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^{1}H NUCLEAR MAGNETIC RESONANCE (NMR)-BASED METABOLOMICS: RAPID ASSESSMENT OF MICROBIAL ACCEPTABILITY OF PORK DURING REFRIGERATED STORAGE

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I. INTRODUCTION

Screening of metabolome changes in meat offers key insights into important indicators of meat status, such as freshness, degree of aging, and authentication [1, 2]. Nuclear magnetic resonance (NMR) spectroscopy is among the most widely used analytical techniques in metabolomics [3]. In our previous study, we demonstrated that NMR-based metabolomics approaches can distinguish between fresh and frozen-thawed meat and assess meat freshness [1]. The established method that uses a water-based solvent to replace deuterium oxide for NMR spectroscopy provides undistorted spectra and allows accurate quantification, but they require lyophilization of meat extracts for NMR experiments [4]. However, the established method is not suitable for monitoring microbial growth in real time because it is too time-consuming. Although water suppression in NMR spectra may cause slight distortion [4], integrating machine learning algorithms could provide a solution to resolve this time-consuming limitation. Therefore, the objective of this study was to evaluate the microbial acceptability of pork during refrigerated storage with minimal sample preparation, differentiating between acceptable and unacceptable microbial conditions using discriminant analysis supported by receiver operating characteristic-based threshold optimization.

II. MATERIALS AND METHODS

Pork loin (*Longissimus lumborum*, n=49), 3 days post-mortem, was purchased in a local butcher's market (Dongtan, Korea) and cut into 100 ± 5 g pieces, wrapped in packaging film with an absorbent pad and stored in refrigerated at 1°C for 16 days. After storage, meat was minced using a chopper to evaluate total aerobic bacterial (TAB) counts and NMR experiment. The chopped pork loins were diluted with distilled and deionized water using a stomacher. After first dilution, the solution was divided for TAB and NMR experiments. For TAB counts, the diluted solution was directly serially diluted with sterile saline (0.85%). Appropriate dilution were prepared in sterile saline and spread on plate count agar. The agar plates were incubated at 37°C for 48 h, and the microbial counts were calculated. Statistical analysis for TAB was performed using general linear model. The significance of differences among mean values were determined by Student-Newman-Keuls multiple range test ($p < 0.05$) using SAS 9.4 software. For NMR experiments, the first dilution solution was centrifuged at 12,000 ×*g* for 10 min. After, 900 μL of supernatant were acquired and mixed with 100 μL of 1 mM 3-(trimethylsilyl)propionic-2,2,3,3-d_4 (TSP) acid deuterium oxide(D_2O) solution for NMR experiment. NMR spectra were recorded in D_2O on a Bruker 850 MHz Cryo-NMR Spectrometer (Bruker Biospin GmbH, Rheinstetten, Baden-Württemberg, Germany), using modified standard noesypr1d (recycle delay of 5 s) pulse program, with lock on the D_2O solvent. The chemical shifts (δ) were algined to the TSP resonance. Phase correction was performed manually using TopSpin 3.6.5 and spectral bining was performed automatically using Chenomx NMR suite (Version 9.02). Discriminant analysis was performed using partial least squares-discriminant analysis (PLS-DA) in MetaboAnalyst 6.0. Data were normalized by median, log-transformed, and autoscaled. receiver operating characteristic (ROC) curve analysis was applied to optimize the classification model.

III. RESULTS AND DISCUSSION

Table 1. Total aerobic bacterial counts of pork loin (*Longissimus lumborum*) during refrigerated storage at 1°C.

	Storage (day)							SEM[1]
	3	6	8	10	12	14	16	
Total Aerobic Bacterial Counts (Log CFU/g)	3.99^{d}	4.28^{cd}	4.17^{cd}	5.07^{c}	5.93^{b}	7.17^{a}	7.70^{a}	0.268

[1] Standard error of the means (n = 49). $^{a-d}$ Different letters in the same row indicate a significant difference ($p < 0.05$).

TAB counts of pork loin were measured to evaluate the meat freshness (Table 1). We set the acceptability threshold at 7 log CFU/g based on microbiological limits established by various countries [5]. The results showed that TAB counts remianed below this threshold until day 12. However, after day 12, the counts exceeded 7 log CFU/g, suggesting microbial spoilage. Based on these findins, we applied a threshold-based bninary classification to determine whether a sample and fast data processing approach could rapidly distinguish between fresh and spoilage samples (Figure 1). Binary classification was performed using PLS-DA, and the number of latent variables was set to 5, which yielded the best performance. Although some fresh samples were misclassified as spoiled, the overall spoilage prediction performance was excellent. To enhance the accuracy of this model, concentrated solutions may be useful because the trace peaks are observed on ^{1}H NMR spectra in peak overlapped regions (data not shown). Additionally, narrow-range spectral binning could improve sensitivity depending on biomarker, allowing for more precise detection of relevant variation.

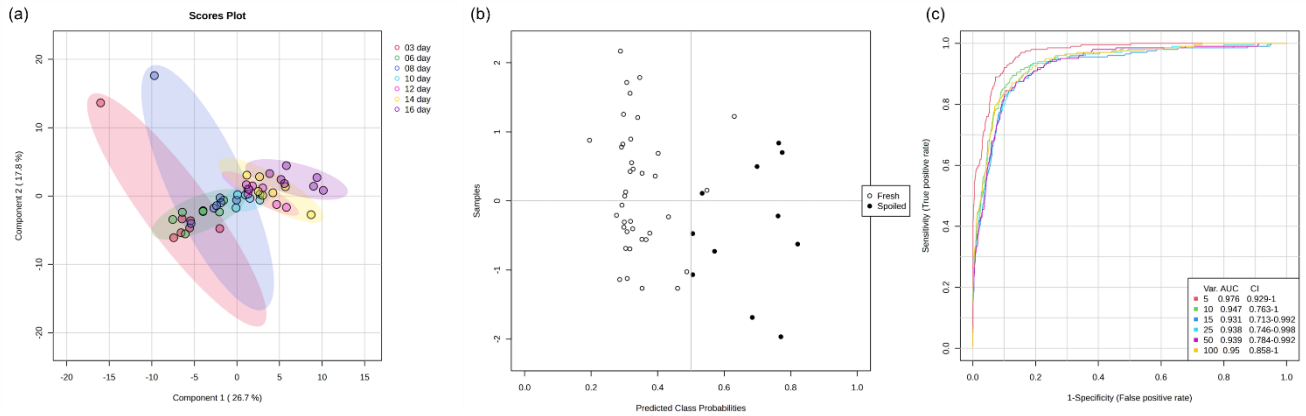


Figure 1. Partial least squares-discriminant analysis (PLS-DA) (a), predicted class probabilities (b), and receiver operating characteristic (ROC) (c) curve from pork loin (*Longissiumus lumborum*) using ^{1}H NMR on 850 MHz cryo-NMR spectrometer.

IV. CONCLUSION

Our results demonstrated the feasibility of ^{1}H NMR analysis, with simple preparation and automated processing, in detecting unacceptable pork before distribution to consumers. However, further optimization of sample preparation and NMR acquisition parameters is necessary for practical application.

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