広島大学学位請求論文

Development of koji using new raw materials and comprehensive analysis of active ingredients

（新規原料を用いた麹の開発とその有効成分の網羅的解析）

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主論文

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Chapter 1. Preface

"Koji fungi (koji-mold)" is a "national fungi" (1), and koji-mold is a general term for molds that include *Aspergillus oryzae*, *A. sojae*, and *A. luchuensis* (as *A. niger* is a different species from the black *Aspergillus* group, it is excluded). Since ancient times, Japan has used these koji-molds to make koji from grains such as rice, wheat, and soybeans, and used it in various traditional fermented foods. For example, sake is made from rice-koji and rice, and alcohol is produced using a method called multiple parallel fermentation, which is rare in the world. Miso is made with rice-koji or barley-koji, soybean-koji, soybean, and salt, while Soy sauce is made from soy sauce-koji (soybean + wheat) and salt. The most important role of koji (koji-mold) in the production of these fermented foods is the supply of enzymes for the degradation of raw materials. It has been clarified that there are approximately 12,000 genes in the genome of *A. oryzae* RIB40 (2, 3), of which 134 are proteolytic enzymes. Furthermore, enzymes of koji-mold are substrate specific and secreted for degrading raw materials (4, 5, 6). In recent years, the fermented food trend has brought back attention to traditional foods such as *amazake* and *shio-koji*, and various foods have been tried to be "koji-made". For example, ginger-koji (7), wine pomace-koji (grape pericarp and seed) (8), and koji cheese (9) are examples of success. These are made possible by the characteristics of koji-mold, which secretes enzymes suitable for the degradation of raw materials (substrates) and cannot be achieved by yeast alone. In addition, the safety of koji-mold and koji is guaranteed by the long eating experience (10). With the diversification of food in recent years, there is a growing need to find new ways to use koji or koji-mold and to develop new materials for koji. Therefore, in this study, I focused on eggs as a new koji material.

Eggs are a nutrient-rich food, and there are a variety of recipes around the world. On the other hand, the high-quality nutritional components (12.3% protein, 10.3% lipid) (11) are only taken as they are, and there is still room for improvement, such as the enzymatic conversion of microorganisms. Therefore, growing koji-mold on eggs was

expected to give flavors that cannot be achieved by cooking, such as decomposing egg proteins to produce peptides and amino acids to enhance umami or decomposing lipids to enhance aroma components. Japan has the second-highest annual consumption of eggs per capita worldwide (12) and improving the flavor of eggs has a great social effect.

The purpose of this paper is to contribute to the development of food culture by developing koji (egg-koji) using new materials (eggs) and investigating its characteristics.

Chapter 2 consists of the egg-koji development method that uses only eggs and koji-mold by selecting and combining Cooked Egg Powder (CEP) and *A. oryzae* AO101 as the most suitable combination (Fig. 1). To suppress the explosive growth of harmful bacteria, I improved the sterilization method, watering method, and amount of water. In addition, it was found that egg-koji has a characteristic enzyme activity balance, in which amylase activity was extremely low and protease activity at pH 6 was high compared to grain koji, such as rice and barley. Egg-koji might produce enzymes suitable for taking in nutrients when growing into CEP and would be expected to give a flavor that could not be achieved by cooking or additives.

In Chapter 3, I investigated the novel functionality of egg-koji by metabolome analysis using GC-MS. Compounds extracted from CEP and egg-koji with methanol aqueous solution were measured by GC-MS, and the measurement data was subjected to principal component analysis, suggesting that the component profiles of CEP and egg-koji were significantly different. Therefore, it was inferred that the egg-derived component was converted into a new substance by koji-mold. Principal component analysis between egg-koji prepared with different *Aspergillus* species suggested the formation of specific components by koji-mold. In particular, the metabolite profile of *A. sojae* AS309 separated from other species in the first principal component.

In Chapter 4, I focused on the components related to "richness" among the component changes caused by the fermentation of whole eggs with egg-koji, and investigated their effects on sensory characteristics. Using AO101 as a standard strain of

egg-koji, the sensory characteristics of whole egg liquid digests (WELDs) prepared with egg-koji and other grain koji were compared. E-101 (WELDs prepared with egg-koji AO101) was the only WELD that enhanced the umami and richness without losing its egg flavor. Additives such as monosodium glutamate and nucleic acid are added to foods to enhance the umami taste, but by using WELD such as E-101, these seasonings and additives can be reduced. Using egg-koji prepared by the different *Aspergillus* strains, WELDs with enhanced umami and richness while retaining the egg flavor of whole eggs could be prepared. Different characteristics of each egg-koji were revealed, such as that E-309 (WELDs prepared with egg-koji AS309) produced more free amino acids and aroma components and E-434 (WELDs prepared with egg-koji *A. luchuensis* AL434) had the potential to take the emulsifying power of free fatty acids, suggesting that seasonings and additives can be reduced in food design. These outcomes have opened the possibility of new egg-related foods.

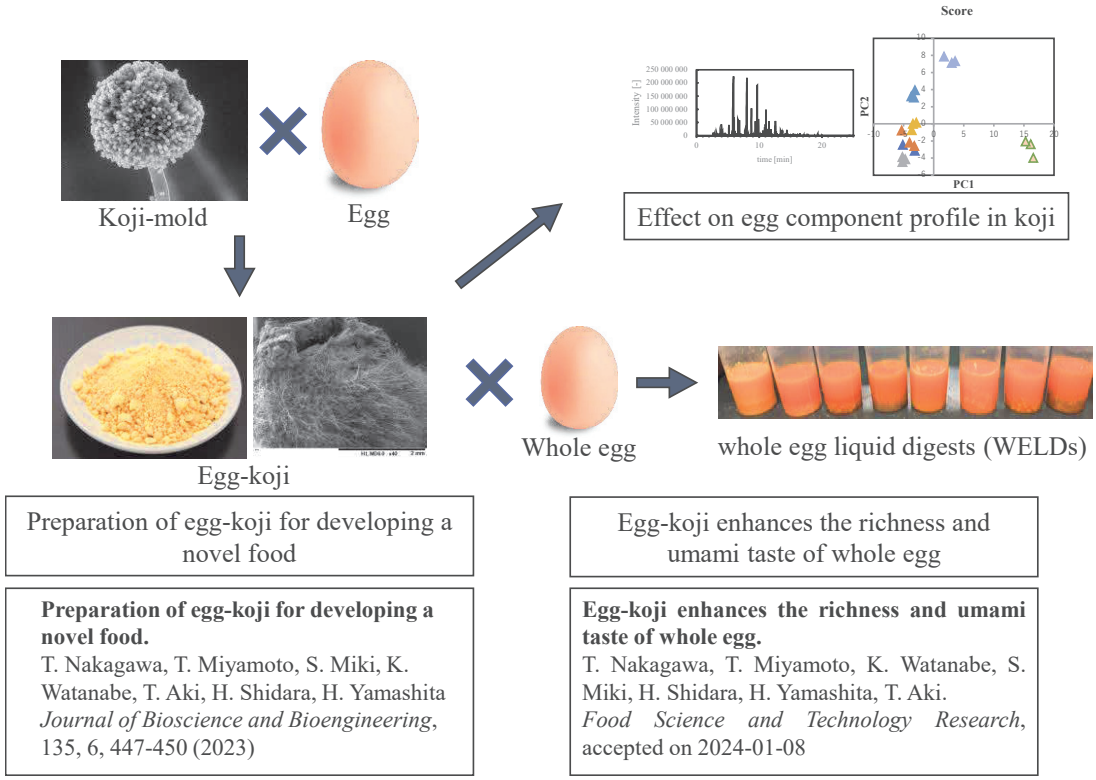

Fig. 1. Preface.

Chapter 2. Preparation of egg-koji for developing a novel food

2.1. Introduction

In this chapter, I aimed to establish a method for stably producing "egg-koji" by growing koji-mold directly on the egg. In a pre-test in which egg yolk was treated with rice-koji and an enzyme agent, it was found that umami and thickness of flavor were improved, while egg flavor was reduced (13). By improving umami and thickness of flavor while maintaining egg flavor, it is expected to have the merit of reducing additives such as monosodium glutamate in food. Therefore, I predicted that "egg-koji", which grew koji mold using egg as a substrate, could decompose and convert egg, and tried to develop it.

There was a report that koji, made from raw material, mixes eggs with carbohydrate sources such as rice and wheat (14, 15). Since a report indicated that de-starch of wheat fusuma changed the balance of secreted enzymes (16), I predicted that growing koji-mold on the eggs themselves without adding carbohydrates such as wheat would stimulate the secretion of enzymes that easily break down nutrients in the eggs and impart new flavors, and I challenged the making of the egg-koji.

2.2. Experimental Procedures

2.2.1. Selection test of egg material

The water content of 12 types of egg materials (1,000 g), including 4 types of dried egg whites (K2, SN, K10, ELS2), 3 types of dried egg yolks (No. 1, No. 11, D1), 3 types dried whole eggs (No. 1, No. 11, D1), Cooked Yolk Powder, and Cooked Egg Powder (CEP) (Kewpie Egg Corporation, Tokyo, Japan), were adjusted to 37.5%. The state of the raw material would confirm whether the water dispersion was uneven, and lumps did not occur, or whether it was liquid or conversely hard like candy. The evaluation of the state of raw materials was rated as good (+), not bad (±), or bad (-) (Table 1). If the moisture could be dispersed evenly and koji-mold could grow easily, it

was rated "good", the "not bad" rating was one in which processing is possible with some effort, and the "bad" rating was one in which processing was difficult due to poor moisture dispersion or hardening of the material.

Conidia of AO101 (0.05 g, 1/20,000 raw egg material weight) (Higuchi Matsunosuke Shoten Co., Ltd., Osaka, Japan) was inoculated and cultured at 30 °C for 42 h. The germination rate of all koji-molds was 90-95%. The growth of koji-mold was judged by the elongation of its hyphae and was rated as good (+), not bad (±), or bad (-) (Table 1). If the mycelium evenly covered the entire material and the state of the material was either good or not bad, it was applied a "good" rating. The "not bad" rating was when mycelial growth was sparsely observed on the surface of the raw material mass out of the "bad" evaluation of the physical properties of the raw material. The "bad" rating indicates that no mycelial growth was observed.

2.2.2. Preparation of the koji extract medium

Water (4,000 mL) and rice-koji (1,000 g) were mixed and held at 58 °C for 18 h. After filtering it through No. 2 filter paper (Toyo Roshi Kaisha, Tokyo, Japan), the liquid was diluted to a Brix of 10° and autoclaved for 15 min.

2.2.3. Measurement of the germination rate

Conidia of koji-mold (0.1 g) and sterile water (100 mL, 0.1% Tween 20) were mixed. The sample (0.2 mL) and 5 mL of koji extract medium were mixed and cultured at 30 °C for 6 h. Cultures were observed under a microscope (200-400x magnification). Elongated mycelia extending from round conidia were counted as germinating conidia, and the germination rate was calculated.

2.2.4. Selection test of koji-mold strain

Conidia (0.05 g) of *A. oryzae* (AO030, AO100, AO101, AO220, AO303), *A.*

sojae AS309, and *A. luchuensis* AL434 (Higuchi Matsunosuke Shoten Co., Ltd., Osaka, Japan) on the market were suspended in 475 mL of sterile water, added to 1,000 g of CEP and mixed. After culturing at 30 °C for 42 h, growth, shape, and aroma in each strain were confirmed (egg-koji is the one in which koji-mold is grown on CEP). From the activity of protease (pH6), the growth of koji-mold was judged. If the activity was higher than that of CEP, koji-mold was considered to be growing. The evaluation of egg-koji with each koji-mold was rated as good (+), not bad (±), or bad (-) (Table 2), and was similar to the growth of koji-mold (Table 1), but in addition, conidia color on the surface of koji and the aroma of koji were also checked.

In addition, I made a prototype of aged egg yolk (13, 17) obtained by digesting egg yolk liquid with egg-koji and evaluated its flavor. Aged egg yolk was obtained by aging egg yolk (925 g) with 120 g of egg-koji, 35 mL of sterile water, and 120 g of salt at 50 °C for 72 h. The evaluation of aged egg yolk was judged by the change in flavor from the yolk and bitterness and rated as good (+), not bad (±), or bad (-) (Table 2). If the products increased umami, or egg yolk flavor and less bitterness, it rated "good". The "not bad" rating was defined as no change in egg yolk flavor, but no fishy or other bad flavors. The "bad" rating was given to those with more bitter tastes.

2.2.5. Extraction of enzymes

Koji (20 g) was added to 0.01 M acetic acid buffer (100 mL, pH 5.0, 0.5% NaCl) and the mixture was extracted at room temperature for 3 h and then filtered using filter paper. Filtrate (10 mL) was dialyzed overnight with 0.01 M acetic acid buffer (pH 5.0), and the solution (20 mL) was obtained as an enzyme solution. By dialysis, sugars and amino acids in the filtrate were removed.

2.2.6. Measuring method of enzyme activity

For each enzyme, the activities were measured (n=3) by selecting representative

enzymes that are important in the koji of sake, miso, and soy sauce (18, 19). Since the water content of each koji was different, all enzyme activities are shown per 1 g of dried koji. α-Amylase activity was defined as 1 U when 1 g of koji decomposed 1 mL of 1% soluble starch solution in 30 min at 40 °C. Glucoamylase activity was defined as 1 U when 1 g of koji produced 1 mg of glucose in 60 min at 40 °C. Protease activity was defined as 1 U when 1 g of koji produced 1 μg of tyrosine in 60 min at 40 °C. Acid-carboxypeptidase (ACP) activity was defined as 1 U when 1 g of koji produced 1 μg of tyrosine in 60 min at 30 °C.

2.2.7. Measurement of the number of bacteria

The sample (1 g) was added to 0.8% NaCl (100 mL, 0.1% Tween 80) and mixed for 20 min. The solution was appropriately diluted and cultured at 30 °C for 48 h using agar medium for detecting bacteria (100 mg titer Kabicidin (antibiotic for mycomycetes, NIHON PHARMACEUTICAL, Tokyo, Japan), 0.5% yeast extract, 0.5% polypeptone, 2.4% glucose, 0.05% KH_2PO_4, 0.125% KCl, 0.125% NaCl, 0.125% $CaCl_2$, 0.125% $MgSO_4$, 0.0001% $FeSO_4$, 0.0001% $MnSO_4$, 0.8% $CaCO_3$, 1.2% agar, pH 6.8). The number of colonies that appeared there was counted.

2.2.8. Sterilization of CEP

Autoclave sterilization conditions were held at 121 °C for 15 min. Steam sterilization conditions occurred when the raw material temperature reached 95 °C and then steamed for 30 min.

2.2.9. Examination of the mixing method

CEP (1,000 g) and 325 mL of water were mixed and sterilized by steaming using a tabletop confectionary mixer (rotation speed 150 rpm) and a cutter mixer (1800 rpm). The tabletop confectionary mixer has two fins installed parallel to the axis of rotation,

while the cutter mixer has three or four blades installed perpendicular to the axis of rotation to mix CEP and water.

2.2.10. Examination of the water addition amount

CEP (1,000 g) and water (290, 325, or 360 mL (addition rate 29.0, 32.5, or 36.0% (V/W)) were mixed using a cutter mixer and then sterilized by steaming.

2.2.11. Preparation of various raw materials koji

To make egg-koji, steamed CEP (100 g) was prepared by mixing CEP with water using a cutter mixer and then steaming for 30 min. To make rice-koji, steamed rice (100 g) was prepared by soaking overnight and steaming for 50 min. To make barley-koji, steamed barley (100 g) was prepared by soaking for 30 min and steaming for 50 min. To make soy sauce-koji, defatted soybeans (20 g) were mixed with 25 mL of water, autoclaved for 30 min, and then mixed with 20 g of cracked wheat. Conidia of AO101 (0.005 g) was inoculated into each of the steamed materials and incubated in a glass Petri dish for 42 h at 30 °C and 90% humidity. Mixing was performed 18 and 24 h after inoculation, and the glass lid was removed and covered with filter paper at 18 h to prevent oxygen deficiency and drying.

2.3. Results

2.3.1. Selection of egg material

In the evaluation of the state of raw material, since dried egg white K2 and K10 became hard like candy, they were rated "bad" (Table 1). Dried egg white SN, ELS2, and dried egg yolk No. 11, D-1 were rated "bad" because of poor moisture dispersion and many soft, large clumps. Although the dried egg yolk No. 1 was a large clump, it was considered that uniform moisture dispersion would be possible with ingenuity, so it was given a "not bad" rating. Dried whole egg No. 1, No. 11, and D-1 were given a "bad"

rating because they became like soft candy. Cooked yolk powder and CEP were rated as "good" because, although small clumps were present, they had good water dispersion and remained in powder form. I evaluated CEP as having the smallest clumps and the easiest raw material processing.

In the evaluation of growth of koji-mold, since dried egg white K2 and K10 were observed no mycelial growth, they rated "bad" (Table 1). Dried egg white SN and ELS2, dried egg yolk No. 11, D-1, and dried whole egg No. 1, No. 11, and D-1 were rated "not bad" because mycelial growth was observed on the surface of the ingredients. On the other hand, no mycelial growth into the mass was observed. Dried egg yolk No. 1, Cooked yolk powder, and CEP were rated "good" because mycelium extended throughout the material and the material was well organized. From these two results, CEP was used, which was predicted to achieve better quality by improvement, as raw materials.

2.3.2. Selection of koji-mold strain

Protease (pH6) activity of CEP was 0 ± 0 (U/g-dry CEP), all koji-mold strains were above the activity of the CEP, so they were determined to be egg-koji (Table 2). In the evaluation of egg-koji, AO100 and AO220 were rated "bad" because of a fishy smell, although mycelial growth was observed (Table 2). Although mycelial growth was observed and the aroma was not bad, AO303 and AS309 were rated "not bad" because green conidia grow on egg-koji. When green or white or brown conidia grow on koji, they look bad as food. Since AO030, AO101, and AL434 were observed mycelial elongation, good aroma, and no conidia on koji, they were rated "good".

In the evaluation of aged egg yolk, AL434 had a "bad" rating because of its bitterness (Table 2), AO100, AO220, and AO303 were given a "not bad" rating because the aroma of the aged egg yolk did not change and no change in flavor was detected. Since AO030, AO101, and AS309 were changed from the raw egg yolk solution, they were rated "good". AO030 had an aroma similar to that of sake. AO101 showed a strong

miso-like flavor, while AS309 showed a flavor that was not as good as AO101. As a result of comprehensively judging both the evaluation of egg-koji and aged egg yolk, AO101 was determined to be the koji-mold strain for egg yolk.

On the other hand, the high number of bacteria (as high as 3.9×10^8 CFU/g) was a problem (Table 2). This was harmful since *Bacillus cereus*, which is one of the typical causative bacteria, for example, is said to cause food poisoning when present in food at 1.0×10^5 CFU/g or more (20, 21). Since egg-koji are expected to be used to enhance umami and aroma, it is not realistic to just heat sterilized egg-koji. Therefore, I decided to search for conditions that can stably produce egg-koji while preventing bacterial contamination.

2.3.3. Comparison of sterilization methods

The number of bacteria in CEP (1.0×10^2 CFU/g) and conidia (less than the detection limit) was significantly lower than the number of bacteria in unsterilized CEP (uninoculated) and egg-koji (Table 3). It was found that the growth of bacteria could not be suppressed only by inoculation of conidia, and therefore, the sterilization of the raw material was examined next.

In a small volume test of unsterilized CEP, the bacteria had already grown 24 h after inoculation, but when autoclave sterilization was performed, the bacteria hardly grew even 42 h after inoculation (Table 4). Next, in a large volume test, the number of bacteria was as low as 6.0×10^2 CFU/g in 10 g culturing of CEP but as high as 6.0×10^6 CFU/g in 1,000 g (Table 4). It was considered a peculiar cause in mass culture.

By autoclave sterilization, CEP was overturned, and off-flavors such as dry odor and oxidized odor were detected. Therefore, the sterilization method was switched to steaming at normal pressure. In the case of uninoculated 10 g of CEP, the number of bacteria after culturing for 42 h was 100 CFU/g or less, so it was judged that it was possible to suppress the growth of bacteria even by steam sterilization.

2.3.4. Examination of the mixing method

There were many small lumps (5 mm or less) in the sterilized CEP at the time of the test in Table 4. Since the lump contains a large amount of water and becomes hard when heated, it is hypothesized that the bactericidal effect of steam is difficult to transmit to the inside and maybe a hotbed for bacterial growth (Table 5). Therefore, I investigated a method of mixing CEP with water and aimed to homogenize CEP as much as possible to reduce lump formation. In the confectionery mixer (8.4×10^4 CFU/g) there were many small lumps, and the texture was poor (Table 6). In contrast, with the cutter mixer (2.7×10^3 CFU/g), there were almost no small lumps, and the texture was smooth. Since a cutter mixer can reduce bacteria by reducing small lumps, it was selected to use for mixing.

2.3.5. Determination of the water content

In Table 6, small lumps were almost reduced, but the number of bacteria remained high. Therefore, the amount of water added was examined next (Table 7). In a moisture content of 29.0%, the number of bacteria was 6.0×10^2 CFU/g, less than 100 CFU/g at 32.5%, and 1.5×10^3 at 36.0%. The growth of koji-mold (mycelium elongation) was better at watering rates of 32.5 and 36.0% ("good") than at 29.0% ("not bad"). From these results, the watering rate of 32.5%, in which the growth of koji-mold was good while suppressing the growth of bacteria, was determined as the amount of water added. By selecting raw materials and koji-mold strains and devising processes such as sterilization, mixing methods, and the amount of water added, it became possible to produce egg-koji using only eggs and koji-mold.

2.3.6. Characteristics of Egg-koji

The characteristics of egg-koji were investigated in terms of enzyme activity. The enzyme potency of egg-koji was compared with those of rice-koji, barley-koji, and

soy sauce-koji used in traditional fermented foods (Table 8). Egg-koji had extremely low activities for both α-amylase and glucoamylase when compared with other koji, and protease had a characteristic balance in which the activity at pH 6 was higher than at pH 3.

2.4. Discussion

In this chapter, the control of the number of bacteria was the biggest challenge. The essential roles of koji are to supply enzymes necessary for the decomposition of raw materials and to impart flavor. Since a sterilization process cannot be established after egg-koji culture to prevent enzyme deactivation, it was necessary to take measures at the raw material processing stage. The factor that causes bacterial proliferation is insufficient sterilization, and if clumps are formed due to uneven moisture dispersion, the clumps are not sterilized enough. Additionally, koji-mold cannot grow inside the clumps, and the quality of koji (enzyme activity, etc.) is not stable. Therefore, I succeeded in developing egg-koji using only eggs (CEP) and koji-mold while suppressing bacterial growth by establishing a method for uniform moisture dispersion, a sterilization method, and the amount of water added to the ingredients (22).

Enzymes of koji-mold are substrate specific and secreted for degrading raw materials. Since eggs contain almost no carbohydrates, the activity of amylase was low, and since the pH of the raw material is approximately 8.5, it is presumed that the protease activity at pH 6 was higher than that at pH 3. These results were considered to represent the substrate specificity. The digestive reaction of egg-koji and egg yolk solution yielded an aged egg yolk with enhanced umami and richness while retaining the unique flavor of egg yolk (23). This change could not be achieved by cooking, nor could it be achieved with conventional rice-koji or enzyme agents. It was expected that egg-koji secreted enzymes necessary for the decomposition of egg components, resulting in the efficient decomposition of egg yolk and enhancement of umami, richness, and flavor. It was found that efficient conversion and decomposition are possible by growing koji-mold on raw

materials in this way. When considering the use of these foods, there is a possible benefit of reducing the number of extra additives and seasonings.

In this chapter, AO101 was only selected, but other strains also produced distinctive egg-koji. In Chapter 3, koji-mold strain-specific changes of ingredient profiles are confirmed, and in Chapter 4, it is confirmed that the sensory and constituent changes of the WELDs of each koji-mold strain are different.

TABLE 1. Selection test of egg material

Egg materials		Evaluation[a]	
		State of raw material	Growth of koji-mold
Dried egg white	K2	-	-
	SN	-	±
	K10	-	-
	ELS2	-	±
Dried egg yolk	No. 1	±	+
	No. 11	-	±
	D-1	-	±
Dried whole egg	No. 1	-	±
	No. 11	-	±
	D-1	-	±
Cooked Yolk Powder		+	+
CEP		+	+

[a] +, Good; ±, Not bad; -, Bad.

TABLE 2. Selection test of koji-mold strain

Koji-mold strains		Protease (pH6) activity (U/g-dry koji)	Evaluation[a]		Number of Bacteria (CFU/g)
			Egg-koji	Aged egg yolk	
A. oryzae	AO030	807 ± 10	+	+	1.1×10^{7}
	AO100	242 ± 1	-	±	1.4×10^{8}
	AO101	312 ± 3	+	+	1.1×10^{8}
	AO220	569 ± 2	-	±	3.9×10^{8}
	AO303	611 ± 2	±	±	3.1×10^{7}
A. sojae	AS309	887 ± 2	±	+	2.2×10^{8}
A. luchuensis	AL434	235 ± 3	+	-	1.9×10^{8}

[a] +, Good; ±, Not bad; -, Bad.

TABLE 3. Number of bacteria in unsterilized raw materials

Raw material	Number of bacteria (CFU/g)
CEP	1.0×10^{2}
Conidia of AO101	N.D.[a]
Unsterilized CEP (uninoculated)	4.2×10^{9}
Egg-koji	5.0×10^{8}

[a] N.D., Detection limit was less than 100 CFU/g.

TABLE 4. Examination of autoclave sterilization

Test volume	Amount of CEP (g)	Mixing with water	Autoclave sterilization	Conidia Inoculation	Culture time (h)	Number of bacteria (CFU/g)
Small	10	-	-	-	24	N.D.[a]
		+	-	-		1.2×10^{7}
		+	-	+		8.0×10^{4}
		+	+	-		N.D.
		+	+	+		N.D.
		-	-	-	42	4.2×10^{2}
		+	-	-		3.4×10^{9}
		+	-	+		5.8×10^{7}
		+	+	-		N.D.
		+	+	+		N.D.
Large	1,000	-	-	-	42	N.D.
	10	+	+	+		6.0×10^{2}
	1,000	+	+	+		6.0×10^{6}

[a] N.D., Detection limit was less than 100 CFU/g.

TABLE 5. Sieving test of egg-koji incubated for 42 h

AO101 Inoculation	Sieving[a]	water content (%)	Number of bacteria (CFU/g)
-	-	18.1	N.D.[b]
+	-	24.5	1.4×10^{3}
+	Powder form	22.4	2.6×10^{2}
+	Lump	30.7	5.0×10^{4}

[a]Egg-Koji was placed on a cooking net (2 mm opening) and sieved by hand. The material that passed through the mesh was collected in powder form, and the material remaining on the mesh was collected as a lump.
[b]N.D., Detection limit was less than 100 CFU/g.

TABLE 6. Examination of the mixing method

Mixing method (Rotation speed)	Evaluation	Number of bacteria (CFU/g)
Confectionery mixer (150 rpm)	A lot of small lumps and bad texture	8.4×10^{4}
Cutter mixer (1,800 rpm)	Smooth touch with almost no lumps	2.7×10^{3}

Table 7. Steam sterilization and examination of the water addition amount

Moisture content (%)	Number of bacteria (CFU/g)	Evaluation of growth of koji-mold	Remarks
29.0	$6.0*10^2$	±	
32.5	N. D.[a]	+	
36.0	$1.5*10^3$	+	
	$5.8*10^7$	+	Not sterilized

[a] N.D., Detection limit was less than 100 CFU/g.

TABLE 8. Comparison of enzyme activity balance between grain koji and egg-koji

Koji (AO101)	U/g-dry koji				
	Amylase		Protease		ACP
	α-	Gluco-	pH 3	pH 6	
Rice-koji	1,722 ± 17	420 ± 13	12,818 ± 369	9,935 ± 66	16,370 ± 849
Barley-koji	2,005 ± 66	282 ± 12	29,171 ± 689	18,110 ± 895	28,623 ± 756
Soy sauce-koji	12,350 ± 595	1,132 ± 30	26,084 ± 944	40,751 ± 1,767	37,873 ± 1,187
Egg-koji	329 ± 17	5 ± 2	21,135 ± 749	27,620 ± 430	21,870 ± 1,042

Chapter 3. Effect on egg component profile in koji

3.1. Introduction

The purpose of this chapter is to track the changes in egg component profiles in the process of egg-koji making. It has been reported that the decomposition and conversion of substances contained in koji raw materials leads to the formation of various components such as oligosaccharides (24), fatty acids, and amino acids, resulting in changes in taste and health effects (25, 26). For example, Oura et al. have reported that amazake made with rice koji suppresses blood pressure increase and amnesia and exhibits anti-obesity effects (27). Yamada et al. also reported that epoxy succinic acid derivatives contained in rice-koji specifically inhibit cathepsin L, a protease involved in the development of allergies and osteoporosis (28). Since the ingredients of koji foods depend on the raw materials and the type of koji-mold used, it is expected that the creation of koji foods with new functionality can be expected by brewing various koji-molds that have not been used as koji raw materials so far.

In this chapter, the components produced in egg-koji were comprehensively analyzed by metabolomics using a gas chromatograph mass spectrometer (GC/MS). Metabolomics is the comprehensive analysis of metabolites (metabolomes) generated by biological activities (29), and in recent years, there has been an increase in the number of reported cases of the quality confirmation of foods and the search for functional ingredients such as cheese (30) and wine (31). The purpose of this chapter was to clarify the new functionality of egg-koji by metabolome analysis by GC/MS, which can measure amino acids, fatty acids, organic acids, etc., which are related to the flavor and nutritional function of foods.

3.2. Experimental Procedures

3.2.1. Investigation of metabolic extraction method using methanol and extraction of components

Steamed CEP and egg-koji grounded in a mortar and pestle to make uniform. The sample (100 mg) was immersed in 1 mL of 80% methanol and inverted at 4 °C for 24 h (from now on referred to as 100 mg, 24 h). Alternatively, 1 mL of 80% methanol was added to a 10 mg sample and stirred with a vortex mixer for 1 min (from now on referred to as 10 mg, 1 min). After adding 12 μg of ribitol as an internal standard to the extract, it was centrifuged at 16,000 ×g at 4 °C for 10 min. The supernatant (200 μL) was transferred to a new loop screw cap tube and lyophilized for 8 h after the removal of the organic solvent in a centrifugal evaporator.

3.2.2. Trimethylsilyl derivatization

Methoxamine hydrochloride solution (100 μL, 20 mg/mL in pyridine) was added to the lyophilized sample and inverted at 28 °C for 90 min to react. After that, 50 μL of MSTFA (Sigma Aldrich) was added and reacted at 37 °C for 30 min.

3.2.3. GC-MS Measurements

TMS reaction solution was transferred to a new glass insert and measured by GC-MS within 12 h. DB-5HT column (Agilent Technology) was connected to a gas chromatograph 7890A (Agilent Technology), and the compounds were separated by a temperature increase program (100 °C, 2 min; 15 °C/min to 330 °C; 330 °C, 6 min). Time-of-flight mass spectrometer JMS-T100CGV (Japan electrons) was used to detect fragment ions generated by the electron ionization method (EI method), and each compound was identified by comparison and analysis with the MS/MS spectral library installed in the MassCenter (Japan electrons).

3.2.4. Data Analytics

The GC-MS acquisition data was converted to netCDF format using the data converter installed in MassCenter, and peaks were picked and aligned using Metalign (32). The metabolite profile of each sample was analyzed for principal component analysis. The data processed by Metalign was opened with AIoutput (33) and principal component analysis was performed.

3.3. Results

3.3.1. Investigation of metabolite extraction method using methanol

Samples extracted by both methods (n=3) were analyzed by GC-MS after derivatization (Fig. 2A-D), and the average number of detected peaks was calculated (Table 9). In the analysis of CEP, 274 peaks were detected at 100 mg, 24 h (Fig. 2A and Table 9), 208 peaks at 10 mg, 1 min (Fig. 2B), and about 60 more peaks were detected at 100 mg, 24 h. In addition, 300 peaks were detected at 100 mg and 24 h (Fig. 2C) and 247 peaks at 10 mg, and 1 min (Fig. 2D), respectively, and about 50 peaks were detected in the 100 mg and 24 h. Therefore, in subsequent experiments, an extraction method in which a 100 mg sample was immersed in 80% methanol and inverted at 4 °C for 24 h was adopted.

3.3.2. Component Analysis by GC-MS

The components extracted from the CEP and egg-koji under the conditions determined in 3-3-1 were TMS derivatized and then analyzed by GC-MS (Fig. 3A-H). The internal standard ribitol was detected around the retention time of 9.6 min, and each component was quantified using this peak area as an index. In particular, the peaks detected in CEP and egg-koji differed significantly after the retention time of 12 min.

3.3.3. Principal Component Analysis

When the quantitative results of each component were subjected to principal component analysis (PCA), the component profiles of CEP and egg-koji were separated by the first principal component (Fig. 4A). In the loading plot, the contribution to the first principal component was high, and when substances were extracted that were increased in egg-koji, it was found that glycerol and sugar alcohols increased markedly in egg-koji (Fig. 4B). In addition, the compound profiles of different species of *Aspergillus* were separated on the second principal component (Fig. 4A), suggesting that species-specific metabolites were generated.

3.3.4. Specific products from each koji-mold

PCA analysis was performed only with the analysis data of each egg-koji, excluding CEP from the results of 3-3-3 (Fig. 4A). As a result, the score plots of each egg-koji were separated (Fig. 5A). Among them, it was speculated that the plot of AS309 was markedly separated from the other koji-mold in the first principal component and had a characteristic metabolic profile. In the loading plot (Fig. 5B), the components that were significantly increased in E-309 (components with a contribution score greater than 0.1332 for PC1) were extracted and found that they contained a variety of amino acids (L-Lys, L-Glu, L-Ile), and malic acids, L-threitol, oxamic acid, and glycerol.

3.4. Discussion

The purpose of this chapter was to investigate whether the koji conversion of eggs, a new koji raw material, produces ingredients that contribute to improving taste and health functions. Compounds extracted from CEP and egg-koji with methanol aqueous solution were measured by GC-MS, and the measurement data was subjected to principal component analysis, suggesting that the component profiles of CEP and egg-koji were significantly different (Fig. 4A). Therefore, it was inferred that the egg-derived component was converted into a new substance by koji-mold. In addition, a significant

increase in glycerol thought to have been converted from neutral lipids, and sugar alcohols thought to have been converted from polysaccharides was observed in egg-koji (Fig. 4B). Since the significant formation of these components was observed regardless of the koji- mold strains, it was suggested that they may be used as biomarkers for evaluating the koji formation of eggs. PCA analysis of egg-koji excluding CEP suggested the formation of a component specific to the koji strain. In particular, the metabolite profile of AS309 separated from other species in the first principal component (Fig. 5A). AS309 contained a variety of amino acids such as L-lysine (Fig. 5B), it was thought to reflect the high protease activity of *A. sojae* (34). As a result of genome sequencing, there are 134 genes involved in the proteolysis of *A oryzae* (35), and it has been reported that the genes of *A. sojae* are equivalent (34). The protease gene ORFs showed high homology (81.7%) to *A. oryzae* 34), and the number of proteases with specific domains was not much different between both species. On the other hand, four types of proteases in *A. sojae* NBRC4239 have one more gene than *A. oryzae* RIB40 (34), and each of the 11 types of proteases in NBRC4239 have one gene less than RIB40. It is thought that such a difference affected the amino acid produced, although slightly. In addition, *A. oryzae* has three copies of the α-amylase gene while *A. sojae* has one copy (34), and it has been confirmed that there is a difference in lipase activity between *A. oryzae* and *A. sojae* on the surface of cheese curds (9), which is thought to affect the difference in products. Since it was inferred in the principal component analysis that not only *A. sojae* but also other koji-mold have a species-specific component profile, I plan to proceed with the analysis while comparing the extraction and measurement methods of metabolites. In addition, if the production of a useful functional component is recognized, it is assumed that molecular breeding of koji-mold that produces a high amount of the component will also be performed.

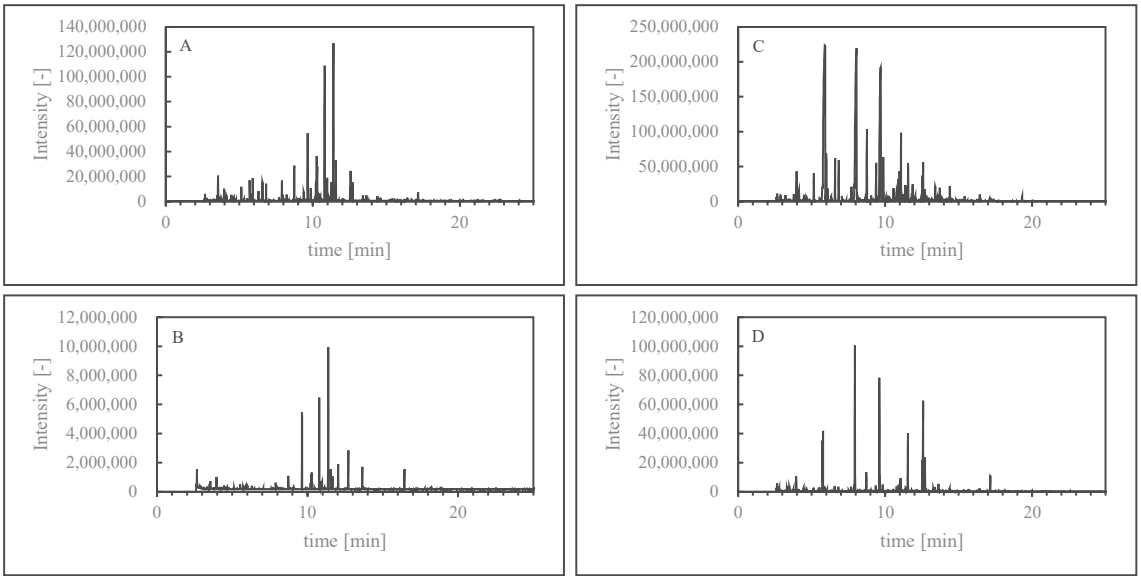

Fig. 2. The GC/MS analysis peaks of methanol extracts.
(A) Steamed CEP (100 mg, 24 h), (B) steamed CEP (10 mg, 1 min), (C) Egg-koji AO220 (100 mg, 24 h), (D) Egg-koji AO220 (10 mg, 1 min).

Table 9. The average number of detected peaks

Extraction method	CEP	Egg-koji AO220
100 mg, 24 h	274	300
10 mg, 1 min	208	247

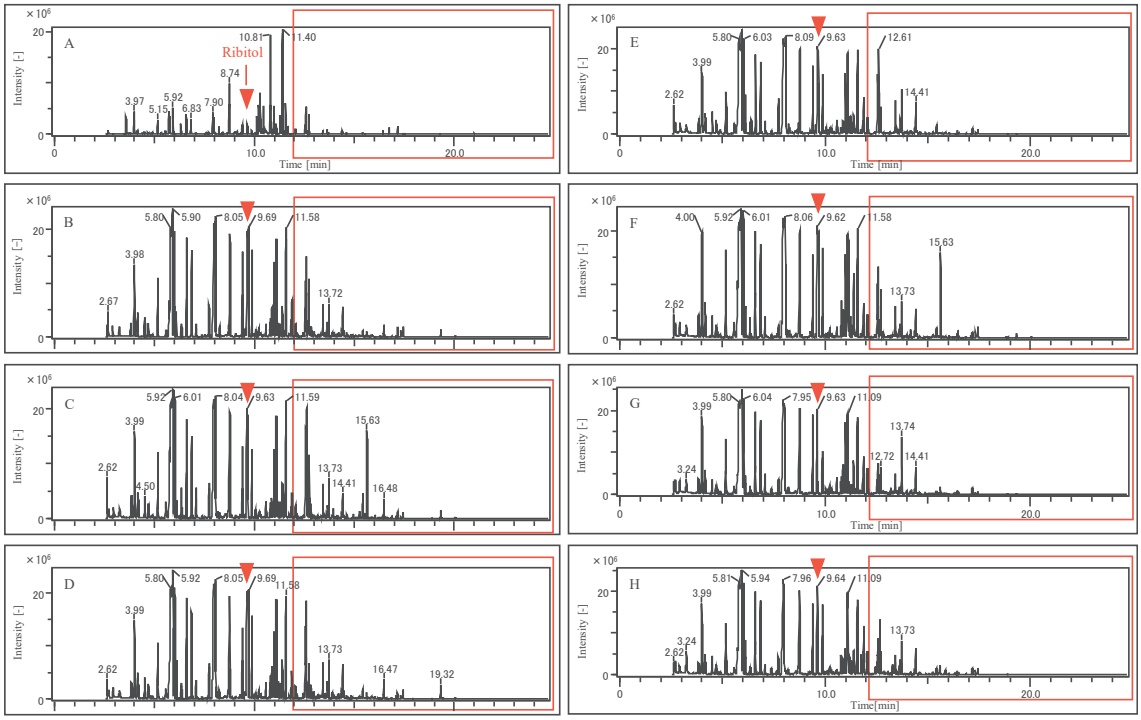

Fig. 3. The GC/MS analysis peaks of methanol extracts in each egg-koji.
The internal standard Ribitol (retention time around 9.6 min) is shown in the triangular position. (A) Steamed CEP, (B) AO220, (C) AO030, (D) AO100, (E) AO101, (F) AO303, (G) AS309, (H) AL434.

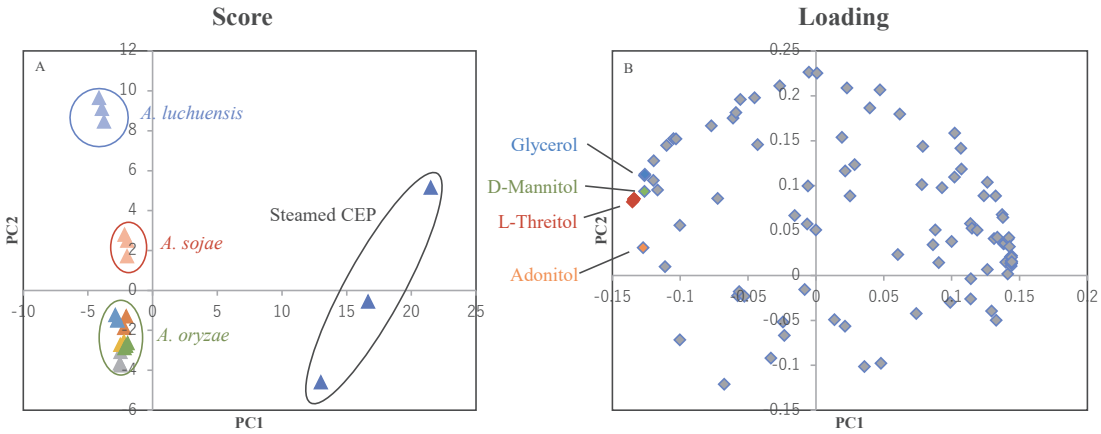

Fig. 4. Principal Component Analysis.
(A) PCA score plot of steamed CEP and egg-koji, (B) loading plot of CEP and egg koji.

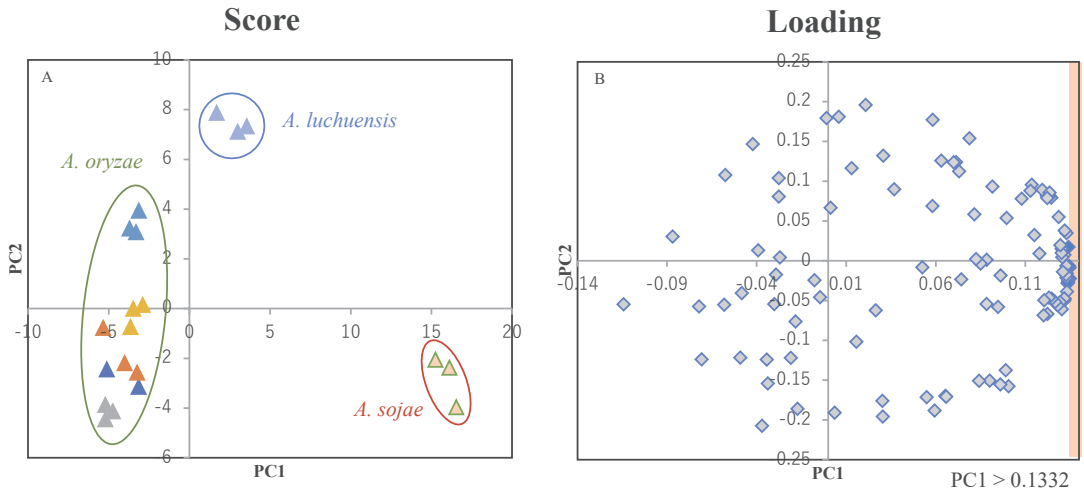

Fig. 5. Specific products from each koji-mold.
(A) PCA score plot of each koji-mold, (B) loading plot of E-309.

Chapter 4. Egg-koji enhances the richness and umami taste of whole egg

4.1. Introduction

In Chapter 2, I developed "egg-koji" made of koji from egg materials (36), and when egg yolk liquid was digested using the egg koji, it had a favorable flavor different from rice-koji (14). It has been clarified that there are approximately 12,000 genes in the genome of *A. oryzae* RIB40 (2, 3), of which 134 are proteolytic enzymes. Since enzymes of koji-mold have substrate specificity (4, 5, 6), growing koji-mold on eggs was expected to give flavors that cannot be achieved by cooking, such as decomposing egg proteins to produce peptides and amino acids to enhance umami or decomposing lipids to enhance aroma components.

"Richness" is one of the characteristics of each food, as well as taste, aroma, texture, color, and luster, and is a factor that determines its deliciousness (37). There are strengths and weaknesses in richness, and deliciousness is given by the strength of richness that is suitable for each food. Richness is a complex characteristic formed from multiple stimuli of taste, flavor, and texture of food. These multiple stimuli are the base part of the taste that determines the characteristics of the food, and richness is felt when persistence and expansion are added. Important ingredients that can give sustainability and expansion to the taste produced from this base are umami substances and oils. Umami substances strengthen the flavor qualities consisting of flavors and aromas, and at the same time, give them spread and persistence. It is known that oils can give persistence, which is a characteristic of richness, by retaining the aroma component. The generation of "richness" of food involves flavor components such as amino acids, aroma components as well as structures and components related to texture (37). In addition to the above components, thickness and persistence are required, and umami substances and oils are important components. In this chapter, I focused on the components related to richness among the component changes caused by the fermentation of whole eggs with egg-koji

and investigated their effects on sensory characteristics. Egg-koji and whole egg liquid were reacted in the presence of salt, and free amino acids, aroma components, and neutral lipids were measured in the digestive product. Furthermore, sensory evaluation of whole egg liquid digests (WELDs) was conducted to investigate whether there was a difference in sensory.

4.2. Experimental Procedures

4.2.1. Preparation of various raw materials koji

Egg-koji and grain-koji (rice, barley, and soy sauce (wheat, skimmed soybeans)) were prepared with slight modifications following **2.2.11**. Conidia of AO101, AS309, or AL434 (0.05 g each, 1/20,000 raw egg material weight) were inoculated into each of the steamed materials and incubated in a glass petri dish for 42 h at 30 °C and 90% humidity. Mixing was performed 18 and 24 h after inoculation, and the glass lid was removed and covered with filter paper at 18 h to prevent oxygen deficiency and drying.

4.2.2. Preparation of WELDs

WELDs were prepared by digesting whole egg liquid (50 g) under 4% NaCl at 60 °C for 24 h by koji (5 g). WELDs prepared with egg-koji AO101, AS309, and AL434 are denoted as E-101, E-309, and E-434, respectively. WELDs prepared with rice-koji AO101, barley-koji AO101, and soy sauce-koji AO101 are denoted as R-101, B-101, and S-101, respectively. CEP, Rice, barley, and Soy sauce are processed in the same way as above by adding only the material before making koji to whole egg liquid. Control was made by adding only NaCl to the whole egg liquid.

4.2.3. Measurement of free amino acids in WELDs

By adding 3.3% trichloroacetic acid, the crude protein in the sample was removed and filtered through a 0.45 μm PVDF filter. The amino acid composition of each

filtered sample was measured using an auto amino acid analyzer (LA 8080, Hitachi High-Tech Corporation, Tokyo, Japan).

4.2.4. Measurement of Aroma components in WELDs

Samples (1.5 g) were diluted (3.0 g) and placed in a glass vial (10 mL) and heated at 40 °C for 15 min. Released volatiles was adsorbed on a solid phase micro extraction (SPME) fiber (polydimethylsiloxane/carboxy/divinylbenzene, Stable Flex 50/30μm, DVB/Carboxen/PDMS, Sigma-Aldrich, St. Louis, MO, USA) at 40 °C for 20 min. The volatiles adsorbed on SPME was separated using a gas chromatography (7890B, Agilent Technologies, Santa Clara, CA, USA) with a capillary column (SOLGEL-WAX, 30 m × 0.25 mm × 0.25 μm; Trajan Scientific and Medical, Victoria, Australia). Volatiles isolated were analyzed using a mass spectrometer (5977A, Agilent Technologies). Operating conditions were as follows: injector temperature, 250 °C; helium flow rate, 1.2 mL/min; oven temperature, 35 °C for 5 min and then programmed to increase at 5 °C/min to 60 °C, and increased at 15 °C/min to 220 °C and held for 9.7 min. Mass spectra were obtained by electron ionization at 70 eV over 29 to 290 mass units, with an ion source temperature of 230 °C. Calibration of the SPME-GC/MS mass scale was regularly performed using perfluorotributylamine.

4.2.5. Lipid analysis of WELDs

The WELD (100 mg) was vortexed with 1 mL of *tert*-butylmethylether/methanol (2:1, v/v) using beads crusher (μT-12, Taitech, Aichi, Japan). Distilled water (500 μL) was added, and mixed solutions were vortexed for 30 seconds using a vortex mixer. An organic layer was collected by centrifugation at 12,000 g for 10 min and was transferred into a 2 mL vial bottle. Total lipids were analyzed using gas chromatography (7890A GC system, Agilent Technologies) equipped with a time-of-flight mass spectrometer (JMS-T100CGV, JEOL, Tokyo, Japan). The components were separated by a capillary column

(DB-5HT, 0.25 mm I.D. × 30 m, Agilent Technologies). The column temperature was held for 2 min at 80 °C, then increased to 330 °C at a rate of 15 °C/min, and held for 6 min. The ionizing voltage was 70 V and the ion chamber temperature was 280 °C. Helium was used as carrier gas at a constant flow rate of 1.12 mL/min.

4.2.6. Sensory evaluation test of WELDs

Sensory evaluation was conducted according to the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects, World Medical Association, Geneva, 2013. Samples were evaluated by seven trained panels consisting of men and women of ages 25-60, a total of 7 parameters of taste and aroma (saltiness, sweetness, umami taste, bitterness, egg flavor, thickness of flavor, and persistence of flavor), were evaluated in ten grades: 1 to 10; and averages were calculated. AO101 was a strain selected during the development of egg koji (Nakagawa *et al*., 2023), and AO101 is used in the industrial production of aged egg yolk. Using AO101 as a standard strain of egg koji, the sensory characteristics of WELDs prepared with egg-koji and other grain koji were compared.

4.3. Results and discussion

4.3.1. Amount of free amino acids in WELDs

The total amino acid content of each WELD was increased 13.9-fold (E-101), 18.1-fold (E-309), 2.7 -fold (E-434), 2.0-fold (R-101), 3.6-fold (B-101), and 16.4-fold (S-101) compared to the control, respectively (Fig. 6A). The total amino acid content was positively correlated with the protease (pH 6) activity of koji ($R^2 = 0.96$) (Fig. 7). Each free amino acid has its taste (38): Gly, Ala, Thr, Pro, and Ser have a sweet taste; Phe, Tyr, Arg, Ile, Leu, Val, Met, and Lys have a bitter taste; Glu and Asp have umami and sour tastes; Gly, Ala, and Ser exhibit umami as well as sweetness at high concentrations. Although amino acids have a variety of flavors, the composition of each amino acid was

almost the same in all samples (Fig. 6B-5G), and it was thought that the higher the total amount of amino acids, the more it affects the umami taste and the thickness of flavor that leads to richness (37).

4.3.2. Aroma components in WELDs

Figure 7 shows the aroma components whose content significantly changed compared to the control, and which are related to richness and umami. 2-Methylbutanal (Fig. 8A) and 3-methylbutanal (Fig. 8B) are aldehydes that enhance the umami taste of aftertaste (39) and are formed by the degradation of isoleucine and leucine, respectively. These compounds were increased in all WELDs. 2-Ethylfuran (Fig. 8C) is a furan compound used to improve the flavor of meat extracts (40). This compound was significantly increased in E-101, E-309, and E-434, decreased in R-101, and unchanged in B-101 and S-101. Hexanal (Fig. 8D) is a green-flavored aldehyde (41), which increased significantly in E-101 and E-309, slightly increased in E-434 and S-101, and decreased in R-101 and B-101. Hexanal was abundant in soybean, the raw material of soy sauce, while it was scanty in S-101, suggesting that this component in soybean was significantly reduced by preparing koji. 1-Penten-3-ol (Fig. 8E) is a green-flavored aldehyde that contributes to richness (42). This compound was increased in E-309, unchanged in E-101, and decreased in the others. Octanal (Fig. 8F) is a citrus-flavored aldehyde (43) and was significantly increased in E-101 and E-309. 1-Octen-3-one (Fig. 8G) is a ketone compound with a mushroom-like or green-like flavor (43) that is formed during heating and aging. It was produced or increased by koji but was not generated only in E-434. Nonanal (Fig. 8H) is a soapy-flavored aldehyde (43) that was increased in all WELDs, but S-101. This compound in E-101 and S-101 were less than those in their respective raw materials. Methional (Fig. 8I), a cheese-flavored sulfide (44) that enhances umami taste, was produced in E-101, E-309, E-434, and S-101, with the highest content in E-309. Furfural (Fig. 8J) is an aldehyde in the almond flavor (45) and

contributes to the richness of miso. This compound was significantly increased in E-101, E-309, E-434, R-101, and B-101, and was decreased in S-101. 1-Octen-3-ol (Fig. 8K), a mushroom-flavored alcohol (44, 45, 46), is produced by enzymatic degradation of linoleic acid, and adding 100 ppt of it enhances the rich taste of bonito and kelp soup (43). It was increased in all WELDs except E-434. Benzaldehyde (Fig. 8L) is a fruity-like or green-flavored aldehyde (39) and was increased in all koji. On the other hand, benzaldehyde in S-101 was significantly reduced compared to that in the koji material. Phenylacetaldehyde (Fig. 8M), a honey-flavored aldehyde that enhances richness (39), was significantly increased in E-101, E-309, E-434, B-101 and S-101. 2-Methylpyrazine (Fig. 8N) is a pyrazine derivative of nutty aroma that is easily produced by heating sugars or amino acids, which is considered to be one of the characteristic flavors of eggs (46). While 2-methylpyrazine was increased in all WELDs compared to that in the control, this compound in WELDs made with egg-koji was less than that in WELD made with CEP, the raw material of egg koji.

From these results, I expected that the increase in various aroma components in WELDs and their involvement with taste components such as amino acids affected the "thickness of flavor" and "persistence of flavor" that led to enhanced umami and richness.

4.3.3. Lipid analysis of WELDs

The lipid content in whole egg liquid is 40.8% (component composition in dry weight) (11), which is the second highest component after protein. Since fats and oils are reported to be involved in richness by the smooth texture and the retention of aroma components, lipids in WELDs were analyzed.

Free fatty acids (FFAs) content was increased in E-101 (24.7 %, Fig. 9A, Table 10), E-309 (28.6%, Fig. 9B), and E-434 (76.9%, Fig. 9C), which are egg-koji-treated WELDs, compared to the control (0.27%, Fig. 9G). Additionally, triacylglycerol (TAG) content in E-101 (53.6%), E-309 (47.6%), and E-434 (12.7%) were significantly lower

than that in the control (75.1%) (Fig. 8). Since only a trace amount of FFAs were detected in R-101 (0.49%, Fig. 9D), B-101 (0.49%, Fig. 9E), and S-101(2.2%, Fig. 9F), the egg-koji, especially egg-koji AL434, was thought to have a higher lipid-degrading capacity than other koji. It has been reported that intracellular TAG lipase in *A. oryzae* functions in neutral lipid degradation, and it is considered that egg-koji secretes a large amount of lipase in this report (47). Therefore, I believe that it is necessary to measure lipase activity in the future. A large amount of FFAs in E-101 and E309 may explain the increase in 1-penten-3-ol, 1-octen-3-one, and 1-octen-3-ol produced during the degradation process of FFAs (Figs. 7E, 7G, and 7K). The egg-koji AL434 was thought to have a different FFA metabolism because those decomposition products were not increased in E-434. FFAs are often at a sensory disadvantage due to their bitterness and astringency. On the other hand, diacylglycerol (DAG) and monoacylglycerol are produced when FFAs are released from TAG and act as emulsifiers and stabilizers (48). It has also been reported that free fatty acids themselves have emulsifying power (49, 50, 51). Therefore, when E-434 is diverted to food, the amount of emulsifier and stabilizer used may be reduced.

4.3.4. Sensory evaluation test of WELDs

AO101 is a strain selected during the development of egg koji (36), and AO101 is used in the industrial production of aged egg yolk. Using AO101 as a standard strain of egg koji, the sensory characteristics of WELDs prepared with egg-koji and other grain koji were compared. Saltiness was significantly reduced in WELDs (Fig. 10A), and it was thought to have been masked by amino acids (Fig. 6A) and aroma components (Fig. 8) produced in WELDs. Umami was significantly elevated in each koji (Fig. 10C). One of the reasons was the increase in Glu, Asp (Fig. 6), 2-methylbutanal (Fig. 8A), 3-methylbutanal (Fig. 8B), and methional (Fig. 8I) related to Umami. Egg flavor increased in E-101 and decreased in R-101, B-101, and S-101 (Fig. 10E). 2-Methylpyrazine is one of the aroma components that forms egg flavor. However, there was no significant

difference in the amount of this compound between E-101 and S-101 (Fig. 8N), suggesting the presence of other components involved in egg flavor. Thickness of flavor and persistence of flavor were significantly increased in each koji (Figs. 9F and 9G). The increase in these flavors was assumed to be due to the increase in amino acids and aroma compounds for the same reason as the increase in umami. No WELDs exhibited significant sweetness compared to the control (Fig. 10B). Glucose, produced by the digestion of carbohydrates, has been reported as a sweetening ingredient in amazake. On the other hand, the carbohydrate content of eggs was very low (1.6%), suggesting that less glucose is produced and that there was little change in sweetness. There was no significant difference in bitterness between each WELDs (Fig. 10D). Bitter compounds include alkaloids, terpenes, amino acids, peptides, and organic acids (50) that exhibit a bitter taste are Phe, Tyr, Arg, Ile, Leu, Val, Met, and Lys (38), and there was no significant difference in the amount of these amino acids among the WELDs (Figs. 5B, 5E, 5F, 5G, and 5H), which may be one reason for the lack of significant difference in bitterness. E-101 was the only WELD that enhanced the umami and richness without losing its egg flavor. Additives such as monosodium glutamate and nucleic acid are added to foods to enhance the umami taste, but by using WELD such as E-101, these seasonings and additives can be reduced.

Next, sensory tests were conducted on E-101, E-309, and E-434 to determine the effect of different strains of koji mold used in the preparation of egg koji on sensory characteristics. Saltiness (Fig. 11A), sweetness (Fig. 11B), umami (Fig. 11C), egg flavor (Fig. 11E), the thickness of flavor (Fig. 11F), and persistence of flavor (Fig. 11G) were not significantly different among E-101, E-309, and E-434. Umami and richness were enhanced while maintaining egg flavor. E-309 had almost the same rating as E-101, but the saltiness rating was lower than that of E-101, and it was thought that the salt habituation had progressed. Since E-309 had a higher total amino acid content than E-101 (Fig. 6A) and has more aroma components such as methional (Fig. 8I), 1-octen-3-ol

(Fig. 8K), and phenylacetaldehyde (Fig. 8M), it was considered to be a composite result of their total. By improving the reaction conditions between E-309 and whole egg liquid, it was thought that it would be possible to develop products with enhanced umami taste compared to E-101. Although E-434 has a large number of FFAs (Fig. 8C) with a negative bitter taste, FFAs as well as DAG and monoacylglycerol generated from TAG have strong emulsifying power. In the case of egg foods, it is important to maintain the emulsified state (47), and emulsifiers such as lecithin and fatty acid esterified with glycerin, propylene glycol, and sorbitan are often added, emphasizing the usefulness of E-434 as an emulsifier adjuster.

Finally, egg white accounts for about 60% of the edible part of the whole egg (11). Therefore, it is expected to reduce food loss by using whole egg liquid instead of egg yolk liquid. This is an advantage toward the realization of a sustainable society.

4.4. Summary

Using egg-koji prepared by the different *Aspergillus* strains, the WELDs with enhanced umami and richness while retaining the egg flavor of whole eggs could be prepared. Different characteristics of each egg koji were revealed, such as that AS309 produced more free amino acids and aroma components and E-434 had the potential to take the emulsifying power of FFAs, suggesting that seasonings and additives can be reduced in food design. It was found that different strains could be used to alter the properties of WELD. By using WELD, which has different properties, we believe that it will be possible to develop products that meet the needs of consumers.

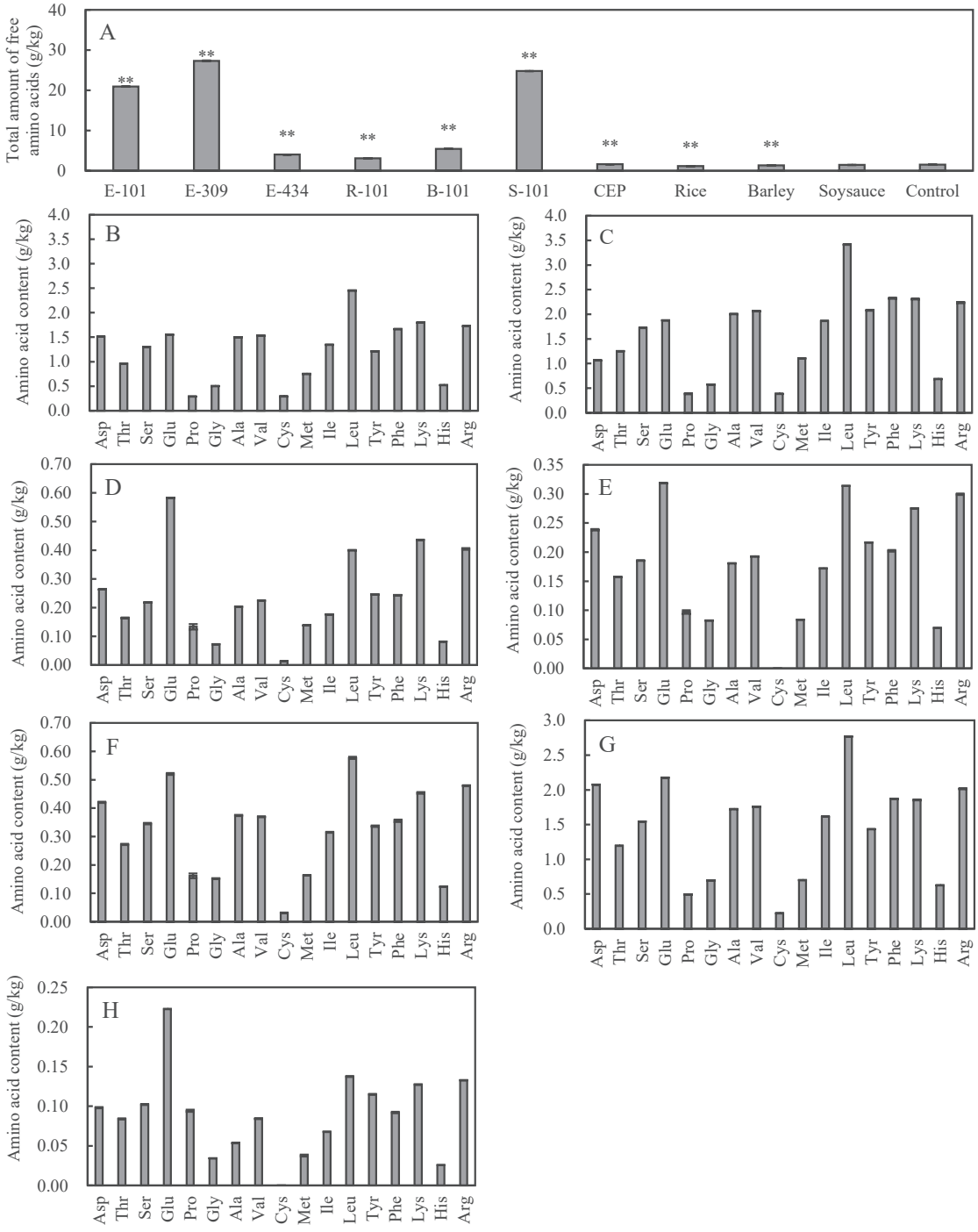

Fig. 6. Amount of free amino acids in WELDs.
The total amount of amino acids in WELDs prepared with different koji (A). Data are expressed as the mean ± SE (n = 3). Values with asterisks differed significantly compared with control in Welch's *t*-tests. $^{**}p < 0.01$. Each amino acid in E-101 (B), E-309 (C), E-434 (D), R-101 (E), B-101 (F), S-101 (G), and control (H) were measured.

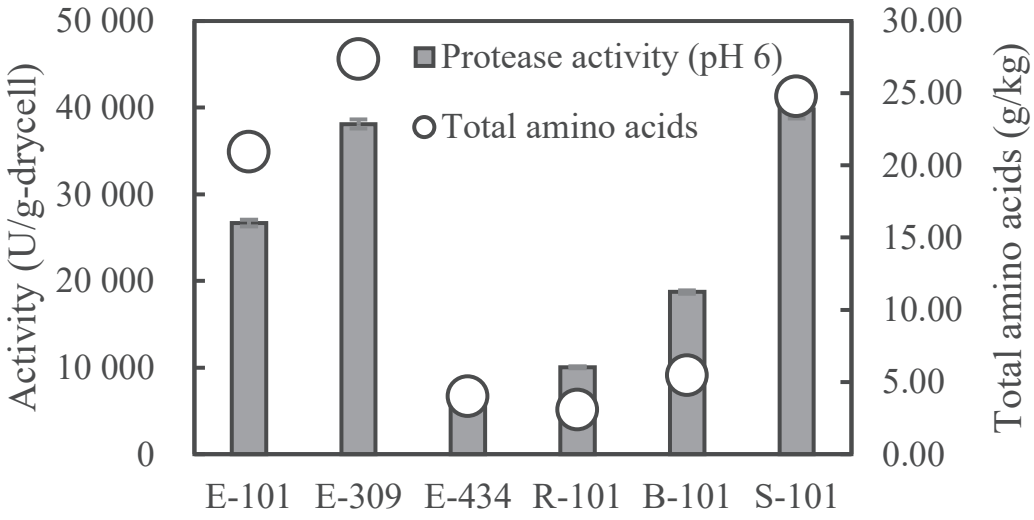

Fig. 7. Protease activity (pH6) and the total amount of amino acids in WELD in each koji.
Data are expressed as the mean ± SE (n = 3).

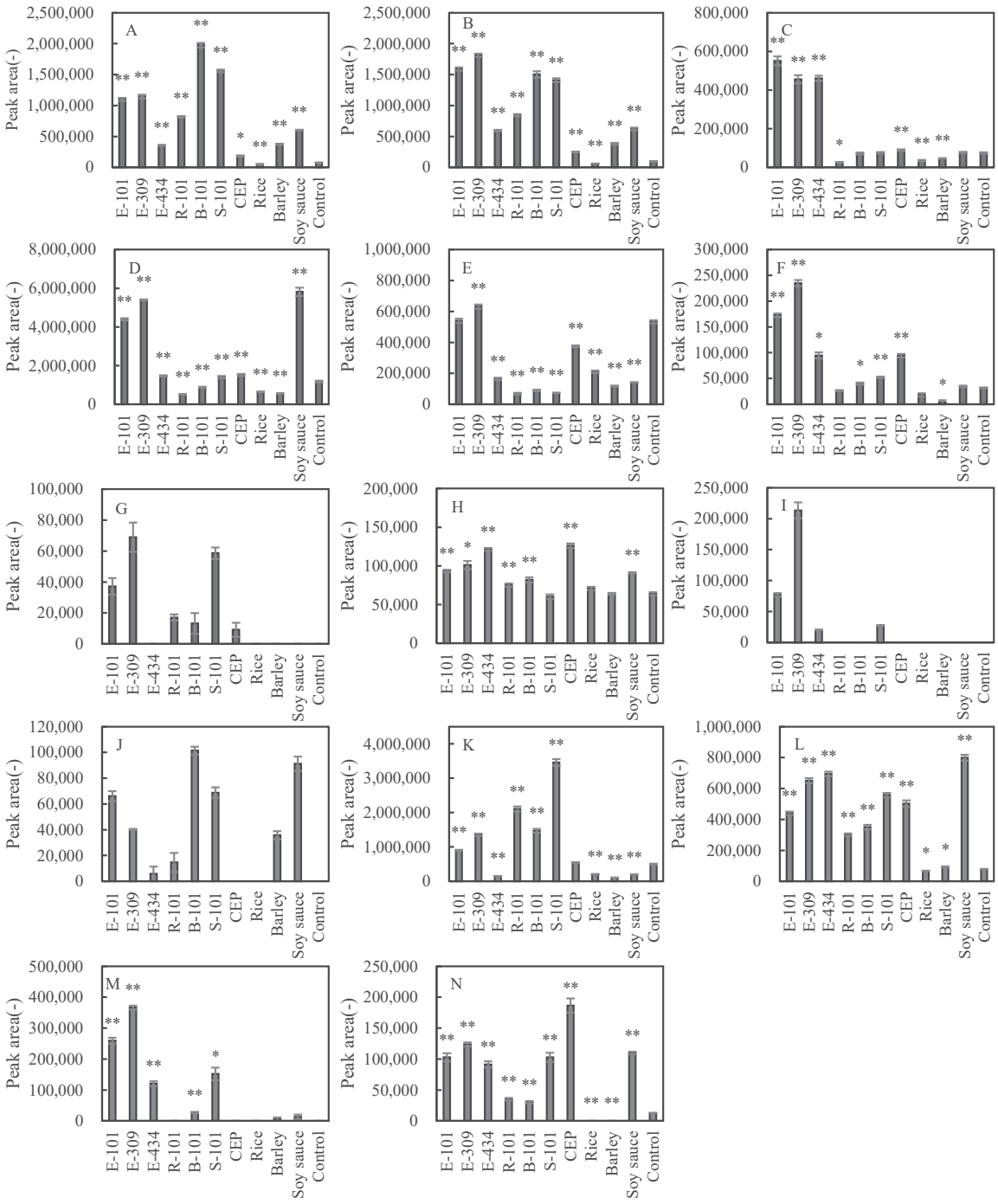

Fig. 8. Aroma components of WELDs.

The peak area of 2-methylbutanal in WELDs prepared with different koji (A). Data are expressed as the mean ± SE (n = 3). Values with asterisks differed significantly compared with control in Welch's t-tests. *p < 0.05; **p < 0.01. 3-Methylbutanal (B), 2-ethylfuran (C), hexanal (D), 1-penten-3-ol (E), octanal (F), 1-octen-3-one (G), nonanal (H), methional (I), furfural (J), 1-octen-3-ol (K), benzaldehyde (L), phenylacetaldehyde (M), 2-methylpyrazine (N).

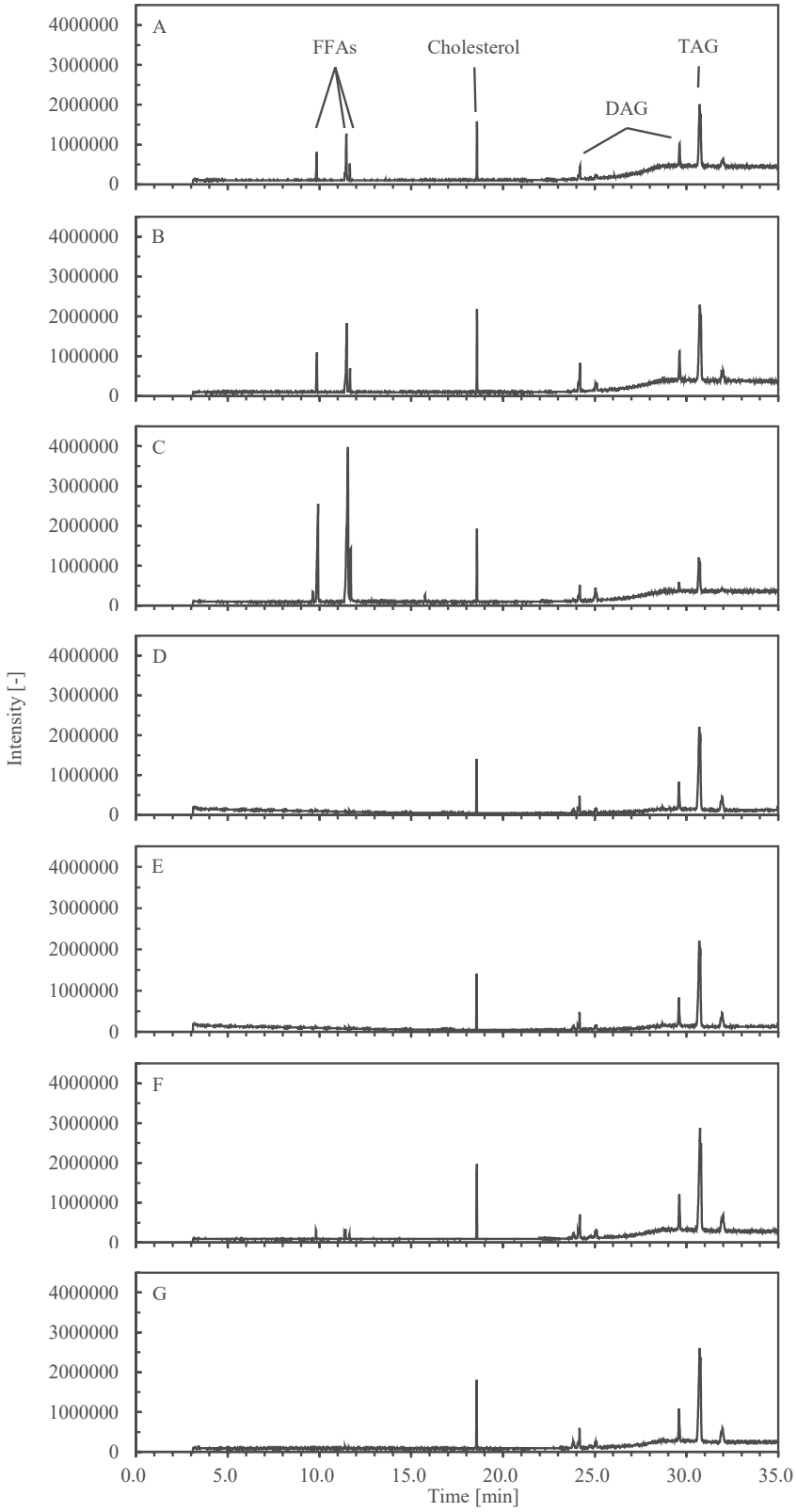

Fig. 9. Lipid analysis of WELDs.
E-101 (A), E-309 (B), E-434 (C), R-101 (D), B-101 (E), S-101 (F), Control (G).

Table 10. Lipid analysis of WELDs

WELD	Comparatively (%)			
	FFAs	DAG	TAG	Cholesterol
E-101	24.7	13.5	53.6	8.3
E-309	28.6	15.9	47.6	8.0
E-434	76.8	4.8	12.7	5.7
R-101	0.48	16.6	75.8	7.2
B-101	0.50	16.6	75.7	7.2
S-101	2.2	17.5	72.2	8.1
Control	0.27	16.6	75.1	8.1

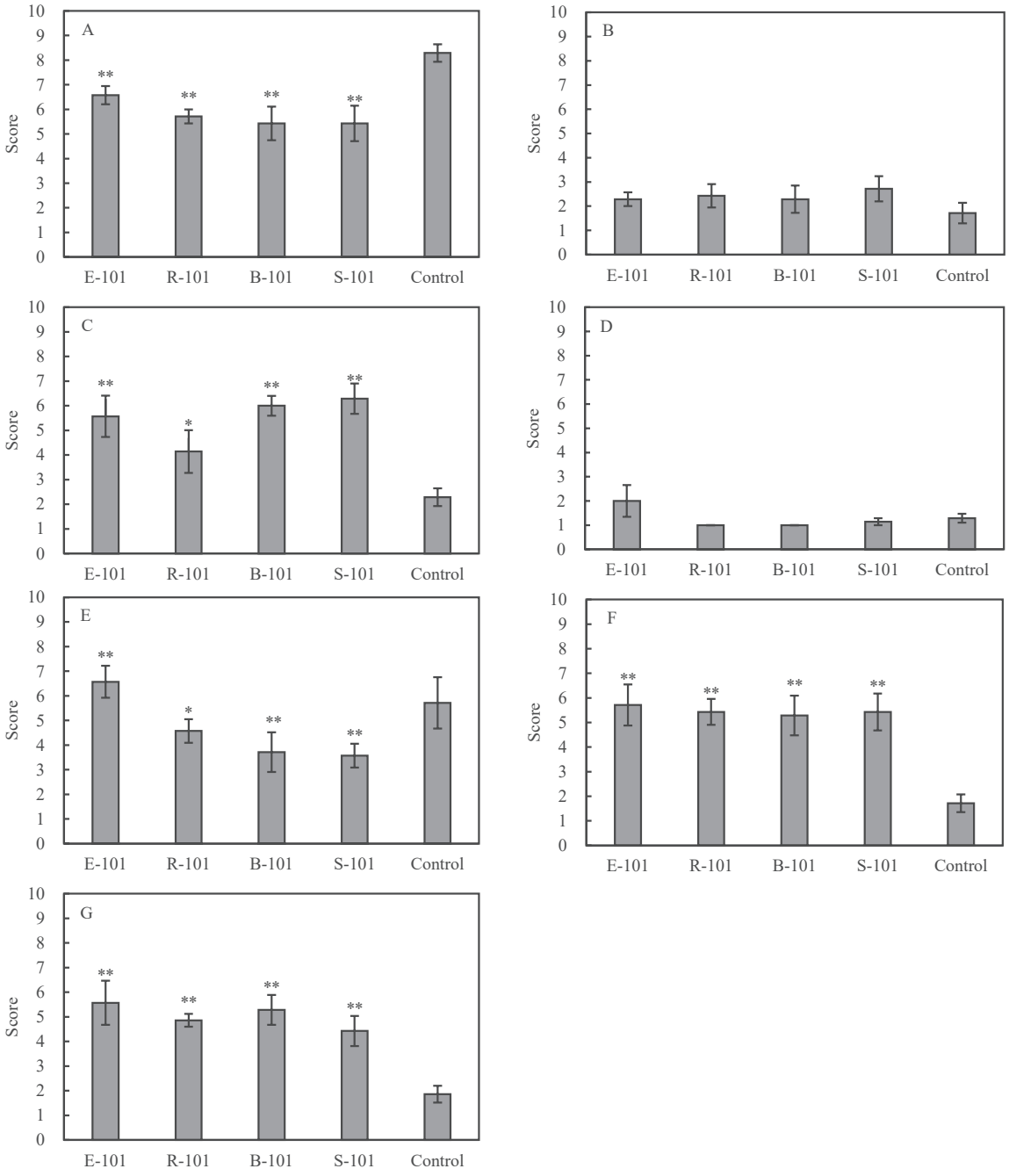

Fig. 10. Sensory evaluation test of WELDs prepared with egg-koji AO101 and grain koji. The average scores for saltiness (A), sweetness (B), umami (C), bitterness (D), egg flavor (E) thickness of flavor (F), and persistence of flavor (G), scored by seven scorers, were calculated. Data are expressed as the mean ± SE (n = 7). Values with asterisks differed significantly compared with control in Welch's *t*-tests. $^{*}p < 0.05$; $^{**}p < 0.01$.

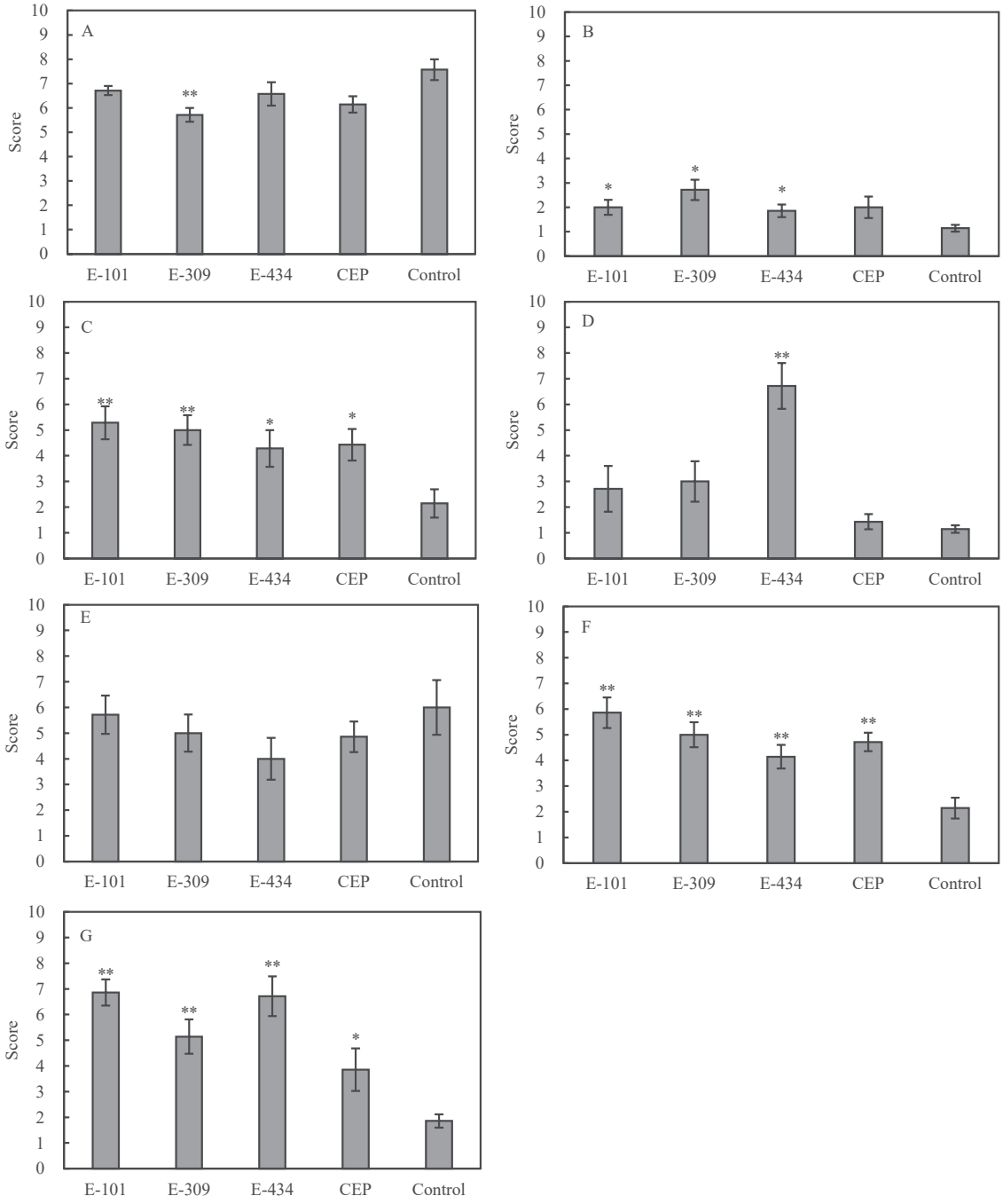

Fig. 11. Sensory evaluation test of WELDs prepared with egg-koji.
The average scores for saltiness (A), sweetness (B), umami (C), bitterness (D), egg flavor (E) thickness of flavor (F), and persistence of flavor (G), scored by seven scorers, were calculated. Data are expressed as the mean ± SE (n = 7). Values with asterisks differed significantly compared with control in Welch's *t*-tests. $^{*}p < 0.05$; $^{**}p < 0.01$.

Chapter 5. Conclusion

With the diversification of food in recent years, there is a growing need to find new ways to use koji or koji-mold and to develop new materials for koji. In this study, I selected eggs as new materials for koji to open up the possibilities of koji-mold (*A. oryzae, A. sojae, A. luchuensis*). The purpose of this study is to contribute to the development of food culture by developing koji (egg-koji) using new materials (eggs) and investigating its characteristics.

In Chapter 2, I succeeded for the first time in developing egg-koji that uses only eggs and koji-mold by selecting and combining Cooked Egg Powder (CEP) and *A. oryzae* AO101 as the most suitable combination. To suppress the explosive growth of harmful bacteria, I improved the sterilization method, watering method, and amount of water. In addition, it was found that egg-koji has a characteristic enzyme activity balance, in which amylase activity was extremely low and protease activity (pH 6) was higher than pH 3, compared to grain-koji (rice and barley). Egg-koji might produce enzymes suitable for taking in nutrients when growing into CEP and would be expected to give a flavor that could not be achieved by cooking or additives.

In Chapter 3, I investigated the novel functionality of egg-koji by metabolome analysis using GC-MS. Compounds extracted from CEP and egg-koji with methanol aqueous solution were measured by GC-MS, and the measurement data was subjected to principal component analysis, suggesting that the component profiles of CEP and egg-koji were significantly different. Therefore, it was inferred that the egg-derived component was converted into a new substance by koji-mold. Principal component analysis between egg-koji prepared with different koji species suggested the formation of specific components by koji-mold. In particular, the metabolite profile of AS309 separated from other species in the first principal component. If this method is used to analyze WELDs (chapter 4), I think it may be possible to extract substances that affect the difference in sensory evaluation of each WELDs.

In Chapter 4, I focused on the components related to "richness" among the component changes caused by the fermentation of whole eggs with egg-koji, and investigated their effects on sensory characteristics. Using AO101 as a standard strain of egg-koji, the sensory characteristics of WELDs prepared with egg-koji and other grain koji were compared. E-101 (WELDs prepared with egg-koji AO101) was the only WELD that enhanced the umami and richness without losing its egg flavor. Additives such as monosodium glutamate and nucleic acid are added to foods to enhance the umami taste, but by using WELD such as E-101, these seasonings and additives can be reduced. Using egg-koji prepared by the different *Aspergillus* strains, WELDs with enhanced umami and richness while retaining the egg flavor of whole eggs could be prepared. Different characteristics of each egg-koji were revealed, such as that E-309 (egg-koji AS309) produced more free amino acids and aroma components and E-434 (egg-koji AL434) had the potential to take the emulsifying power of free fatty acids, suggesting that seasonings and additives can be reduced in food design.

Taken together, in Chapter 2, I established a manufacturing method for egg-koji that enables actual production on a certain scale, and in Chapter 3, I clarified some of the characteristics of egg-koji. Currently, egg-koji is used as an ingredient in the production of aged egg yolk, which is added to various foods and sold. Compared to grain (plant) raw materials, animal-derived raw materials tend to contain less carbohydrates and more proteins and fats (lipids), so they retain their unique flavor and physical properties, and it was thought that koji making was difficult until now. Abundant nutrient sources are more susceptible to bacterial contamination, so scaling up production is often an issue. In food development, there are not many examples of consistent development from the lab level (research) to actual production, and I was able to obtain important knowledge of foods using koji, a new raw material. In Chapter 4, as a basis for the future development of new foods, I prototyped WELDs, analyzed their components, and evaluated their sensory properties. In addition, compared to egg-koji's standard AO101 (E-101), AS309 (E-309)

and AL434 (E-434) exhibited distinctive components and organoleptic properties. It can be said that this is very effective for the development of product lines. I hope that the findings of this study will serve as an important foundation for future research and development of koji and new materials koji.

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References

1. Brewing Society of Japan.: Declaration "Koji fungi (Koji-kin) as the National Fungi" (2013).
2. Machida, M., Asai, K., Sato, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., and other 53 authors.: Genome sequencing and analysis of *Aspergillus oryzae*, *Nature*, 438, 1157-1161 (2005).
3. Iwashita, K.: Genome Biology Will Open the Door for the View World of Koji Mold Science, *Kagaku to seibutsu,* 47, 329-337 (2009) (in Japanese).
4. Yoshii, H.: Miso Brewing and Enzymes (III), *Journal of Brewing Society of Japan*, 68, 895-901 (1973) (in Japanese).
5. Kusumoto, K., Matsushita, M., Furukawa, I., Yamagata, Y., Koide, Y., Ishida, H., Takeuchi, M., Kashiwagi, Y.: Efficient production and partial characterization of aspartyl aminopeptidase from *Aspergillus oryzae*, *J. Appl. Microbiol*., 105, 171-1719 (2008).
6. Yasui, M., Oda, K., Masuo, S., Hosoda, S., Katayama, T., Maruyama, J., Takaya, N., Takeshita, N.: Invasive growth of *Aspergillus oryzae* in rice *koji* and increase of nuclear number, *Fungal. Biol. Biotechnol*., 7, 1-15 (2020).
7. Kawabata, Y., Kanaoka, M., Bogaki, C., Suzuki, K., Iguchi, T., Takaoka, M., Watanabe, T.: Effects of ginger koji intake on skin surface temperature for young women sensitive to cold temperatures. J. Brew. Sos. Japan, 108, 778-786 (2013).
8. Kanzaki, H.: Creation of highly functional Japanese wine pomace-koji using koji-mold solid culture technology. Newsletter of Okayama foundation for science and technology, 122, 1-3 (2021).
9. Suzuki, S., Ohmori, H., Hayashida, S., Nomura, M., Kobayashi, M., Hagi, T., Narita, T., Tomita, S., Yamashita, H., Arakawa, Y., and other 3 authors: Lipase and protease activities in Koji cheeses surface-ripened with *Aspergillus* strains,

Food Sci. Technol. Res., 27, 543-549 (2021).

10. Machida, M., Yamada, O., Gomi, K.: Genomics of *Aspergillus oryzae*: Learning from the History of Koji Mold and Exploration of Its Future, *DNA RESEARCH*, 15, 173-183 (2008).
11. Ministry of Education, Culture, Sports, Science and Technology.: Standard tables of food composition in Japan (Eighth revised edition), *Tsutatomo Printing*, Nagano (2020).
12. Sugano, M.: Attraction of Eggs, *Japan Egg Science Society*, Tokyo (2020).
13. Miyamoto, T., Kihara, A., Shidara, H.: The Society for Biotechnology, Japan annual meeting abstract, 71st, p.185 (2019).
14. Sho, S., Ueno, Y., Hatta, H., Narita, H.: High-density Cultivation of Koji Using Sponge Cake Made from Egg White and Development of Fermented Egg White Sauce, *Nippon Shokuhin Kagaku Kogaku Kaishi*, 61, 77-84 (2014).
15. Sho, S., Kikushima, N., Hatta, H., Ueno, Y.: Egg Koji-derived seasoning and its production method, Japanese Patent JP5467289, (2014).
16. Kaneko, A., Ozeki, K.: Selective production of cellulase and hemicellulase from *Aspergillus oryzae* using starch-free wheat, *J. Brew. Soc. Japan*., 112, 77-81 (2017).
17. Miyamoto, T., Kihara, A., Sasahara, R.: Aged egg yolk, processed food using same and preparing a method of aged egg yolk, Japanese Patent, JP6473855 (2019).
18. Brewing Society of Japan.: The 4th revised prescribed analysis commentary of National Tax Agency, 212-228, *Brewing Society of Japan*, Tokyo (1993) (in Japanese).
19. Editorial Committee for Soy Sauce Testing Methods, Japan Soy Sauce Research Institute.: Soy Sauce Test Method, 287-289, *Japan Soy Sauce Research Institute*, Tokyo (1985) (in Japanese).

20. Food Safety Commission of Japan.: *Bacillus cereus* foodborne poisoning (Factsheet), *Food Safety Commission of Japan*, Tokyo (2011) (in Japanese).
21. Ueda, S.: Food poisoning and testing for *Bacillus cereus*, *The Chemical Times*, 228, 11-18 (2013) (in Japanese).
22. Miyamoto, T., Inami, S., Nakazawa, M., Yamashita, H., Nakagawa, T.: Egg-koji production method and egg-koji, Japanese Patent, JP7038561, (2022).
23. Shidara, H.: Recent food information on processed egg products, *Journal of Home Economics of Japan*, 71, 193-199 (2020).
24. Yamashita, H *et al*.: Koji Starter and Koji World in Japan. *Journal of Fungi,* 7, p. 569 (2021).
25. Yongwoo Jo *et al*.: Changes of Physiochemical and Enzymatic Activities of *Doenjang* Prepared with Different Amounts of Rice *koji* during 30 Days of Fermentation. *Foods*, 10, 372 (2021).
26. Yang Y *et al*.: Improvement of flavor profiles in Chinese rice wine by creating fermenting yeast with superior ethanol tolerance and fermentation activity. *Food Res. Int.*, 108, 83-92 (2018).
27. Oura, S., Suzuki, S., Hata, Y., Kawato, A., Abe, Y.: Evaluation of physiological functionalities of amazake in mice. *Journal of the Brewing Society of Japan*, 102, 781-788 (2007) (in Japanese).
28. Yamada, T., Hirakata, J., Aikawa, M., Suizu, T., Saito, Y., Kawato, A., Suginami, K., Oda, J.: Cysteine Protease Inhibitors Produced by the Industrial Koji Mold, *Aspergillus oryzae* O-1018. *Biosci. Biotech. Biochem.,* 62, 907-914 (1998).
29. Yoshida, Y., Kubara, T., Kikuchi, A.: Metabolomics / Metabonomics. *Bunseki*, 7, 371-378 (2009) (in Japanese).
30. Ochi, H., Naito, H., Iwatsuki, K., Bamba, T., Fukusaki, E.: Metabolomics-based component profiling of hard and semi-hard natural cheeses with gas chromatography/time-of-flight-mass spectrometry, and its application to sensory

predictive modeling. *Biosci. Bioeng*, 113, 751-8 (2012).

31. Chloe R-G *et al*.: High precision mass measurements for wine metabolomics. *Front Chem.*, 2, 1-9 (2014).

32. Lommen, A: MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Anal. Chem.*, 81, 3079 (2009).

33. Tsugawa H *et al*.: GC/MS based metabolomics: development of a data mining system for metabolite identification by using soft independent modeling of class analogy (SIMCA). *BMC Bioinformatics*, 12, 131 (2011).

34. Sato A *et al*.: Draft genome sequencing and comparative analysis of *Aspergillus sojae* NBRC 4239. *DNA Res*, 18, 165 (2021).

35. Yamagata, Y.: Proteolytic Enzyme of *A. oryzae*, *Kagaku to seibutsu,* 54, 109-116 (2016) (in Japanese).

36. Nakagawa, T., Miyamoto, T., Miki, S., Watanabe, K., Aki, T., Shidara, H., Yamashita, H. Preparation of egg-koji for developing a novel food. *J. Biosci. Bioeng.* Vol. 153, 6. 447–450 (2023).

37. Nishimura, T., Egusa, A. "Koku" Involved in Food Palatability an Overview of Pioneering. *Kagaku to seibutsu*, Vol. 54, 2, 102–108 (2016).

38. Ninomiya, T. Research on the taste of amino acids. *Journal of Cookery Science of Japan*, 1, 185–197 (1968).

39. Inoue, Y., Watanabe,. Cooked Aroma Components Contributed to Koku of Soy miso. *Journal of the Brewing Society of Japan*, 113, 9–12 (2018) (in Japanese).

40. Fujimoto, A., Yamase, N., Oki, H. Meat extract, Japanese Patent, JP193968 (2008).

41. Migita, K., Takahama, Y., Takahagi, Y., Sugiyama, N., Kikuchi, K., Matsuishi, M., Okitani, A. Analysis of Aroma Compounds of Heated Fats from Wagyu Beef and Other Cattle Using Headspace SPME. *Food Sci. Technol.* Res, 59, 3, 127–

138 (2012).

42. Hartvigsen, K., Lund, P., L. F. Hansen., Holmer, G. (2000). Dynamic Headspace Gas Chromatography/Mass Spectrometry Characterization of Volatiles Produced in Fish Oil Enriched Mayonnaise during Storage. J. *Agric. Food Chem*. 48, 10, 4858–4867 (2000).

43. Inagaki, S. Study about the characteristics of branched chain aldehydes which are the potent odorants of cheese and beef. Ph. D. thesis, Tokyo University of Agriculture, Tokyo (2018).

44. Kawabata, S. Characteristic aroma compounds of surface mold-ripened cheese and their generating pathway. *Milk Science*, 59, 303–307 (2010).

45. Hayase, F., Watanabe, H. Volatile Components Contributed to Koku Taste. *Journal of the Brewing Society of Japan*, 109, 161–167 (2014) (in Japanese).

46. Takano, Y., Tanizawa, J. Aroma components that improve egg sensation and egg sensation enhancers, Japanese Patent, JP194128 (2013).

47. Anantayanon, J., Jeennor, S., Panchanwaporn, S., Chutrakul, C. Significance of two intracellular triacylglycerol lipases of Aspergillus oryzae in lipid mobilization: A perspective in industrial implication for microbial lipid production, *Gene*, 793, 1–10 (2021).

48. Takahashi, Y. Control of emulsification and demulsification in food emulsions. *Japan journal of food engineering*. Vol. 16, 3, 171–175 (2015) (in Japanese).

49. Ota, S., Yuki, E. Hydrolysis of Edible Fats and Oils. *Journal of Oleo Science.* Vol. 26, 3, 150–164 (1977) (in Japanese).

50. Hagiwara, K. Bitter components of food. *Journal of Cookery Science of Japan.* Vol. 13, 1, 21–26 (1980).

51. Watanabe, T., Kawai, T., Nonomura, Y. Effects of Fatty Acid Addition to Oil-in-water Emulsions Stabilized with Sucrose Fatty Acid Ester. *Journal of Oleo Science.* Vol. 67, 3, 307–313 (2018).