiments. Sensory evaluations of the taste, smell, texture, and color were conducted by the panels, using a 5-point hedonic scale method. Very poor was corresponded to 1.0 and very good to 5.0. The panelists were selected from members of the Department of Food and Biotechnology, Woosuk University (28). The results of the sensory evaluations were expressed as the mean ± standard deviation (SD) of 16 panelists. The significance was verified via Duncan's multiple range tests, using the SPSS software package (29,30).

Results and Discussion

Isolation and identification of strain To make *kimchi*, with high-GABA content and enhanced nutritional functions, lactic acid bacteria with good GABA generating capability were isolated from *kimchi*. Extracted lactobacilli from *kimchi* for the test of GABA generating capability were in quantity of approximately 1,300 in total. The strain, initially isolated by smearing on a medium containing 0.002% BCP in MRS broth was cultured in MRS liquid medium with 1% MSG. The result showing highly productive GABA lactobacillus was confirmed by TLC as in Fig. 1. As Fig. 1 shows, the spot corresponding to the OPK 2-59 strain is in the same location as the standard GABA spot. After examining the biochemical characteristics of OPK 2-59 strain through the API test, it was identified as a *Lactobacillus* strain (Table 3). As a result, the isolated strain was named *Lactobacillus* sp. OPK 2-59. 16S rDNA sequencing analysis revealed that it was *Lactobacillus sakei* (data not shown).

GABA production capacity of the selected strain To characterize the GABA forming capacity of the selected strain, GABA generated from *Lactobacillus* sp. OPK 2-59 in MRS medium containing 1% of MSG was compared to the official strains (Table 4). GABA forming capacity of OPK 2-59 strain was substantially higher than that of the official strains such as *Lactobacillus plantarum* kctc3103 (17).

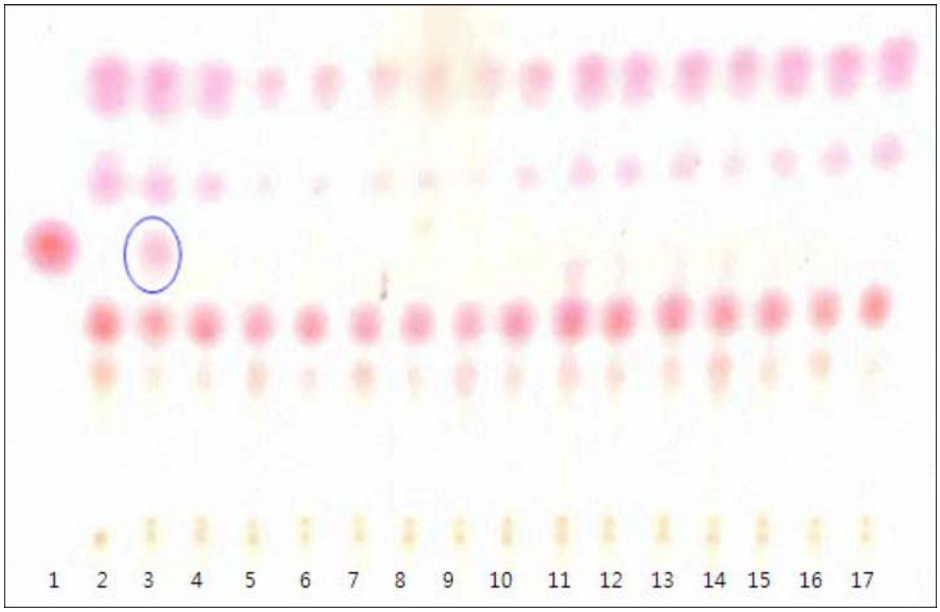

Fig. 1. Comparison of TLC spots between *Lactobacillus* sp. OPK 2-59 and GABA standard. The strain was cultured in an MRS liquid medium with 1% MSG. The spot with circle corresponding to the OPK2-59 strain is in the same location as the standard GABA spot. Lane 1, spot of standard GABA; lane 2-17, spots of lactic acid bacteria cultures.

Table 3. Characterization of *Lactobacillus* sp. OPK 2-59 (KFCC 11392)[1)]

Characteristics	OPK 2-59
Gram-staining	+
Form	Rod type
Spore production	-
Gas production ability in glucose broth	CO_2 production
Catalase production	-
Glucose	+
Ribose	-
Galactose	+
Fructose	+
Maltose	+
Sucrose	+
Xylose	-
Glycerol	-
Starch	-
Acetic acid	-
Arabinose	+
Melezitose	-
Melibiose	+
Cellobiose	-
Trehalose	-
Viability at 15°C	+
Viability at 45°C	-
Vialbility at 50°C	-
Size	1.0-1.5 μm

[1)]Fermentation characteristics and biochemical features of the isolated strains were tested by using API 50 CHL kit (BioMerieuxsa).

Table 4. Comparison of GABA content in different strains of *Lactobacillus*[1)]

Train	GABA content (nmol/mL)
Lactobacillus sp. OPK 2-59	15,266.1 (±233.1)
Lactobacillus plantarum kctc3103	27.6 (±5.4)
Lactobacillus brevis kctc 41028	3.7 (±2.5)
Lactobacillus brevis kctc 41029	5.2 (±2.3)

[1)]GABA content of each strain was analyzed for comparison of *Lactobacillus* sp. OPK 2-59 and different strains of *Lactobacillus*.

***Kimchi* pH and acidity changes** The increase in acid content generated by fermentation of lactic acid was influenced for the biggest component change in *kimchi* fermentation. Therefore, measuring acidity and pH has been used as a benchmark to identify the fermentation stage of *kimchi* (31). In this experiment, changes in pH and acidity were measured after *kimchi* matured and confirmed stimulation of lactic acid bacteria which was added as a starter. There was little difference in pH among the groups right after making *kimchi*. The pH of the MSG+starter added group (M&S group) was 5.4, the pH of the starter added group (S group) was 5.3 and that of the MSG added group was 5.2. Every group's pH decreased after maturing for 24 hr at 15°C and the range of pH was similar at 4.75-4.86. After storing *kimchi* for 3 days following fermentation for 24 hr, the M&S group's measured pH was relatively lower than other groups. In comparing the M group and S group, the pH of the S group was lower than the M group after 6 days of storage (Fig. 2). Acidity of the M&S group before fermentation was slightly higher than the others at 0.4%, S group was 0.35% and M group was 0.32%. In early stages after fermentation, it showed a similar pattern (Fig. 3). After 12 days storage, the acidity of each group was quite different with the M&S group at 0.96%, the S group at 0.76%, and the M group at 0.55%. After by 18 days of storage, every group's acidity increased, but tended to fall from the 21st day. According to the results of pH and acidity tests, the pH and acidity of the group with starter were lower and higher, respectively, compared to the group without starter. Among them, the M&S group had the lowest pH and the highest acidity. In time when *kimchi* is ripening, pH decrease and acidity increase is obvious due to the augmentation of lactic acid bacteria. In case with M&S, when pH decreases and acidity increases, the added OPK 2-59 does not become extinct, but actively multiplying and converts MSG into GABA and as a result the GABA content increase possibility is supposed to be, rather than it was in other groups. Because of the added OPK 2-59 active growing, the result of low level of pH and high level of acidity was supposed to be.

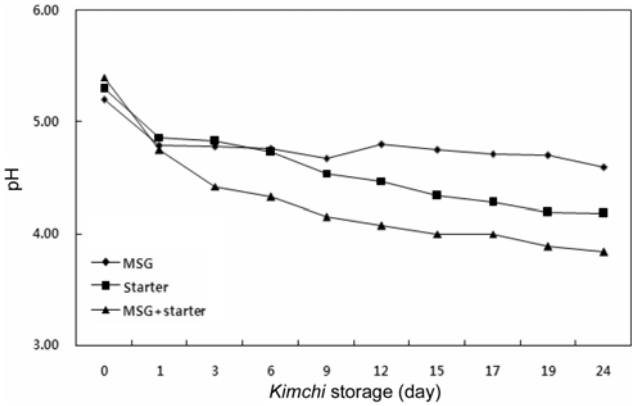

Fig. 2. Changes of pH in *kimchi* samples during storage.

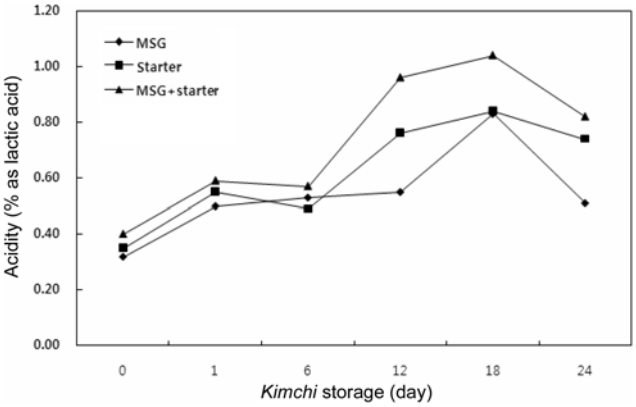

Fig. 3. Changes of acidity in *kimchi* samples during storage.

Reducing sugar Reducing sugar in *kimchi* is used as a nutritive element for microorganism during fermentation of *kimchi* and is metabolized to lactic acid, alcohol, and CO_2.

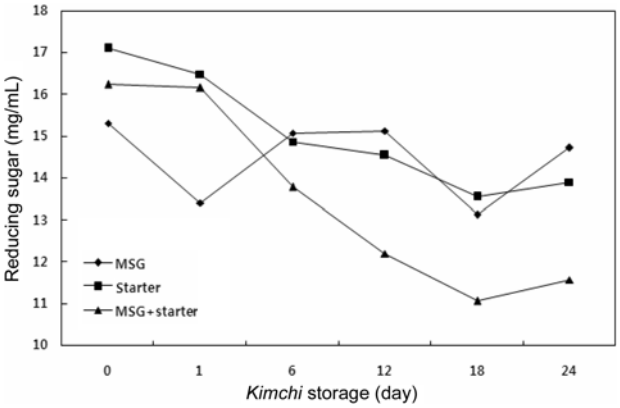

Fig. 4. Changes of reducing sugar contents in *kimchi* samples during storage.

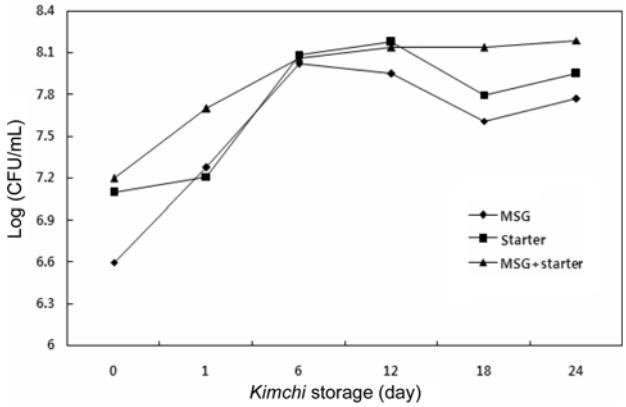

Fig. 5. Changes in number of lactic acid bacteria in *kimchi* samples during storage.

Because of this, reducing sugar in *kimchi* decreases and as a result of the metabolism, it contributes to the unique flavor and fragrance of *kimchi*. By examining changes in reducing sugar, one can measure the ripening of *kimchi*, fermenting of microorganisms, and change in flavor (32). When measuring reducing sugar right after making *kimchi,* the S group had the highest at 17.11 mg/mL, M&S group had 16.24 mg/mL, while the M group had 15.32 mg/mL. After fermentation, the content of reducing sugar decreased in all groups. After storage the M&S group had the lowest reducing sugar from the 6th day, while there was little change in the M group. During storage after fermentation, M&S group's reducing sugar content was lower than the two other groups and continued to decline as the storage period proceeded (Fig. 4), indicating that a continuation of the proliferation of lactic acid bacteria was active.

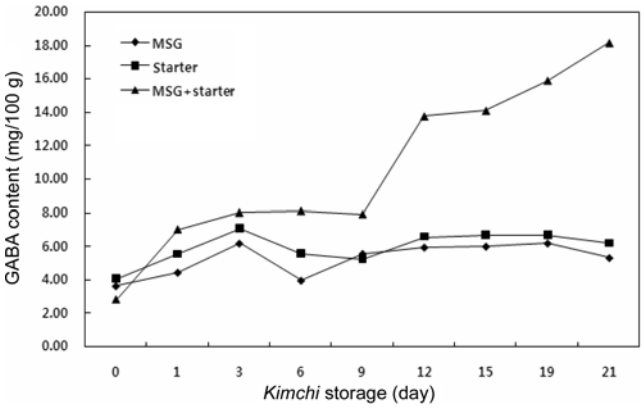

Fig. 6. Changes of GABA contents in *kimchi* samples during storage.

Change in number of lactic acid bacteria It is reported that the process of ecological succession of microorganisms, where various microorganisms repeatedly proliferate and vanish, occurs during the fermentation process of *kimchi* (23,33). In this study, it was determined that the number of lactobacilli in the S and M&S group was higher than in the M group. After ripening, the number of lactic acid bacteria in the M&S group was higher than in the 2 other groups, while M and S group's lactic acid bacteria counts were similar. After 6 days of storage, every group's counts of lactic acid bacteria were similar, but after the 12th day, the M and S group's counts fell, while the M&S group's counts increased (Fig. 5). As a result, the M group's counts were expected to be smaller, but they were only smaller before fermentation. After storage, there was little difference in the counts. This shows that a lot of microorganisms proliferate after fermentation in the group with added MSG. But microorganisms were not estimated to have the ability to convert MSG into GABA.

Change in *kimchi* GABA content GABA contents of the experimental groups are shown in Fig. 6. Prior to fermentation, the S group was found to have a slightly higher content at 4.06 mg/100 g, while the M group had 3.66 mg/100 g and the M&S group had 2.84 mg/100 g. After fermentation, it was observed that the GABA content of every group increased, but the change in GABA content of the M&S group was the greatest. After the 13th day of storage, the GABA content of the M&S group was 13.76 mg/100 g, clearly rising compared to other groups. Moreover, after the 21st day of storage, the M&S group was determined to have a GABA content of 18 mg/100 g, while the M and S group's *kimchi* was measured to have GABA contents of less than 6 mg/100 g, clearly distinguishing themselves from the M&S group. The activity of starter for the M&S group was robust and, as a result, GABA's precursor MSG was transformed into GABA. In fact, the MSG content of the M&S group at the 21st day of storage was clearly reduced in half (data not shown). For M group, the number of lactic acid bacteria was measured as similar to other groups, but the rise in GABA content was not apparent, so it was concluded that lactic acid bacteria other than added starter strain mostly proliferated. Moreover, for S group's *kimchi*, it was concluded that the rise in GABA content did not occur because MSG, which is the precursor to be converted to GABA, wasn't added.

Sensory evaluation of *kimchi* The results of sensory evaluation for taste, smell, texture, and color to examine the impact of the addition of MSG and starter on the organoleptic properties of *kimchi* are shown in Table 5. For the M, S, and M&S group, there were no significant differences in taste, smell, and texture during the sensory evaluation, but in terms of color, there was a significant

Table 5. Sensory evaluation of *kimchi* [1)]

Test	M group	S group	M&S group
Taste	2.75±1.29	2.43±0.81	3.06±1.12
Smell	3.43±0.89	2.93±1.23	3.56±1.03
Texture	3.43±0.89	2.75±1.06	3.37±1.02
Color	3.56±1.31[ab2)]	2.81±1.10[b]	3.81±1.10[a]

[1)]21 days aged *kimchi* was selected to sensory test after some repeated experiment.
[2)]Means with different letters are significantly different as determined by Duncan's multiple range test ($p<0.05$).

change with S group's 2.81±1.10 and M&S group's 3.81±1.10. (Table 5). Generally, M&S group had the highest sensory evaluation scores, followed by M and S group (Table 5).

By using *Lactobacillus* sp. OPK 2-59, which has a high GABA producing capacity, isolated from *kimchi* as a starter and adding MSG, *kimchi* with higher GABA content and enhanced functionality was manufactured. In general MSG, a form of glutamic acid commonly used as a flavor enhancer, is used as a seasoning source in making *kimchi* (34). Glutamic acid is amino acid we get in the most abundance through the meal, and it exists in the form of free glutamate or L-glutamate with protein in natural foods. Isolated glutamate which is especially abundant in such foods as cheese, tomato, tangle, and mushroom is added to cooking as the components of delicate flavor. Ingested glutamate goes through a complicated metabolic process, and in most living creatures, the concentration of free glutamate is much higher in brain tissue than in plasma. As L-glutamate can't go through the blood-brain barrier, it is known to be biosynthesized in the brain tissue, where L-glutamate turns into GABA by glutamate decarboxylase (GAD) (35). MSG is often viewed as a harmful food additive. However, side effects from overeating MSG have been only reported in the Chinese restaurant food syndrome, while no side effects stemming from eating it within the boundary of allowed quantity have been reported thus far (36). The use of MSG in *kimchi* for producing GABA during the storage period after fermentation will reduce the content of MSG and help overcome consumer objections. Moreover, as almost all MSG used as seasoning is made with fermenting technology, using an appropriate amount of MSG is expected to help buoy taste and functionality at the same time. During the sensory evaluation test, the M&S group generally received higher scores confirming that M&S group's *kimchi* with higher GABA content did not have a problem in taste (37). Using this producing method, we will see in the future if the GABA content increases and *Lactobacillus* sp. OPK 2-59 grows in *kimchi* which is added to pickled fish. We will also examine functional characteristics and the differences from *kimchi* which is currently sold in the market. Moreover, we will continue to examine starter and MSG content which can maximize GABA content and other useful amino acids.

Acknowledgments

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