INTRODUCTION

With increasing incomes, meat and meat-based product consumption has been increasing worldwide because of its superior taste and nutritional value. Chicken is a popular source of meat among health-conscious consumers due to the perception that it contains low fat and cholesterol content. Chicken meat has a short shelf life because it is easily spoiled by microorganisms, which result in the deterioration of meat quality through the production of odoriferous end products such as volatile basic nitrogen (VBN) and lipid peroxides, which deteriorate the sensory quality of meat. Because of its short shelf life, chicken meat is often stored frozen. However, frozen/thawed (FT) chicken has diminished quality, resulting in a lower price than unfrozen chicken. Sometimes, FT chicken meat can be illegally mixed with unfrozen chicken and distributed when the product is in high demand. FT meat is more susceptible to microbial spoilage owing to leakage of fluid that contains nutrients for microorganisms. Microbial contamination level is recognized as an important criterion for evaluating meat freshness and safety. When microbial contamination occurs, metabolites are consumed differently, depending on the characteristic metabolisms of the microorganisms, resulting in different metabolomes that can be distinguished.

RESULTS AND DISCUSSION

Total aerobic bacteria (TAB) and physicochemical properties

Microbial and physicochemical properties were measured to evaluate the freshness of chicken breast meat (Table 1). The FT breast meat had significantly higher microbial contamination than that in unfrozen meat (p < 0.05). The high microbial contamination levels of the FT breast meat seemed to result from the leakage of exudates, which occurs during the structural destruction of meat during freezing and thawing. The exudate is composed of free amino acids, sugars, and nucleotides, and it can be a good nutrient source for microorganisms. The collapse of muscle cell structures during freezing and thawing can result in cavities and meat exudates, which provide optimal conditions for microbial growth (Table 1). According to previous studies that used the minimal spoilage criterion TAB > 6.0 log CFU/g, the shelf life of chicken breast meat was less than 10 and 7 days for unfrozen and FT breast meat, respectively. In unfrozen breast meat, only VBN demonstrated a significant increase along with TAB on day 10, whereas increases in a-value, drip loss, and 2-thiobarbituric acid reactive substances (TBARS) values were not
There was no significant difference in pH between unfrozen and FT breast meat on different storage days. Likewise, a previous study showed that pH of approximately 6.0, which is similar to that used in the present study, was suitable for microbial growth. According to Rukchon et al., the initial spoilage level of skinless chicken breast was 6.0 log CFU/g. Based on the results, only changes of VBN are closely related to initial spoilage level, demonstrating that VBN can be used as a freshness indicator of chicken breast meat in both unfrozen and FT breast meat. In addition, some relationships are still unclear. Further experiments are needed to investigate this relationship between unfrozen and FT breast meat. It would have been more helpful if we knew the initial state of the FT.

Metabolites
For the evaluation of freshness indicators in chicken breast meat, 2D \(^{1}H\)-\(^{13}C\) HSQC NMR spectra were acquired and processed by multivariate analysis (Fig. 1; Supplementary Table 2). The correlation analysis was performed to determine common trends in both unfrozen and FT breast meat in a time-dependent manner (Fig. 2). The two-way analysis of variance (ANOVA) results (Fig. 1d, e) demonstrated that the quantities of most metabolites were increased gradually to the storage time. The quantities of organic acid, hypoxanthine, and free amino acids metabolites were increased gradually to the storage time. The quantities of organic acid, hypoxanthine, and free amino acids were highly correlated with storage time, whereas those of proline, inosine 5’-monophosphate (IMP), and N,N-dimethylglycine (DMG) demonstrated highly negative correlations (Fig. 2).

Table 1. Total aerobic bacteria and physicochemical properties of unfrozen and frozen/thawed (FT) chicken breast meat during storage.

| Storage (day) | Unfrozen | FT | SEM* |
|--------------|----------|----|------|
|              |          |    |      |
| Total aerobic bacteria (log CFU/g) | 3.01* | 3.05* | 0.110 |
| Drip loss (%) | 3.87* | 4.21* | 0.136 |
| SEM² | 0.098 | 0.108 | 0.096 |
| FT breast meat showed high | 1.00* | 2.99* | 0.482 |
| l* (Lightness) | 53.81* | 54.25* | 0.777 |
| SEM² | 0.521 | 1.559 | 1.223 |
| a* (Redness) | 4.44* | 5.03* | 0.350 |
| SEM² | 0.426 | 0.309 | 0.281 |
| b* (Yellowness) | 13.35* | 14.69* | 0.588 |
| SEM² | 0.725 | 0.759 | 0.545 |
| pH | 6.16* | 6.17* | 0.066 |
| SEM² | 0.048 | 0.042 | 0.076 |
| TBARS (mg malondialdehyde/kg) | 0.30b | 0.30b | 0.016 |
| SEM² | 0.015 | 0.017 | 0.023 |
| VBN (mg/100 g) | 10.10c | 10.55c | 0.753 |
| SEM² | 0.230 | 0.343 | 0.658 |

1Standard of the mean (n = 35).
2(SEM = 10).
3The value represents thawing loss (%) on day 0.
4Different letters in the same storage day indicate a significant difference (p < 0.05).
5Different letters in the same group indicate a significant difference (p < 0.05).

The CIE values, only the a* value significantly increased in a time-dependent manner. The L* value was not different in either treatment group, except on the initial storage day. Regardless of storage day, FT breast meat showed high b* value than unfrozen breast meat. According to previous study, color a* value only affected in a time dependent manner, whereas b* value only affected by freezing methods such as freezing rate and air composition. There was no significant difference in pH between unfrozen and FT breast meat on different storage days. Likewise, a previous study showed that pH of approximately 6.0, which is similar to that used in the present study, was suitable for microbial growth. According to Rukchon et al., the initial spoilage level of skinless chicken breast was 6.0 log CFU/g. Based on the results, only changes of VBN are closely related to initial spoilage level, demonstrating that VBN can be used as a freshness indicator of chicken breast meat in both unfrozen and FT breast meat. In addition, some relationships are still unclear. Further experiments are needed to investigate this relationship between unfrozen and FT breast meat. It would have been more helpful if we knew the initial state of the FT.
These results indicate that metabolic changes can be used as evidence of microbial growth. Succinate and acetate were representative organic acids produced by microorganisms during microbial fermentation. Increases in acetic acid and succinic acid can result from glucose fermentation by microorganisms, and this change can be observed in muscle-based foods during storage. A recent study by Bórquez et al. indicated that acetic acid can suppress microbial counts in fish flesh, but the microorganisms steadily grow in a time-dependent manner, even in 5 mg/kg acetic acid.

An increase in hypoxanthine with a decrease in IMP during storage is a typical change that allows prediction of the freshness of chicken meat using K-value (%). In addition to changes in nucleotides, free amino acids (aspartic acid, valine, isoleucine, lysine, tyrosine, leucine, glutamic acid, methionine, alanine, glycine, and threonine), and biogenic amines (tyramine and cadaverine), there can be evidence of proteolysis by both meat enzymes and microbial activity during storage. Increases in VBN and biogenic amines are normally accompanied by food spoilage and are treated as indicators of freshness. In addition, DMG and proline are commonly used as indicators of freshness.
can be consumed by microorganisms as carbon or nitrogen sources, and a decrease in DMG and proline is evidence of microbial growth\(^1,12,33\). As indicated above and in agreement with previous studies, DMG and proline levels decreased during storage \((p < 0.05)\). VBN levels were more strongly correlated with metabolites (especially acetic acid, hypoxanthine, lysine, and succinic acid) than that with TAB (Fig. 2a, b). This may be a result of stagnating microbial growth in the later storage stage (after minimal spoilage TAB level); TAB did not correlate with VBN and acetic acid at different storage times (Fig. 2a). Considering the physicochemical properties of chicken breast meat, acetic acid had a strong correlation with VBN (\(r = 0.97\)), and could act as an appropriate freshness indicator (Fig. 2b).

### Unfrozen and FT breast meat

**Overall metabolic trends by multivariate analysis.** To delineate and identify the relationships among metabolite changes, storage time, and freshness, Interactive principal component analysis (iPCA), ROC analysis, and two-way ANOVA were performed (Fig. 1). iPCA results revealed a strong correlation between PC1 (47.4\%) and storage time. In addition to PC1, the use of the PC1–PC2 (12.8\%) plane explained the difference better by treatment (unfrozen and FT breast meat) and storage time than the use of PC1 only (Fig. 1a; Supplementary Table 1). Unfrozen breast meat was clearly distinguished from the other clusters until day 1. From day 4, the clusters of unfrozen breast meat moved in a similar direction to FT breast meat over time but did not overlap. The clusters of FT breast meat moved along PC1 and PC2 over time. In PC3 (7.4\%), the unfrozen breast meat cluster had a negative correlation, whereas the FT breast meat cluster was positively correlated over time. PC3 supports the explanation of overall variation, but it cannot be used independently. Prior to selecting metabolic indicators for distinguishing both groups, receiver operating characteristic (ROC) curve analysis and two-way ANOVA were used for the selection of indicators (Fig. 1b, c).

The highest predictive accuracy was the 92.7\% for the model made with 50 features. Among them, a total of 41 metabolites that were significantly different were placed on the Venn diagram \((p < 0.05)\) (Fig. 1d, e). For the selection of indicators, we used a two-way ANOVA. In the Venn diagram, four metabolites were significant only in unfrozen vs. FT breast meat, and interactions were chosen as candidates for ROC curve analysis because the top 15 metabolites with average importance among the 50 features were not directly related to the results of the two-way ANOVA.

### Enhancing discriminant ability with selected metabolites

Among the various combinations of candidates, 11 metabolites were selected based on their prediction score, and ROC analysis was performed (Fig. 3). The ROC curves consisting of the selected metabolites had higher predictive accuracy than that of the original model using the whole metabolome, and the accuracy was highest when 10 features were used (Fig. 1b; Fig 3a–c). The metabolites are listed in order of mean importance from most to least important in Fig. 3d. Quantifying the metabolites is important for evaluating meat quality such as flavor-active and bioactive compounds\(^34\). Furthermore, this information can also be used for distinguishing between groups using a machine-learning algorithm\(^3\). The quantity of glutamine rapidly decreased after day 10 in unfrozen breast meat (0.48-fold) but remained constant throughout the storage period in FT breast meat \((p < 0.05)\). In contrast, asparagine gradually increased in unfrozen breast meat but decreased after day 13 in FT breast meat. Asparagine demonstrated a similar trend in both groups, but it was higher in FT breast meat during initial and terminal storage than in unfrozen breast meat. Proline content decreased gradually in both groups but it was higher in unfrozen breast meat, at 1.28-fold change on day 0 followed by a 3.81-fold change by day 16, than in FT breast meat \((p < 0.05)\). Glutamine, arginine, asparagine, and proline can be utilized by microorganisms, and the utilization of free amino acids varies depending on microbial species\(^12,35–37\).
However, some free amino acids demonstrated different behavior in unfrozen meat compared with that in FT meat. It can be inferred that freezing and thawing could change the microbial composition through the competitive exclusion of microorganisms, resulting in the survival of cold-resistant species and altering the metabolite consumption trend.

Previous studies reported that 2,6-diaminopimelic acid (DAPA) and γ-aminobutyric acid (GABA) are direct evidence of microbial proliferation. DAPA is a component of cell wall peptidoglycan in various bacteria and is a precursor of lysine. The presence of DAPA can be used to evaluate microbial contamination and the existence of lactobacilli. Based on these results, FT breast meat is more vulnerable to microbial spoilage than unfrozen breast meat because of the exudates during thawing. They contain suitable nutrients for microbial growth, resulting in a high TAB and pH. IMP was gradually decreased and could be used to clearly distinguish between unfrozen and FT breast meat, whereas inosine could be used to distinguish unfrozen meat compared with that in FT breast meat. In addition, it can be used to differentiate unfrozen from FT breast meat based on different metabolic patterns.

Fig. 4 Schematic representation of overall physicochemical and metabolic changes in unfrozen and frozen/thawed chicken breast meat. Red colored letters represent the most suitable candidates for identifying chicken freshness based on the microbial spoilage level.

A summarized schematic illustration of the present study is represented in Fig. 4. Among all physicochemical properties, only VBN could predict the spoilage of chicken breast meat at the initial TAB level. Generally, proportional increases in organic acids, free amino acids, biogenic amines, and hypoxanthine were observed, while proline and DMG decreased proportionally during storage. Acetic acid was highly correlated with VBN, which could be a simple indicator of freshness in chicken breast meat without VBN analysis for both unfrozen and FT breast meat. Unfrozen and FT breast meat can be differentiated by uniform concentration of carnosine, β-alanine, and histidine levels, consistent changes in nucleotides by storage time, and changes in microbial metabolism patterns that are reflected by some free amino acids. Based on these results, 2D NMR-based metabolomics could be useful for evaluating the freshness of chicken breast meat. In addition, it can be used to differentiate unfrozen from FT breast meat based on different metabolic patterns.

METHODS
Sample preparation
Five packages (1 kg/package) for unfrozen and frozen experiment groups of skinless chicken (cobb 500) breast meat were purchased on the same day from the same slaughterhouse (Harim Co. Ltd. Iksan, South Korea), respectively. All chickens were slaughtered in an automated commercial slaughterhouse system. Carbon dioxide stunning and air-chilling (carcasses were chilled to 2°C and the temperature was kept at about 3.5 h during rigor mortis) were used. For preparation of FT breast meat, each breast meat was rapidly frozen at −35°C immediately after slaughter, with the aim of reaching −20°C in 40 min, packed randomly, and subsequently stored at −20°C. Breast meat (5 packages/group) was packaged by polystyrene and styrofoam tray, transferred to the laboratory using a cooler with ice. It took about 3 h from chicken processing plant to the laboratory. For simultaneous analysis with unfrozen breast meat, frozen breast meat was thawed 24 h before the unfrozen breast meat samples were collected, and both samples were prepared at the same facility. From the same package (8–9 breasts/package), seven breasts were randomly selected, and these were packaged aerobically and individually in low-density polyethylene/nylon bags (oxygen permeability of 4.7 g/m² for 24 h at 100% RH/25°C) and marked with the same number. The breast meat was stored for up to 16 days (0, 1, 4, 7, 10, 13, and 16) at 2°C. After drip loss and color were evaluated daily in quintuplicate, each chicken breast meat was minced using a mini chopper (CH180, Kenwood Appliances Co., Ltd., Dongguan, China). TAB and pH analyses were performed...
immediately. Samples for TBARS, VBN, and NMR analyses were weighed, vacuum-packaged, and stored at −70 °C until further analyses.

**TAB counts**

TAB counts were performed according to the method described by Yong et al. Each sample (3 g) was diluted in 27 mL of sterile saline (0.85%) for 2 min using a stomacher (BagMixer® 400 P, Interscience Ind., St. Nom, France). Appropriate dilutions were prepared in sterile saline and spread on plate count agar (Difco Laboratories, Franklin Lakes, NJ, USA). The agar plates were incubated at 37 °C for 48 h, and the microbial counts were calculated. The results are expressed as log numbers of colony-forming units per gram (log CFU/g).

**Drip loss**

The breast meat was weighed before packaging. Each day during storage, drips were collected to evaluate drip loss. At the first day, in case of FT breast meat, thawing loss was evaluated using each whole pack instead of drip loss and each FT breast meat was packaged aerobically for further storage. Drip loss was represented as a percentage and calculated as follows:

\[
\text{Drip loss(%) = } \frac{\text{original sample weight} - \text{sample weight after storage}}{\text{original sample weight}} \times 100
\]

**Color**

Surface color measurements (CIE \(L^*, a^*, b^*\) values representing lightness, redness, and yellowness, respectively) of chicken breast meat samples (skin side of Pectoralis major) were obtained using a colorimeter (CM-5, Konica Minolta Censing Inc., Osaka, Japan) in the condition of illuminant D65 and 10° standard observer with a 30 mm aperture size plate. Calibration was performed using a calibration plate. The color of each sample was measured three times along the skin side to minimize error and the average value was used.

**pH**

Chicken breast meat (1 g) was homogenized with 9 mL of distilled water using a homogenizer (T25 Ultra, Ika Works, Staufen, Germany). The homogenates were centrifuged (Continental 512 R, Hanil Co., Ltd., Incheon, South Korea) at 2,265 × g for 10 min and filtered (Whatman No. 4, Whatman PLC, Middlesex, UK) in the condition of pH 7.0. K2CO3 was added to the inner space, and the filtrate was measured using a pH meter (Seven2Go fi, Mettler-Toledo International Inc., Schwerzenbach, Switzerland). Prior to measuring the pH, the pH meter was calibrated using standard buffers (pH 4.01, 7.00, and 9.21).

**TBARS**

Chicken breast meat (5 g) was homogenized with 15 mL of distilled and deionized water (DDW) and 50 μL of butylated hydroxy toluene (in ethanol) for 30 s at 1,513 × g. The homogenate (2 mL) was transferred to a 15 mL test tube and mixed with 4 mL of TBA and trichloroacetic acid (20 mM TBA in 15% trichloroacetic acid). The test tubes were heated in a water bath at 90 °C for 30 min, cooled, and centrifuged (Continental 512 R, Hanil Co., Ltd.) at 2,265 × g for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (X-ma 3100, Human Co. Ltd., Gwangju, South Korea). The TBARS value was calculated as mg malondialdehyde/kg sample.

**VBN**

The VBN content was evaluated using the Conway micro-diffusion technique with slight modifications46. Chicken breast meat (5 g) was homogenized with 20 mL of DDW for 30 s at 1,130 × g. The homogenate was filtered using a filter paper (Whatman No.1, Whatman PLC, Middlesex, UK). Further, 1 mL of filtrate and K2CO3 were placed at both end sides separately into the outer space of the Conway tube (Sibata Ltd., Saitama, Japan), and 1 mL of 0.01 N H2BO3 and 100 μL of Conway reagent (0.066% methyl red/0.066% bromocresol green, 1:1) were added to the inner space, and the Conway tube was sealed with grease.

\[
\text{VBN mg%} = \left( \frac{\text{sample weight after storage} - \text{sample weight during storage}}{\text{original sample weight}} \right) \times 100
\]

where \(\phi\) is the titration volume of 0.01 N HCl (mL) in the sample and \(b\) is the titration volume of 0.01 N HCl (mL) in the blank.

**Preparation of chicken breast meat for NMR analysis**

Chicken breast meat (5 g) was thawed at 4 °C for 30 min. Thawed chicken breast meat was homogenized at 1,720 × g for 30 s (T25 basic, Ika Co., Kg, Staufen, Germany) with 20 mL of 0.6 M perchloric acid. The homogenate was centrifuged (Continental 512 R, Hanil Co., Ltd., Incheon, South Korea) at 3,086 × g for 15 min at 4 °C. Each supernatant was transferred to a new test tube and neutralized with KOH. The neutralized extracts were centrifuged again under the same conditions. After centrifugation, the supernatant was filtered using a filter paper (Whatman No. 1, Whatman PLC) and lyophilized (Freezer dryer 18, Labco Corp., Kansas City, MO, USA). The lyophilized extracts were stored at −70 °C until NMR analysis. Prior to NMR analysis, lyophilized extracts were reconstituted by 1 mL of 1 mM 3-(trimethylsilyl)propionic-2, 2, 3, 3-d4 acid (TSP) deuterium oxide solution [20 mM phosphate buffer system titrated with pH 7.0]. TSP was used for the purpose of aligning the NMR spectrum to 0.00 ppm. Reconstituted extracts were kept at 35 °C for 10 min to dissolve metabolites well and then centrifuged at 3,086 × g for 15 min at 4 °C. The 800 μL of supernatant was transferred to microtube and centrifuged at 17,000 × g for 15 for 10 min (HM-150IV, Hanil Co., Ltd.). The 600 μL of final supernatant were transferred to 5 mm NMR tube for NMR analysis.

**NMR analysis**

All NMR spectra were recorded in D2O at 298 K using a Bruker 850 MHz Cryo-NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany). 1H NMR was performed by applying standard zg30 with slight modification (recycle delay of 1 s) default in Topspin 3.6.2 (Bruker Biospin GmbH). The 1H NMR experiment was performed using 64 k data points, a sweep width of 17,007.803 Hz, and 128 scans. According to Kim et al., two-dimensional NMR analyses [1H-1H correlation spectroscopy (COSY), 1H-13C total correlation spectroscopy (TOCSY), 1H-13C heteronuclear single quantum coherence (HSQC), and 1H-13C heteronuclear multiple-bond correlation spectroscopy (HMBC)] were performed to quantify and qualify the metabolites (Supplementary Table 2). COSY and TOCSY experiments were performed with 2 k data points in the \(f_2\) domain and 256 increments in \(f_1\), each with 8 and 16 scans, respectively. Spectral widths of 11 ppm were used for TOCSY experiments. HSQC and HMBC experiments were performed with 2 k data points in the \(f_2\) domain and 512 increments in \(f_1\), each with 8 and 32 scans, respectively. The spectral widths were 11 ppm for the \(f_2\) dimension and 180 and 240 ppm for the \(f_1\) dimension. Coupling constant values of 145 and 8 Hz were employed to set the delay durations for short-range and long-range correlations, respectively. To quantify the metabolites, acquired HSQC spectra were processed using Analysis of MIXtures software v3.9 (Bruker Biospin GmbH). Among assigned peaks of each metabolite (Supplementary Table 1), non-overlapped peak with good intensity was selected as candidate. To quantification of peak, all HSQC spectra were loaded and overlapped on Analysis of MIXtures software. Selected peak ranges were set manually for...
automation process, then all spectra were quantified at once. After quantification, we reviewed the dataset and organized it according to the format for further analysis.

Statistical analysis

The acquired integrated HSQC data was used for multivariate analysis. Multivariate analysis was performed using MetaboAnalyst 4.0. Prior to multivariate analysis, integrated data were filtered interquartile range to eliminate outlier, log-transformed without normalization and mean-centered and divided by the standard deviation of each variable for all multivariate analyses. iPCA and two-way ANOVA were performed to distinguish between unfrozen and FT meat based on treatment and storage time. Additionally, quantified metabolites, physicochemical properties, and TAB were combined for correlation analysis using pattern hunter analysis. Discriminant analysis was completed using ROC curve analysis with linear support vector machine (SVM) classification. The feature metabolites were ranked based on the SVM’s built-in ranking method. Statistical analysis for selected metabolites in the figure of discriminant analysis was performed using the procedure of the general linear model for the comparison of quantified metabolites from HSQC. Significant differences were determined using the Student–Newman–Keuls multiple range test with the SAS software (SAS 9.4, SAS Institute, Cary, NC, USA) at a confidence level of p < 0.05. All experiments were conducted in quintuplicate. TAB counts, color, TABARS, and VBN were conducted with two or three observations per replication and the values were averaged for statistical analysis.

DATA AVAILABILITY

The authors declare that all relevant data supporting this study has been included within the paper. Raw data will be made available by corresponding authors upon reasonable request.

Received: 27 March 2022; Accepted: 31 August 2022; Published online: 22 September 2022

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ACKNOWLEDGEMENTS
This work was supported from “Cooperative Research Program for Agriculture Science and Technology Development (PJ01620502)” Rural Development Administration, Republic of Korea.

AUTHOR CONTRIBUTIONS
Conceptualization, methodology, formal analysis, software, data curation, visualization, writing—original draft (H.C.K.); conceptualization, methodology, validation, investigation (K.H.B.); formal analysis, investigation, visualization (Y.E.L.); investigation, formal analysis (T.K.); data curation, validation (H.J.K.); validation, visualization (D.L.); conceptualization, project administration, funding acquisition, supervision, resources, writing—review and editing (C.J.).

COMPETING INTERESTS
The authors declare that they have no known competing financial or nonfinancial interests that could have appeared to influence the work reported in this paper.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41538-022-00159-x.
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