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Garlic Fermentation by Lactic Acid Bacteria

Yu Sun Kim[1], Hyung Hee Baek[2], Ill Min Chung[3], Bin Kwon[4], and Geun Eog Ji[1,4]*

[1]*Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Seoul 151-742, Korea*
[2]*Department of Food Engineering, Dankook University, Cheonan, Chungnam 330-714, Korea*
[3]*Department of Applied Life Science, College of Life & Environmental Sciences, Konkuk University, Seoul 143-701, Korea*
[4]*Research Center, Bifido Inc., Hongcheon, Gangwon 250-804, Korea*

Abstract Garlic has been used for condiments and also for medicines to cure various diseases since ancient times. Many studies on the processing of garlic have been published, however, few of them were related with fermentation because of the antimicrobial action of the garlic. In this study, to conduct garlic fermentation, 4 lactic acid bacteria (LAB) strains with growth abilities in garlic medium were selected. Addition of various nitrogen, carbon, and mineral sources generally did not improve the growth of experimental strains during garlic fermentation except for *Lactobacillus casei* KFRI 704 by yeast extract and *Lactococcus lactis* subsp. *cremoris* ATCC 19257 by mineral sources. High performance liquid chromatography (HPLC) analysis of 32 phenolic compounds during fermentation showed that formononetin was decreased time dependently. The concentrations of volatile compounds and alliin did not change during fermentation. The results of this study would provide the basic understanding of garlic fermentation by selected strains of LAB.

Keywords: garlic fermentation, lactic acid bacteria, phenolic compound, volatile compound, alliin

Introduction

Since ancient times, garlic (*Allium sativum* L.) has been used as an ingredient of medicine, condiments, seasonings, and health foods all over the world (1,2). Recent studies have reported the beneficial functions of garlic such as antimicrobial effect (3), anticancer effect (4,5), antioxidative effect (6,7) as well as cholesterol-lowering effect (8). Currently, most of the commercial garlic products in Korea are being sold as dried garlic powder, whereas oil-macerate products are being distributed as medical supplies in Germany, the largest commercial garlic market in Europe. In America, dried garlic powder and garlic oil are being sold as dietary supplements in the market (9). Recently so-called black garlic produced by aging around 70°C and high humidity (90 RH) condition for about one month is being newly introduced in Korean and Japanese markets as a health product. Even though black garlic is being claimed as fermented garlic, it should not be regarded as authentic fermented garlic.

When garlic is cut or crushed, the unique tastes and flavors of garlic are generated by allicin which is converted from alliin by vacuolar enzyme, alliinase (10,11). Allicin is known as a major antimicrobial agent preventing the growth of various microorganisms including lactic acid bacteria (LAB) (3,10,11). For that reason, the development of fermented garlic products by LABs has not been tried yet.

The objectives of this study were to screen LAB strains which can survive in garlic medium and to characterize the fermented garlic with respect to the bacterial growth, changes in phenolic compounds, volatile compounds, and alliin during fermentation.

*Corresponding author: Tel: +82-2-880-8749; Fax: +82-2-884-0305
E-mail: geji@snu.ac.kr
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Materials and Methods

Sample treatments Garlic (*Allium sativum* L.) was purchased from a farm in Daeseo-myeon, Goheung-gun, Jeollanam-do, Korea. Peeled and trimmed garlic cloves were packed in a commercial polyethylene bag and stored at −80°C. The garlic cloves were heated to inactivate alliinase at 180°C for 10 min in oven (SK-E12; Sanyo, China) and dehydrated using freeze dryer. The sample was ground to powder by an electric blender and stored at −20°C. For the preparation of garlic medium, the dried garlic powder was dissolved into the distilled water at 10%(w/v) concentration and heat-treated at 95°C for 30 min.

Microorganisms and culture conditions Twenty lactic acid bacteria (LAB) strains (14 *Lactobacillus*, 4 *Bifidobacterium*, 2 *Lactococcus*, and 1 *Leuconostocs* strains) were cultured in garlic medium for preliminary examination to screen the ones which can grow in the garlic medium. Among them, 4 LAB strains (*Lactobacillus plantarum* BIF, *L. bulgaricus* KCTC 3188, *L. casei* KFRI 704, *Lactococcus lactis* subsp. *cremoris* ATCC 19257) were selected and used for the experiment. The selected bacteria were pre-cultured anaerobically in de Man Rogosa and sharpe (MRS) broth medium (Difco, Lawrence, KS, USA) containing 0.05%(w/v) L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 18 hr except for *Lac. cremoris* which was cultured at 26°C for 18 hr. The activated culture was inoculated at 1%(v/v) into heat-treated garlic medium. The garlic cultures were incubated aerobically at 37°C for 24 hr except for *Lac. cremoris* which was incubated aerobically at 26°C for 24 hr. pH was measured at every time interval (0, 12, 24, 36, and 48 hr) for the observation of bacterial growth. The growth of LABs was determined by counting viable cells plated on MRS agar plates (Difco, Lawrence, KS, USA). Plates were

incubated at 37°C for 48 hr with the exception of *Lac. cremoris* which was incubated at 26°C for 48 hr. For the preparation of various garlic medium, yeast extract (Difco, Sparks, MD, USA) was used as a nitrogen source, maltose (Sigama-Aldrich) was used as a carbon source. Mineral sources [0.1%(v/v) Tween 80, 0.01%(w/v) magnesium sulfate, 0.01%(w/v) calcium chloride, 0.01%(w/v) manganese sulfate, 0.2%(w/v) sodium carbonate, 0.2%(w/v) ammonium acetate, 0.19%(w/v) dipotassium phosphate] were added to the garlic medium.

Analysis of phenolic compounds by high performance liquid chromatography (HPLC) Thirty-two phenolic compound standards (gallic acid, 5-sulfosalicylic acid, pyrogallol, homogentisic acid, protocatechuic acid, gentisic acid, chlorogenic acid, *p*-hydroxybenzoic acid, (+)catechin, vanillic acid, syringic acid, caffeic acid, vanillin, *p*-coumaric acid, rutin, ferulic acid, *m*-coumaric acid, salicylic acid, hesperidin, *o*-coumaric acid, myricetin, resveratrol, quercetin, *t*-cinnamic acid, naringenin, hesperetin, formononetin, biochaninA, β-resorcylic acid, naringin, kaempferol, veratric acid) were purchased from Sigma-Aldrich. The standard stock solutions (50, 100, 250, and 500 ppm) were made with dimethylsulfoxide (DMSO). All standard calibration curves showed high degrees of linearity (R^2>0.99). The freeze-dried fermented garlic medium powders (2 g each) were mixed with 10 mL of acetonitrile and 2 mL of 0.1 N HCl agitated in shaking incubator for 2 hr at room temperature. The suspension was filtered through Whatman No. 42 filter paper and water was removed by vacuum rotary evaporator. The residues were dissolved in 10 mL of 80% methanol (HPLC grade) (J. T. Baker, Philipsburg, NJ, USA). HPLC analysis was performed with an Agilent 1100 series G1311A Quaternary pump and G1315B DAD detector (Hewlett-Packard, Waldbronn, Germany). Compounds were separated on a YMC-Pack ODS-AM-303 (5 μm, 4.6×250 mm i.d: YMC, Kyoto, Japan) column. The absorbance of each sample solution was measured at 280 nm. The mobile phase was distilled water with 0.1% glacial acetic acid (solvent A) and acetonitrile with 0.1% glacial acetic acid (solvent B). Run time was 63 min using a flow rate of 1 mL/min. The 32 standards and all solvents used were of HPLC grade.

Identification of volatile compounds by gas chromatography (GC) To extract the sample, solid phase microextraction (SPME) apparatus (Supelco, Bellefonte, PA, USA) was used. Homogenized garlic medium cultured by LABs (10 mL) and 9.72 μg of internal standard (3-heptanol) were put into the 20-mL of vial and then tightly sealed and allowed to stand at 50°C for 30 min. After 30 min of sampling at 50°C, the fiber was retracted and immediately inserted into the inlet of GC for thermal desorption. Divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used for absorption of volatile compounds. Tentative identification of the volatile compounds extracted by SPME was performed using Agilent 6890N GC/Agilent 5973 network mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). Volatiles were separated on a DB-5 ms column (60 m length× 0.25 mm i.d.×0.25 μm film thickness: J & W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was set at 40°C for 5 min, raised to 200°C at 5/min and held at this temperature for 20 min. The detector and injector temperatures were 250 and 200°C, respectively. The ionizing energy of the mass selective detector was 70 eV with a scanning mass range of *m/z* 33-330.

Quantification of alliin by HPLC Freeze-dried garlic medium (1 g) and 20 mL of 50% methanol containing 0.1% formic acid were extracted at room temperature. The extractions were filtered through Whatman No. 5 filter paper and filtered extract was evaporated *in vacuo*. The concentrated extractions were fractionated with ethylether, precipitated into the ethanol solution for elimination of carbohydrate during 12 hr. The supernatants were centrifuged at 1,900×g for 15 min and evaporated *in vacuo*. After addition of pre-frozen methanol (2 mL) and 50°C acetone to the sample, it was crystallized for 24 hr. The crystallized samples were centrifuged at 1,900×g for 15 min and then the residues were dried to obtain crude alliin extract in desiccators. The crude alliin extract was diluted in distilled water for HPLC analysis. HPLC analysis was performed with a Dionex P680 HPLC pump and Dionex UVD170U detector (Dionexsofotron, Germering, Germany). Compounds were separated on a Phenomenex Luna C18 5 μ 1,005Å column (4.6×150 mm i.d., Phenomenex, Torrance, CA, USA) and Dionex TCC-100 column oven (Dionexsofotron) at 25°C. The absorbance of each sample solution was measured at 210 nm. The mobile phase was 60% methanol containing 0.01% trifluoroacetic acid (TFA). Injection volume was 20 μL and run time was 12 min using a flow rate of 0.55 mL/min.

Statistical analysis Statistical analysis was performed using the one-way analysis of variance (ANOVA) of the SPSS (Statistical Package for the Social Science 17.0 version, Chicago, IL, USA) program. All measurements were repeated at least 3 times and samples of volatile compounds were assayed in duplicate. Differences between treatment means were determined using the Duncan's multiple range tests at a probability level of 0.05.

Results and Discussion

Phenolic compounds, volatile compounds, and alliin in garlic medium Phenolic compounds are commonly found in some vegetables, fruits, and herbs. Phenolic compounds are well known for health-beneficial properties, which include free radical scavenging activity, inhibition of hydrolytic and oxidative enzymes, and anti-inflammatory action (12). To our knowledge, there have been no studies about the contents of phenolic compounds other than quercetin and myricetin in garlic. In the present study, 27 phenolic componuds were detected (Table 1) while the contents of gallic acid, syringic acid, *m*-coumaric acid, *t*-cinnamic acid, and hesperetin were minimal. Among 32 phenolic compounds, the content of 5-sulfosalicylic acid and chlorogenic acid (36 μg/g) were the highest and *p*-coumaric acid (0.4 μg/g) was the lowest. Most of the phenolic compounds were observed at higher concentration in raw garlic than in the garlic medium except for chlorogenic acid, (+)catechin, and vanillic acid (Table 1). The difference

Table 1. Contents of phenolic compounds and alliin in garlic medium

	Phenolic compound (μg/g)[1]	Raw garlic	Garlic medium
1	Gallic acid	-[2]	-
2	5-Sulfosalicylic acid	32.0	36.0
3	Pyrogallol	49.5	20.1
4	Homogentisic acid	20.5	25.6
5	Protocatechuic acid	15.9	10.5
6	Gentisic acid	59.1	21.5
7	Chlorogenic acid	16.4	36.0
8	*p*-Hydroxybenzoic acid	1.3	0.5
9	(+)Catechin	4.8	7.1
10	Vanillic acid	0.6	3.2
11	Syringic acid	-	-
12	Caffeic acid	20.0	11.1
13	Vanillin	3.7	1.4
14	*p*-Coumaric acid	0.5	0.4
15	Rutin	7.1	6.8
16	Ferulic acid	7.1	2.6
17	*m*-Coumaric acid	-	-
18	Salicylic acid	5.2	4.4
19	Hespsridin	6.4	6.6
20	*O*-Coumaric acid	10.6	9.2
21	Myricetin	73.6	18.6
22	Resveratrol	8.4	3.8
23	Quercetin	16.7	16.5
24	*t*-Cinnamic acid	0.2	-
25	Naringenin	4.1	2.0
26	Hesperetin	0.4	-
27	Formononetin	37.2	6.1
28	BiochaninA	1.8	1.9
29	*b*-Resorculic acid	6.2	5.8
30	Naringin	8.1	4.7
31	Kaempferol	4.5	3.4
32	Veratric acid	5.0	5.7
	Alliin (mg/g)	4.5	5.6

[1]Values represent mean of 3 measurements.
[2]Detected below 0.1 μg/g.

in quantities of phenolic compounds between raw garlic and garlic medium was presumably due to the heating procedures during sample preparation.

Nine volatile compounds were detected in garlic medium (Table 2). As allicin is a very unstable compound, it was easily degraded into diallyl disulfide, vinyl dithiins, and ajoenes within 24 hr (13). Concentrations of volatile compounds in garlic medium were decreased compared with those in raw garlic except for diallyl sulfide and allyl methyl trisulfide (Table 2). Jeong *et al.* (14) reported that di-1-propenyl disulfide and 2-vinyl-4H-1,3-dithiin was not found in the garlic. In contrast, both were detected in garlic medium in this study. This result might have been due to the differences in the heating time and temperature which might have affected the quantities of volatile compounds and production of other compounds. The main organic sulfur compounds of garlic medium were revealed as allyl sulfide. The mono, di, and trisulfide compounds in garlic

Table 2. Contents of volatile compounds in raw garlic and garlic medium (mg/L)

Volatile compound	Raw garlic	Garlic medium
Diallyl sulfide	13.4[1]	12.3
Allyl methyl sulfide	8.4	5.6
Unknown	11.4	10.4
Diallyl disulfide	277.2	145.5
Allyl 1-propenyl disulfide	9.6	4.0
Di-1-propenyl disulfide	35.4	10.4
Allyl methyl trisulfide	27.3	29.5
3-Vinyl-3,4-dihydro-1,2-dithiin	39.0	16.8
2-Vinyl-4H-1,3-dithiin	66.4	33.3
Diallyl trisulfide	335.3	245.2
Unknown II	8.4	3.1
Unknown III	20.6	21.7

[1]Values represent means of 2 measurements.

medium have been reported to have multiple biological effects, including antioxidant effect, anticancer effect, cholestrerol lowering effect, and antiplatelet aggregation effect (5,7,15-17).

The amount of alliin was 5.6 mg/g in garlic medium and there was no difference between raw garlic and garlic medium (Table 1). When garlic was heated at 120°C, alliin in garlic was thermally degraded to smaller compounds including allyl alcohol (2-propen-1-ol) which was reported to have antimicrobial effects (18,19). However, the amount of alliin did not change at a significant level during heating in this study. Since garlic cloves were heat-treated at 180°C for 10 min, the production of allyl alcohol became minimal in garlic medium, which might have allowed the inoculated LABs to grow.

Growth of LABs in various garlic medium Among 20 bacteria, bifidobacteria, and yeasts showed weaker growth than *Lactobacillus* strains in garlic medium (data not shown). This suggested that *Lactobacillus* had a strong resistance than bifidobacteria or yeasts to the antimicrobial garlic compounds. The heating step might have inactivated the alliinase resulting in the inhibition of the production of allicin and allyl alcohol from alliin, which might have allowed *Lactobacillus* to grow in garlic medium. Microbial growth and changes of pH in the garlic medium from 0 to 48 hr of fermentation are shown in Fig. 1 and 2. The effect of nitrogen, carbon, and mineral sources on growth was assessed. Initially the experimental bacteria were inoculated with about 10^7 CFU/mL bacterial cells. After fermentation for 24 hr in garlic medium, the number increased up to 2-5×10^8 CFU/mL for *L. plantarum* and *L. bulgaricus*. For *L. plantarum* and *L. bulgaricus,* the addition of yeast extract, maltose, and mineral sources did not show growth promotion compared to the unsupplemented garlic medium during fermentation. In contrast, the addition of yeast extract enhanced the growth of *L. casei* up to 1×10^9 CFU/mL and mineral sources enhanced the growth of *Lac. cremoris* up to 2×10^9 CFU/mL. No growth promotion was shown in *L. casei* by maltose and mineral sources and in *Lac. cremoris* by yeast extract and maltose, respectively. Our data indicated that garlic medium itself contained

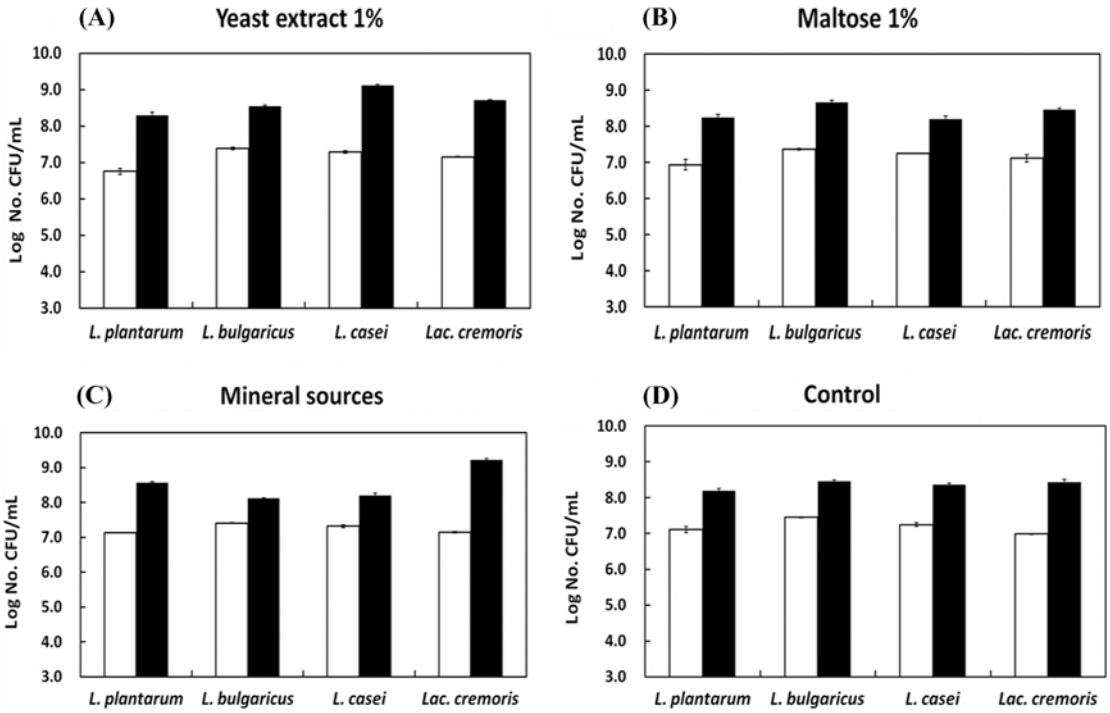

Fig. 1. Growth of LABs in garlic medium containing various supplements during fermentation for 24 hr. Each bar represents mean±SD of 3 measurements. Fermentation time (□ 0 hr, ■ 24 hr).

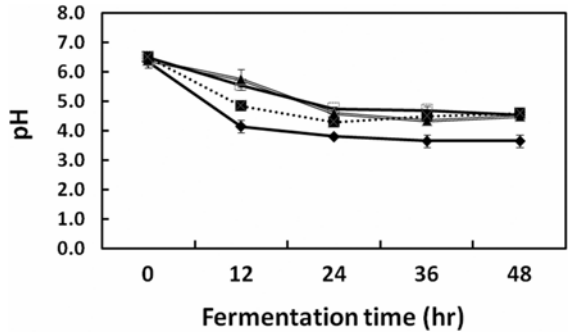

Fig. 2. Changes of pH in garlic medium by LABs during fermentation for 48 hr. -◆-, *L. plantarum*; -□-, *L. bulgaricus*; -▲-, *L. casei*; ···■··· *Lac. cremoris*. Each bar represents mean±SD of 3 measurements.

enough nutrient factors for growth of bacteria in general. Initial pH level was about 6.4 and it decreased down to about pH 4.6 by *L. bulgaricus, L. casei,* and *Lac. cremoris* and pH 3.7 by *L. plantarum* after fermentation for 48 hr (Fig. 2).

Changes in the concentration of formononetin during garlic fermentation As described above, 32 phenolic compounds were detected in garlic medium. Among them, the concentration of formononetin decreased time dependently during fermentation (Fig. 3). However, the concentration of volatile compounds and alliin did not change at a significant level during fermentation (data not shown). Initial concentration of formononetin in garlic medium was 6.1 μg/g. After fermentation for 48 hr, the concentration of formononetin was decreased to undetectable level by *L. plantarum, L. bulgaricus,* and *L. casei.* In comparison, *Lac. cremoris* decreased the formononetin only 30% after 48 hr. Formononetin is a phytoestrogen that exhibits variable degrees of estrogen receptor agonism (21).

Rodriguez *et al.* (21) found that *L. plantarum* CECT 748^T had an ability to metabolize not only *p*-coumaric acid, caffeic acid, and ferulic acid which were classified as hydroxycinnamic acids but also gallic acid and protocatechuic acid which were classified as hydroxybenzoic acids. All these phenolic compounds possessing a carboxylic acid functional group were metabolized by phenolic acid decarboxylase in *L. plantarum* CECT 748^T. However in this study, the concentrations of protocatechuic acid and caffeic acid did not decrease during fermentation and initial amounts of *p*-hydroxybenzoic acid and *p*-coumaric acid were very low (data not shown). The discrepancy between Rodriguez *et al.* (21) and our present study might have been originated from the difference of experimental bacterial strains, culture condition, and/or media composition.

As we know, phenolic compounds exist in many fruits and vegetables. There are a few studies on phenolic compounds metabolized by LAB in wine. *Lactobacillus hilgardii* 5w isolated from wine could metabolize gallic acid and catechin in various medium (22). However there has been no information on the metabolism of formononetin by bacteria in garlic medium. In conclusion, fermentation of garlic by selected strains of lactic acid bacteria may provide a vehicle for the potential use of both garlic and lactic acid bacteria in the development of functional food products.

Acknowledgments

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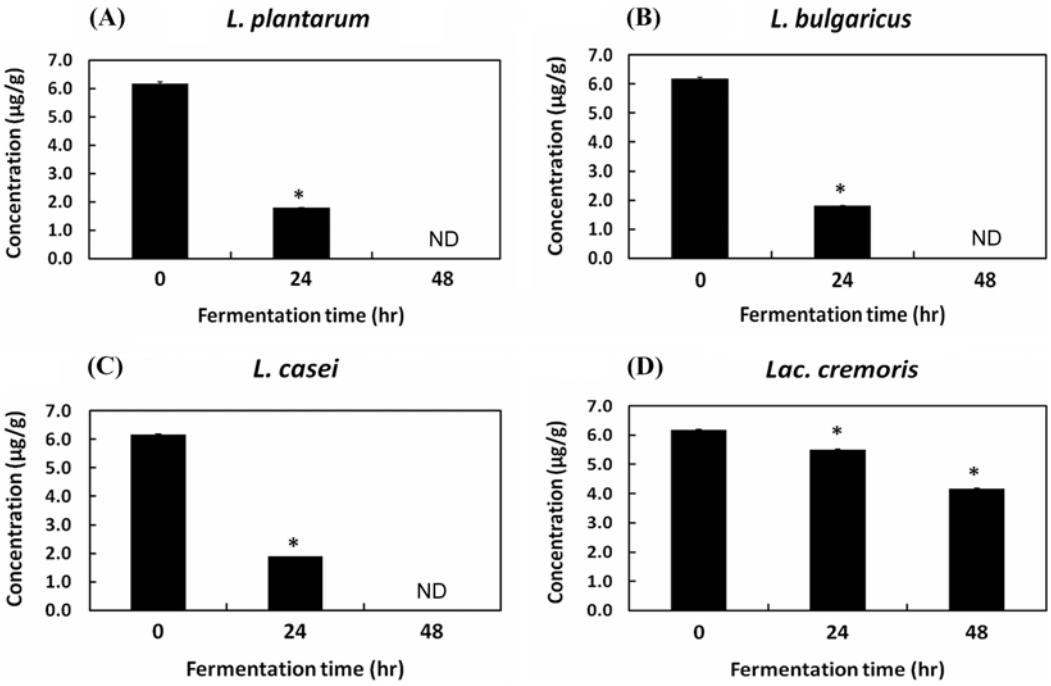

Fig. 3. Changes in the concentration of formononetin during garlic fermentation by LABs for 48 hr. Means were significantly different at $*p<0.05$ and each bar represents mean±SD of 3 measurements. ND, not detected.

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