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Development and Metabolite Profiling of Elephant Garlic Vinegar

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Elephant garlic (*Allium ampeloprasum* var. *ampeloprasum)*, which belongs to the Alliaceae family along with onion and garlic, has a flavor and shape similar to those of normal garlic but is not true garlic. Additionally, its properties are largely unknown, and its processing and product development have not been reported. In this study, we focused on using elephant garlic to produce a new type of vinegar, for which the market is rapidly growing because of its health benefits. First, we evaluated the effects of elephant garlic addition on acetic acid fermentation of rice wine by *Acetobacter pasteurianus*. In contrast to normal garlic, for which 2% (w/v) addition completely halted fermentation, addition of elephant garlic enabled slow but successful fermentation of ethanol to acetic acid. Metabolite analysis suggested that sulfur-containing volatile compounds were less abundant in elephant garlic than in normal garlic; these volatile compounds may be responsible for inhibiting acetic acid fermentation. After acetic acid fermentation, vinegar with elephant garlic did not have any sulfur-containing volatile compounds, which could positively contribute to the vinegar flavor. Moreover, the amino acid profile of the vinegar suggested that nutritional and sensory properties were more enhanced following addition of elephant garlic. Thus, elephant garlic may have applications in the development of a new vinegar product with improved flavor and quality and potential health benefits.

Keywords: Solid-phase microextraction gas chromatography/mass spectrometry, aroma components, allicin, diallyl trisulfide, free amino acids, gamma-amino butyric acid

Introduction

Elephant garlic is three times as large as normal garlic and has a similar shape and flavor, but is classified as a type of leek (*Allium ampeloprasum*). In contrast to normal garlic and onion, which also belong to the *Allium* genus, elephant garlic contains both allicin and *syn*-propanethial-*S*-oxide, which are only found in garlic and onion, respectively [1]. Although elephant garlic is used for cooking in some countries such as Thailand [2] and has been studied for its antibacterial [2, 3] and antioxidant activities [4−6], its chemical composition and biological properties are largely unknown. In contrast, garlic has long been studied for its various biological properties, including antibacterial, anticancer, and antitoxic effects [7−11]. However, garlic is rich in allicin, which inhibits the growth of microorganisms [8, 12], making it difficult to apply in the development of fermented foods.

Vinegar, which mainly consists of organic acids, amino acids, and volatile components, is traditionally used as a food seasoning and preservative [13]. The functional properties of vinegar have been studied to evaluate its antioxidant activity and for body fat reduction and fatigue recovery [13−15]. Vinegar can be synthesized or produced by fermentation [13]. Inexpensive commercial vinegar products are produced by acetic acid fermentation of distilled and diluted ethanol [16]. In contrast, traditional vinegar is produced by ethanol fermentation of fruits or grains followed by acetic acid fermentation [17]. Depending on the type of raw materials, additives, and fermentation processes, the nutritional and functional benefits of vinegar can be improved [15]. Accordingly, studies of traditionally fermented vinegar, which involves ethanol fermentation with natural resources, such as apples [18], grapes [15, 19, 20], and onions [16], followed by acetic acid fermentation, have been conducted. To develop a new vinegar variant, we examined the effects of elephant garlic addition on acetic acid fermentation of rice wine. Through metabolite profiling, elephant garlic vinegar was characterized and evaluated as a new type of vinegar product.

Materials and Methods

Raw Material Preparation

Elephant garlic samples were provided by the Gunwi Agriculture Technology Center, Korea in 2016, and normal garlic samples were purchased from a local market in Daegu, Korea in 2016. Elephant garlic and normal garlic samples were sliced, freeze-dried, ground, and stored at −80°C until analysis. Fresh commercial rice wine (Palgongsan makgeolli, Daegu, Korea) containing 6% (w/v) ethanol was purchased from a local market in Daegu (Korea) and used before its expiration date.

Vinegar Starter Preparation

Acetobacter pasteurianus (ATCC 33445) was precultured in YPDE (glucose 2% (w/v) and ethanol 1% (w/v)) at 30°C and 80 rpm (0.14 ×*g*) for 48 h. Cells were harvested and resuspended in 20 ml of rice wine in a 100-ml flask and then incubated at 30°C and 80 rpm (0.14 ×*g*) for 72 h. The culture was used as a vinegar starter. The starter was stored at 4°C for 7 days without losing its activity.

Acetic Acid Fermentation

Two milliliters of the vinegar starter was added to 20 ml of rice wine in a 100-ml flask with or without garlic powder. Acetic acid fermentation was performed at 30°C and 80 rpm (0.14 ×*g*) until the ethanol was exhausted.

Sample Preparation for Analysis

Samples were mixed with an equal volume of distilled water and centrifuged for 10 min at 3,134 ×*g*. The supernatant was filtered using a syringe filter (13 mm, 0.45 μm), and the flow-though was stored at −80°C until analysis.

Analysis of Ethanol and Acetate by High-Performance Liquid Chromatography (HPLC)

The concentrations of ethanol and acetate were determined by HPLC (1260 Series; Agilent Technologies, USA) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex, Inc., USA). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 ml/min at 50°C.

Analysis of Volatile Compounds by Solid-Phase Microextraction (SPME)-Gas Chromatography/Mass Spectrometry (GC/MS)

From 5-ml samples, volatile compounds were extracted using SPME fibers coated with divinylbenzene/carboxen/polydimethyl-siloxane. The extracted compounds were injected using a split mode (1:20) into a GC/MS system (7890A-5975C; Agilent Technologies) equipped with an MPS autosampler (Gerstel, Germany) and a DB-WAX column (60 m, 250 μm, 0.25 μm; Agilent Technologies). The oven temperature was initially held at 40°C for 2 min, increased to 220°C at 2°C/min, and then increased to a final temperature of 240°C at 20°C/min, which was maintained for 5 min. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The mass selective detector was operated in scan mode with a mass range of 50–550 *m/z*.

Analysis of Free Amino Acids Using an Amino Acid Auto-Analyzer

Twenty-microliter samples, which were hydrolyzed with 6 N HCl, were injected into an amino acid auto-analyzer (L-8900; Hitachi, Japan). Free amino acids were separated on an ion exchange column (2622 SC PF; Hitachi) at 50°C and eluted with a buffer set (PF-1, PF-2, PF-3, PF-4, PF-6, PF-RG, R-3, and C-1) as a mobile phase. The eluted free amino acids were reacted with ninhydrin solution (Wako, Japan) at 135°C in a post-column. The detected ninhydrin-labeled free amino acids, which were measured at wavelengths of 570 and 440 nm, were quantified. A mixture of type ANII and type B (Wako) was used as an internal standard for free amino acid quantification.

Data Processing and Statistical Analysis

The metabolites were evaluated by principal component analysis (PCA) using STATISTICA software (ver. 7.0; Stat Soft, USA). The presented data were based on significance ($p < 0.05$) by Scheffe's *t*-test, using SPSS software (ver. 23.0; SPSS, Inc., USA).

Results

Effects of Elephant Garlic Addition on Acetic Acid Fermentation

We first tested how elephant garlic addition (0–4.5% (w/v)) affected the acetic acid fermentation of rice wine by *A. pasteurianus*. After 96 h of fermentation, the acetate yield (g acetate/g ethanol) was highest for samples with 0% or 1.5% elephant garlic addition, followed by that with 2.0% and 3.0% addition, with the lowest acetate yield observed following addition of 4.5% elephant garlic (Fig. 1). Because elephant garlic addition over 3.0% significantly resulted in low acetate yield, we conducted subsequent experiments with 2.0% elephant garlic addition.

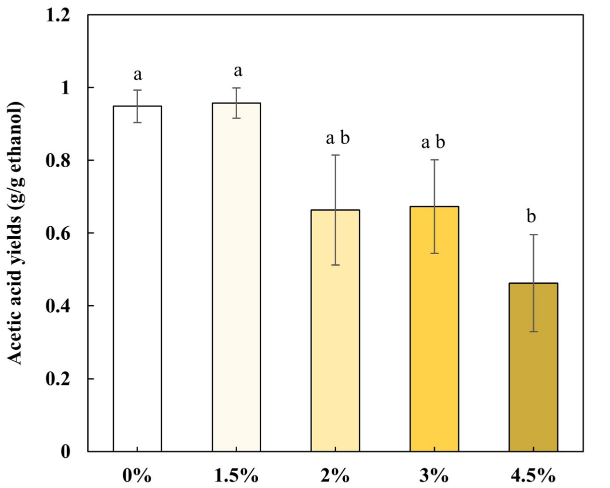

Fig. 1. Effect of elephant garlic addition on acetic acid yields (g/g ethanol) by *Acetobacter pasteurianus*.

Fermentation was performed at 30°C and 80 rpm for 96 h. Different letters (a and b) represent significant differences ($p < 0.05$).

Comparison of Acetic Acid Fermentation with Elephant Garlic and Normal Garlic Addition

Next, we determined the differences between acetic acid fermentation with elephant garlic and normal garlic addition. Without additives (control) or with 2% (w/v) elephant garlic, ethanol was completely fermented into acetate within 3 and 7 days, respectively (Fig. 2). However, with 2% (w/v) normal garlic, only a small amount of ethanol was consumed. These results suggest that acetic acid fermentation by *A. pasteurianus* was strongly inhibited by normal garlic, but not by elephant garlic, under the same conditions tested.

Sulfur-Containing Volatile Compounds of Elephant Garlic and Normal Garlic

Most plants belonging to the *Allium* genus have sulfur-containing volatile compounds, which are known to inhibit microbial growth [4, 8, 9, 21, 22]. We predicted that the volatile metabolite profiles of elephant garlic and normal garlic may differ, affecting the fermentation results. Thus, rice wine samples containing 2% (w/v) elephant garlic or normal garlic at 0 h of fermentation were subjected to headspace analysis by SPME-GC/MS. Among the detected metabolites, 15 were sulfur-containing compounds, as listed in Table 1. As expected, the metabolite profiles differed between the elephant garlic and normal garlic samples; only seven compounds were detected in the normal garlic samples. Moreover, most other compounds shared in both samples were present at lower levels in the elephant garlic than in the normal garlic samples. Notably, diallyl trisulfide, the major form of hydrolyzed allicin with strong antimicrobial activity [2, 7, 8, 12], was barely detected in the elephant garlic samples. Therefore, the low content of diallyl trisulfide in elephant garlic may explain why acetic acid fermentation was possible following addition of elephant garlic.

Analysis of Flavor Components of Elephant Garlic Vinegar

As described above, elephant garlic has sulfur-containing volatile compounds, although the quantity is lower than

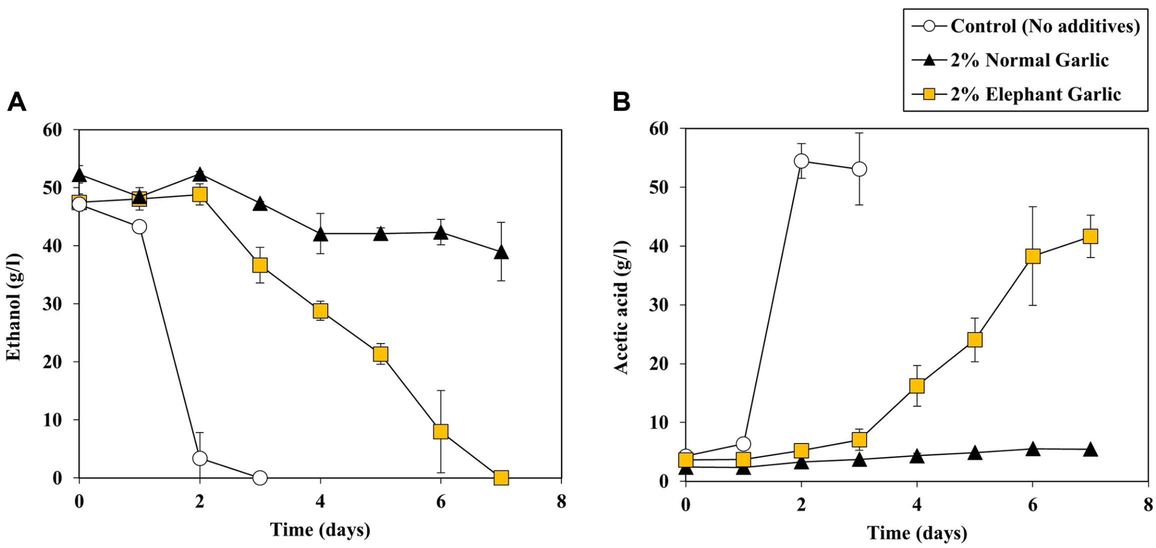

Fig. 2. Effects of 2% (w/v) garlic and 2% (w/v) elephant garlic addition on ethanol consumption (**A**) and acetic acid production (**B**) by *Acetobacter pasteurianus*.

Table 1. Sulfur-containing volatile compounds that differed among normal garlic, elephant garlic, and elephant garlic vinegar.

	Before acetic acid fermentation		After acetic acid fermentation
	Normal garlic	Elephant garlic	Elephant garlic vinegar
Diallyl trisulfide	**18.99 ± 2.45**	**0.40 ± 0.08**	**ND**
Diallyl disulfide	**12.86 ± 2.47**	**10.09 ± 2.18**	**ND**
(Z)-Allyl-1-propenyl disulfide	2.56 ± 0.55	1.82 ± 0.37	ND
(E)-Allyl-1-propenyl trisulfide	2.04 ± 0.22	ND	ND
3-Vinyl-1,2-dithiacyclohex-4-ene	1.01 ± 0.01	ND	ND
Methyl allyl trisulfide	**0.94 ± 0.14**	**0.18 ± 0.01**	**ND**
3-Vinyl-1,2-dithiacyclohex-5-ene	0.88 ± 0.02	ND	ND
3H-1,2-Dithiole	0.48 ± 0.04	ND	ND
5-Methyltetrathiane	0.43 ± 0.05	ND	ND
Methyl allyl disulfide	**0.26 ± 0.05**	**0.86 ± 0.14**	**ND**
4H-1,2,3-Trithiine	0.25 ± 0.03	ND	ND
Diallyl monosulfide	**0.20 ± 0.03**	**0.15 ± 0.01**	**ND**
1-Allyl-3-propyltrisulfane	0.09 ± 0.01	ND	ND
(E)-Propenyl methyl disulfide	ND	0.25 ± 0.05	ND
(E)-Propenyl propyl disulfide	ND	0.28 ± 0.05	ND

Values are the means of relative peak heights (%) ± standard deviations of triplicate determinations using GC-MS.
Compounds known to have antimicrobial activity are highlighted in bold font.
ND, not detected.

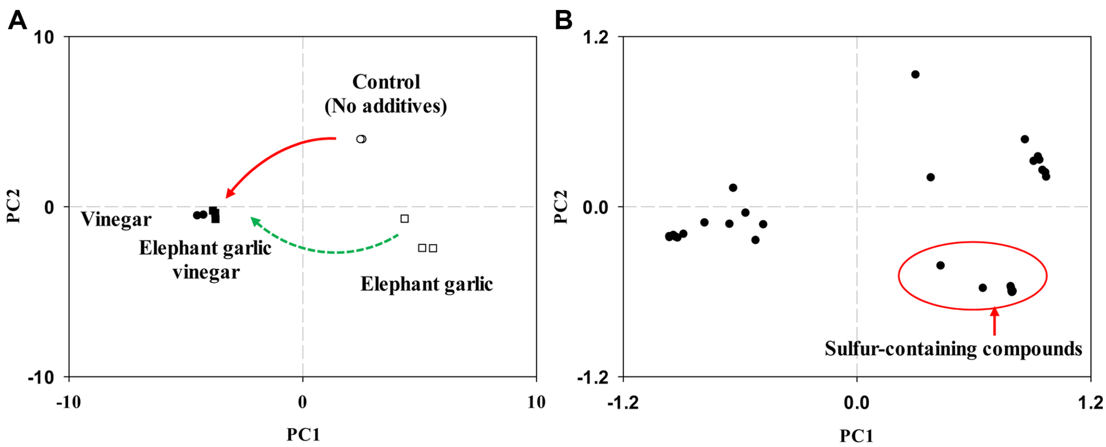

Fig. 3. Changes in volatile compound profiles during acetic acid fermentation with 2% (w/v) elephant garlic addition.
The relative abundance of volatile compounds is presented by principal component analysis. Score plot **A** shows that volatile compound profiles altered by addition of 2% (w/v) elephant garlic became negligible after acetic acid fermentation. Loading plot **B** shows that contents of sulfur-containing volatile compounds were reduced dramatically during acetic acid fermentation.

that in normal garlic. These compounds have not only antimicrobial activities but also strong off-flavors [23, 24]. Therefore, flavor components in elephant garlic vinegar were analyzed to determine how elephant garlic addition altered vinegar flavor. Rice wine samples (before fermentation) and vinegar samples (after fermentation) with or without addition of 2% (w/v) elephant garlic were analyzed by SPME-GC/MS, and the results were statistically evaluated by PCA. The samples were clustered into three groups on the basis of principal components 1 and 2, which explained the variations among the samples by 62.92% and 16.56%, respectively (Fig. 3A). PC1 contributed primarily to the separation of rice wine and vinegar samples. PC2 contributed primarily to the separation of rice wine samples with elephant garlic from rice wine samples without elephant garlic. As highlighted in Fig. 3B, separation by PC2 was driven by sulfur-containing volatile compounds present in elephant garlic. Notably, the vinegar samples were clustered together, regardless of elephant garlic addition. None of the eight sulfur-containing volatile compounds was detected in vinegar samples containing elephant garlic (Table 1). Additionally, the other flavor components, such as esters and alcohols, showed no significant difference between the elephant garlic vinegar and the control vinegar samples ($p > 0.05$; Fig. 3A). These results suggested that elephant garlic addition before acetic acid fermentation could yield vinegar without off-flavors.

Free Amino Acid Profiles of Elephant Garlic Vinegar

The free amino acid content in vinegar is directly determined by the raw materials and fermentation processes used and is therefore used as a criterion for vinegar quality control [25]. Elephant garlic addition is expected to alter the amino acid contents and profiles of vinegar, impacting the taste and nutritional aspects of vinegar. We found that 2% (w/v) elephant garlic addition increased the total free amino acid content of rice wine by 163% (Fig. 4). Moreover, acetic acid fermentation contributed to an additional increase in the free amino acid content; with elephant garlic addition, vinegar contained 5.6 mg/ml total free amino acids, which was higher than that in control vinegar by 176%. This suggested that elephant garlic vinegar contained more free amino acids than those initially released from elephant garlic.

To understand how acetic acid fermentation synergistically increases the free amino acid contents of vinegar with elephant garlic addition, an amino acid auto-analyzer was used to determine the amino acid profiles of rice wine samples (before fermentation) and vinegar samples (after fermentation) with or without 2% (w/v) addition of elephant garlic. The results of 27 amino acids were statistically evaluated by PCA, as shown in Fig. 5. PC1 (67.61%) and PC3 (6.30%) clearly distinguished the samples into four groups (Fig. 5A). PC1 represented changes during acetic acid fermentation; the amino acids highlighted in Fig. 5B were rich in the samples after fermentation. Interestingly, the changes towards positive PC1 were greater with elephant garlic addition than without it. The result suggested that

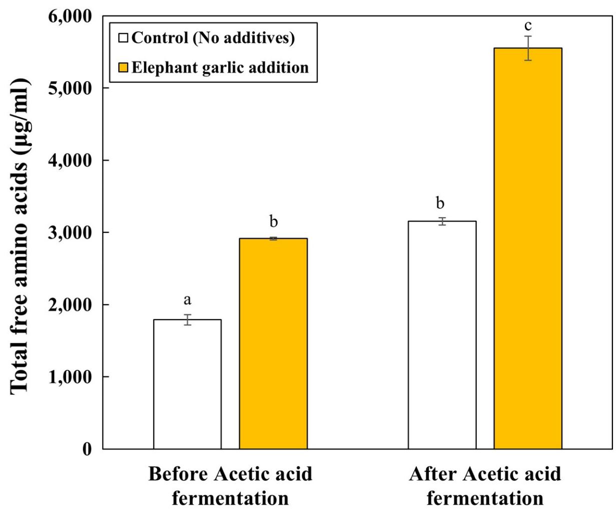

Fig. 4. Effect of elephant garlic addition on the total free amino acid content before and after acetic acid fermentation.
Values are the means ± standard deviations of duplicate determinations. Different letters (a–c) represent significant differences ($p < 0.05$).

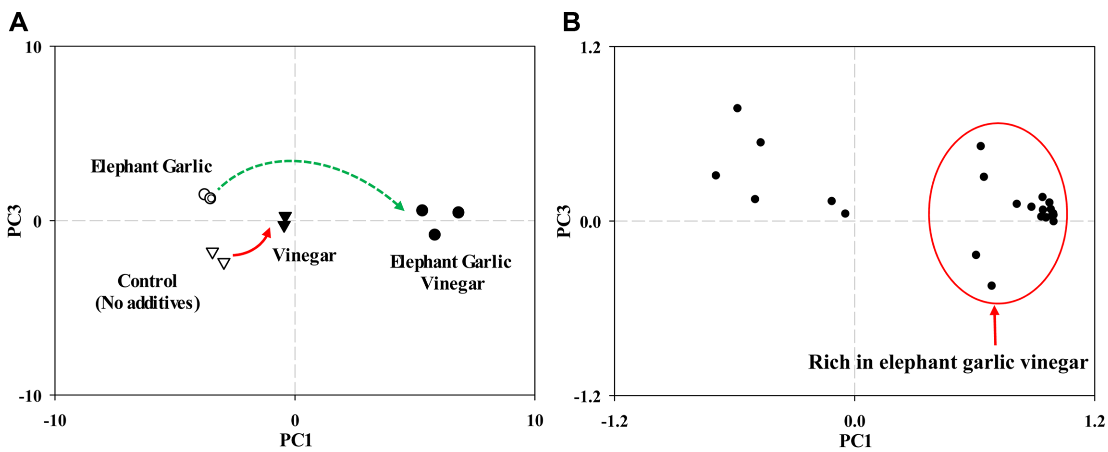

Fig. 5. Changes in amino acid profiles during acetic acid fermentation with addition of 2% (w/v) elephant garlic.
All data were evaluated by principal component analysis. Score plot **A** indicates changes in free amino acids during acetic acid fermentation for each group. Loading plot **B** is presented by each variable (free amino acids), explaining the correlation with the data shown in the score plot.

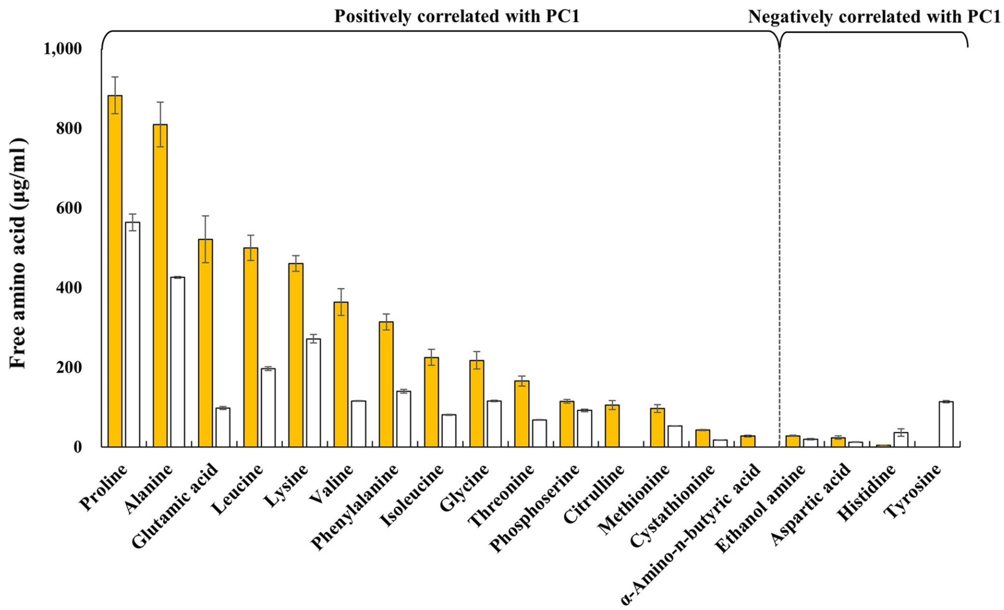

Fig. 6. Effect of elephant garlic addition on the free amino acid content after acetic acid fermentation, showing a significant difference ($p < 0.01$) between elephant garlic vinegar (■) and vinegar (□).

elephant garlic addition may have promoted acetic acid fermentation, leading to the increase in some free amino acids in its vinegar.

For detailed analysis of the amino acid profiles, 19 amino acids showing significantly different concentrations ($p < 0.01$) between elephant garlic vinegar and control vinegar samples were examined (Fig. 6). Tyrosine was detected only in control vinegar samples, whereas citrulline, α-amino-*n*-butyric acid, and hydroxyl proline were detected only in elephant garlic vinegar samples (Table 2). Proline and alanine were the most abundant amino acids in both elephant garlic and control vinegar samples. Most free amino acids were rich in elephant garlic vinegar compared with that in control vinegar. Glutamic acid, which has the greatest effect on savory flavor (umami), was richer in elephant garlic vinegar than in the control vinegar by 535%. Additionally, glycine, which also confers a savory taste, was richer than in control vinegar by 189%. Gamma-amino butyric acid, which has various physiological functions [33], was richer in elephant garlic vinegar by 287% following acetic acid fermentation (Table 2). In summary, the changes in amino acid contents and profiles of vinegar with elephant garlic addition were unique and could positively affect its nutritional and sensory properties.

Discussion

Plants in the *Allium* genus contain bioactive sulfur compounds with strong antioxidant and antimicrobial activities [2, 4, 8, 9, 12, 21, 22, 26]. Sulfur-containing amino acids mainly found in cloves are enzymatically converted to *S*-allyl-L-cysteine sulfoxide (alliin) and alk(en)yl thiosulfinates (allicin). Because of its low stability, allicin immediately changes into a volatile compound, but is converted to stable forms, such as diallyl disulfide, diallyl trisulfide, and methyl allyl trisulfide [7, 22, 27]. These sulfur-containing volatile compounds contain the disulfide (R-S-S-R) group, which inhibits microbial growth. Furthermore, increased sulfur levels have additional inhibitory activities; diallyl disulfide and diallyl trisulfide have 20- and 150-fold higher antimicrobial activity than diallyl monosulfide, respectively [7]. In the present study, five types of sulfur-containing compounds were detected in both elephant garlic and normal garlic (Table 1); however, most of these compounds were more abundant in normal garlic. Thus, compared with normal garlic, elephant garlic contained reduced levels of sulfur compounds and had great potential for applications in the development of new fermented foods such as vinegar, as demonstrated in this study.

The volatile sulfur compounds present in elephant garlic and normal garlic are also responsible for strong off-flavors with low thresholds [23]. Thus, for food quality assurance, methods for detecting sulfur-containing volatile compounds, such as SPME-GC/MS, have been well-established [24, 28]. Additionally, various processes, such as roasting, crushing, drying, and autoclaving, have been reported to alleviate

Table 2. Free amino acid concentrations (μg/ml) before and after acetic acid fermentation with the addition of elephant garlic.

	Before acetic acid fermentation		After acetic acid fermentation	
	Control (no additives)	2% (w/v) Elephant garlic	Control (no additives)	2% (w/v) Elephant garlic
Alanine	140.6 ± 2.5^{a}	157.6 ± 2.4^{a}	425.7 ± 2.5^{b}	809.6 ± 56.0^{c}
α-Amino-*n*-butyric acid	ND	ND	ND	27.4 ± 2.1
β-Alanine	7.6 ± 0.2^{a}	8.1 ± 1.0^{a}	14.0 ± 0.7^{b}	13.9 ± 1.0^{b}
β-Amino isobutyric acid	79.5 ± 2.4^{a}	79.8 ± 1.8^{a}	112.7 ± 5.9^{b}	121.0 ± 9.2^{b}
γ-Amino-*n*-butyric acid	38.8 ± 0.6^{a}	49.1 ± 0.9^{a}	103.5 ± 2.0^{b}	141.1 ± 20.7^{c}
Arginine	150.2 ± 7.0^{a}	1057.8 ± 46.2^{a}	380.7 ± 8.9^{b}	65.0 ± 15.73^{c}
Aspartic acid	24.4 ± 0.3^{a}	48.8 ± 1.1^{b}	11.9 ± 0.4^{b}	23.4 ± 3.9^{c}
Citrulline	ND	68.5 ± 3.1^{a}	ND	105.4 ± 11.3^{b}
Cystathionine	5.9 ± 2.4^{a}	4.5 ± 2.4^{a}	17.0 ± 0.3^{b}	42.4 ± 1.5^{c}
Cystine	34.7 ± 1.5^{a}	33.2 ± 1.2^{a}	62.0 ± 2.9^{b}	73.7 ± 4.1^{c}
Ethanol amine	28.5 ± 0.1^{a}	30.2 ± 0.2^{b}	18.9 ± 1.8^{b}	27.9 ± 1.5^{b}
Glutamic acid	73.5 ± 1.5^{a}	86.8 ± 0.3^{a}	97.4 ± 3.6^{a}	521.2 ± 58.4^{b}
Glycine	55.5 ± 1.2^{a}	60.8 ± 0.5^{a}	115.2 ± 1.7^{b}	217.3 ± 21.9^{c}
Histidine	16.2 ± 1.0^{a}	27.8 ± 0.4^{b}	35.9 ± 9.3^{c}	4.6 ± 0.1^{c}
Hydroxy proline	3.2 ± 4.5^{a}	ND	ND	5.9 ± 2.4^{a}
Isoleucine	23.8 ± 3.6^{a}	31.2 ± 0.2^{a}	80.4 ± 1.1^{b}	224.8 ± 20.2^{c}
Leucine	59.7 ± 5.1^{a}	69.1 ± 0.4^{a}	196.4 ± 5.0^{b}	499.2 ± 31.7^{c}
Lysine	55.2 ± 1.0^{a}	102.1 ± 4.0^{b}	271.3 ± 10.7^{c}	460.3 ± 19.4^{d}
Methionine	16.0 ± 1.4^{a}	17.4 ± 0.4^{a}	52.7 ± 0.4^{b}	96.4 ± 9.7^{c}
3-Methylhistidine	ND	ND	6.5 ± 4.4	ND
Phenylalanine	39.2 ± 1.6^{a}	50.4 ± 0.4^{a}	139.8 ± 4.5^{b}	313.9 ± 20.1^{c}
Phosphoserine	41.1 ± 0.1^{a}	44.8 ± 1.0^{a}	91.6 ± 2.9^{b}	114.4 ± 4.4^{c}
Proline	778.9 ± 23.7^{a}	703.2 ± 33.1^{b}	563.7 ± 20.8^{b}	882.2 ± 46.2^{c}
Serine	27.0 ± 1.0^{a}	38.8 ± 0.3^{ab}	60.2 ± 1.3^{b}	159.1 ± 20.8^{c}
Threonine	22.5 ± 1.2^{a}	28.0 ± 0.6^{a}	68.0 ± 0.9^{b}	165.3 ± 12.6^{c}
Tyrosine	29.6 ± 8.7^{a}	49.6 ± 1.7^{b}	113.5 ± 3.2^{c}	ND
Valine	39.2 ± 0.8^{a}	68.1 ± 1.5^{ab}	115.4 ± 0.9^{b}	363.3 ± 33.6^{c}

Values are the mean ± standard deviations of at least duplicate determinations using an amino acid auto-analyzer.
Different letters (a–d) in the same row represent significant differences (Scheffe's test, $p < 0.05$).
ND, not detected.

the pungent flavor [24, 29]. We detected eight sulfur-containing compounds in elephant garlic, but none were detected in elephant garlic vinegar after acetic acid fermentation. Therefore, the fermentation process can successfully improve elephant garlic flavors by eliminating sulfur compounds.

Free amino acids are a source of nitrogen for acetic acid bacteria [30]. As reported previously [31], arginine is abundant in elephant garlic. We also found that the arginine concentration increased by 7-fold after elephant garlic addition in rice wine samples (Table 2). Interestingly, after acetic acid fermentation, the arginine content in elephant garlic vinegar decreased significantly. Instead, glutamic acid and citrulline, which are metabolites of arginine, increased by 6- and 1.5-fold, respectively. Moreover, tyrosine and histidine were reduced significantly after acetic acid fermentation when elephant garlic was added. These results suggested that acetic acid bacteria may metabolize nutrients in elephant garlic, influencing the amino acid profiles of vinegar [32].

In this study, we have developed a new type of vinegar through acetic acid fermentation of rice wine with 2% (w/v) elephant garlic. In contrast to normal garlic, elephant garlic addition resulted in the complete conversion of 5% (w/v) ethanol to acetic acid. We observed that the reduced abundance of sulfur-containing volatile compounds in elephant garlic compared with that in normal garlic may be related to successful acetic acid fermentation. Additionally, acetic acid fermentation was effective in eliminating sulfur-containing volatile compounds from elephant garlic, which could cause an unpleasant flavor. Finally, the contents of flavor-enhancing or nutritionally important amino acids as well as total free amino acids in elephant garlic vinegar were much higher than those in control vinegar. On the basis of these results, we conclude that elephant garlic vinegar has positive sensory properties with high nutritional value.

Acknowledgments

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Conflict of interest

The authors have no financial conflicts of interest to declare.

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