Research Paper (Original Article)

Dry-aged beef manufactured in Japan: Microbiota identification and their effects on product characteristics

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Abstract (100-160 words)

We aimed to determine the mold, yeast, and bacterial distributions in dry-aged beef (DAB) manufactured in Hokkaido, Japan, and to study their effects on meat quality compared to wet-aged beef (WAB). Two rump blocks from Holstein steer were dry- and wet-aged for 35 days at 2.9 °C and 90% RH. The psychrophilic molds *Mucor flavus* and *Helicostylum pulchrum* and other fungi (*Penicillium* sp. and *Debaryomyces* sp.) appeared on the crust of DAB, while lactic acid bacteria and coliforms were suppressed in the inner part of the meat. The composition of C16:0, C18:0, and C18:1 fatty acids did not differ between DAB and WAB, while more C17:0 fatty acids were detected in DAB. Dry aging suppressed acids and increased the production of various aroma compounds with mushroom-like, nutty, and other pleasant flavors. The meat quality and free amino acid (FAA) contents of DAB and WAB did not differ significantly. In this study, we identified major molds on DAB, which might contribute to an increase in aroma. Keywords: dry-aged beef; *Mucor flavus*; *Helicostylum pulchrum*; psychrophilic mold; meat quality; volatile aroma compounds

1. Introduction

Dry-aged beef (DAB) is gaining popularity in many countries, especially in Asia (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016). Dry aging is a traditional method of storing beef carcasses or prime/subprime cuts at refrigerated temperatures without vacuum packaging (Khan, Jung, Nam, & Jo, 2016). DAB is exposed to air flow during the entire aging period, drying the surface and forming a crust on which microorganisms, especially molds and yeast, have been observed to grow (Kim et al., 2020). Molds and yeast isolated from DAB have been reported to possess proteolytic and lipolytic activities and can induce the breakdown of myofibrils in DAB (Oh et al., 2019). Therefore, the tenderness of DAB has been attributed to both the endogenous enzymatic activity in beef muscle (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010) and the presence of molds and yeast (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016; Hanagasaki & Asato, 2018; Oh et al., 2019).

DAB is characterized by its unique flavors, which have often been described as beefy, buttery, nutty, and earthy (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016). These flavors develop through reactions between flavor compounds (taste-related compounds and aroma volatiles) during aging (Lee et al., 2019). Taste-related compounds include inosine 5′-monophosphate, reducing sugars, and free amino acids (FAAs), whereas aroma volatiles are derived from the oxidation of lipids, such as triglycerides, phospholipids, and free fatty acids, and/or the Maillard reaction between reducing sugars and FAAs (Lee et al., 2019). During the dry aging process, flavor compounds become concentrated as moisture evaporates (Kim, Kemp, & Samuelsson, 2016; Lee et al., 2019). Warren and Kastner (1992) reported that dry-aged strip loins had higher levels of beefiness and brown-roasted flavors than vacuum-aged (wet-aged) strip loins. Furthermore, dry aging with molds produced different levels of flavor compounds compared to dry aging without molds (Hanagasaki & Asato, 2018). These findings indicate that the dry aging process coupled with mold growth may improve the meat quality of DAB.

A few studies have been published on the identification and isolation of molds and yeast in DAB. Specifically, *Penicillium camemberti*, *Debaryomyces hansenii* (Ryu et al., 2018), *Pilaira anomala* (Lee et al., 2019) and *Mucor flavus* (Hanagasaki & Asato, 2018) are present on the crust of DAB produced in Asian countries. However, DAB-related fungi and their impacts on meat quality are not well understood. Therefore, in this study, we aimed to first isolate and identify microorganisms, especially molds and yeast, from DAB produced in an aging room in Hokkaido, the northernmost prefecture in Japan. Second, we evaluated the meat quality characteristics of DAB by comparing them to those of wet-aged beef (WAB), which does not form a crust.

2. Materials and Method

2.1 Aging process

Two blocks of rump (approximately 8 kg each) obtained from the right and left sides of an individual Holstein steer (18 months old) were used for dry and wet aging. The dry-aged block was processed uncovered, while the wet-aged block was vacuum-packed in a plastic bag. Aging was performed in an aging room (Goshima Thermal Engineering Co., Ltd., Hokkaido, Japan) (it has been used for 16 months) installed in the Kitaichi Meat Co., Ltd. (Hokkaido, Japan) under the following conditions: average temperature, 2.9 °C; relative humidity, 90%; and air flow, 1.8–2.5 m/s. We used a 35-day aging period in terms of the juiciness and tenderness of DABbased on the recommendations from a study by Smith et al. (2008). Wet aging also served as a control without fungal colonization in the same aging room. After aging, the beef blocks were transported to the laboratory and processed within one day for further analyses. The weights of the DAB and WAB were recorded before and after aging, and weight loss is expressed using the method of DeGeer et al. (2009). Aging loss was calculated as (weight loss during aging/weight before assigned aging) × 100. Trim loss of

the DAB was calculated as (weight loss due to trimming/untrimmed weight) × 100. The combined loss of the DAB was calculated as (weight before aging – trimmed weight)/(weight before aging) × 100.

2.2 Microbial analysis

2.2.1 Preparation of microbial suspension

Microbiological analyses were performed on the crust and inner part of the DAB and the inner part of the WAB. Microbial samples were collected from the DAB crust by wiping a 10 × 10 cm surface area square with a moistened cotton swab. The swab was then immersed in 10 mL of a sterilize 0.9% NaCl solution. Then, 10 g of the inner parts of the DAB and WAB were placed inside a Filtrate bag II (Eiken Chemical Co. Ltd., Tochigi, Japan) and homogenized with 90 mL of a sterilize saline solution in a stomacher (Exnizer 400; Organo Corp., Tokyo, Japan) for 5 min at 260 rpm. Serial dilutions of the swab samples and homogenate were prepared and used for further analyses, including fungal isolation and bacterial quantification.

2.2.2 Identification of fungal species on the DAB crust

Fungi on the crust of the DAB were isolated and cultured under aerobic conditions at 4 °C and 25 °C on potato dextrose agar (PDA) (Eiken Chemical Co. Ltd., Tochigi, Japan) for 5–7 days. Morphologically distinct colonies were restreaked, and a resulting single colony was isolated and stored at −80 °C for further experiments. The morphology of the colonies was examined on PDA after 14 days at 4 °C. DNA was extracted using the method of Bok and Keller (2012) with slight modifications. Fungal cells were cultured in potato dextrose broth (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) supplemented with 0.1% yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan) at 4 °C or 25 °C. After culturing, the collected fungal cells were disrupted using a Disruptor Genie (Scientific Industries, Inc., Bohemia, NY,

USA). The internal transcribed spacer (ITS) and D1/D2 regions of the extracted DNA were amplified using the primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC -3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG -3') (White, Brun, Lee, & Taylor, 1990) and NL1 (5'-GCATATCAATAAGCGGAGGAAAAG -3') and NL4 (5'- GGTCCGTGTTTCAAGACGG -3') (Kurtzman & Robnett, 1997), respectively. The primers Act-1 (5'-TGGGACGATATGGAIAAIATCTGGCA-3') and Act-4r (5'-TCITCGTATTCTTGCTTIGAIATCCACAT -3') (Voigt & Wöstemeyer, 2000) were also used to confirm the identity of *Helicostylum* sp. PCR products were sequenced (3500 Genetic Analyzer, Thermo Fisher Scientific, Waltham, MT, USA) and analyzed using BLAST (National Center for Biotechnology Information) to identify the fungal species.

2.2.3 Mycotoxin analysis on DAB crust

Mycotoxins (aflatoxins B_1, B_2, G_1, and G_2; patulin; deoxynivalenol; and zearalenone) on the DAB crust were analyzed by the Mycotoxin Research Association (Yokohama, Japan). Aflatoxins were analyzed as per the "Test Methods Related to Total Aflatoxin" in Notice Syoku-An No. 0816-2, dated August 16, 2011. Patulin and deoxynivalenol were analyzed by LC/MS, and their quantification limits were 0.05 and 0.1 ppm, respectively. Zearalenone was analyzed by HPLC, and the quantification limit was 0.1 ppm.

2.2.4 Bacterial quantification

Diluted microbial suspensions were cultured on selective media to count specific bacterial species by the viable plate count method. The total bacterial abundance was determined on Pearlcore plate count agar (Eiken Chemical Co. Ltd., Tochigi, Japan), while lactic acid bacteria and staphylococci were cultured and counted on Man, Rogosa, and Sharpe agar (CM 0361; Oxoid, Hampshire, England) and Pearlcore mannitol salt agar (MSA) (Eiken Chemical Co. Ltd., Tochigi, Japan), respectively. Coliforms were counted on Chromocult coliform agar

(Merck KGaA, Darmstadt, Germany). The coliform agar plates were incubated under aerobic conditions at 37 °C for 24 h. All other agar plates were incubated under aerobic conditions at 37 °C for 48 h. The visual counts of colonies were used to calculate the decadic logarithm of colony-forming units per gram sample ($\log_{10}$ colony forming unit (CFU)/g).

The presence of *Staphylococcus aureus* on the surface of the DAB was determined by a tube coagulase test according to the method of Mizobuchi et al. (1994) with slight modifications. Yellow bacterial colonies growing on the DAB were cultured on MSA and then inoculated into rabbit plasma (Eiken Chemical Co. Ltd., Tochigi, Japan) to detect coagulation.

2.3 Proximate composition

Moisture, crude protein, crude fat, and ash contents of DAB and WAB were determined according to Association of Official Analytical Chemists (AOAC) methods (2002). The moisture content was measured according to AOAC method 934.01. The crude protein and crude fat contents were determined by the Kjeldahl (AOAC method 928.08) and Soxhlet (AOAC method 991.36) methods, respectively. The ash content was determined using a muffle furnace at 550 °C (AOAC method 923.03). Carbohydrate content was calculated as carbohydrate content = 100 − (moisture + crude protein + crude fat + ash content). The calorie contents (kcal) of WAB and DAB were calculated using the Atwater values to convert the corresponding fat (9 kcal/g), protein (4.02 kcal/g), and carbohydrate (3.87 kcal/g) contents.

2.4 Instrumental color

One-inch-thick cuts of WAB and DAB were bloomed for 1 h at 4 °C; then, instrumental color was measured at 10 different locations using a spectrophotometer (CM-2600d; Konica Minolta, Inc., Tokyo, Japan) with a D65 illuminant and a 10 ° standard observer. The device was calibrated with a white tile prior to measurements. The lightness (CIE L^*), redness (CIE a^*), and yellowness (CIE b^*) of the samples were determined; then, vividness (chroma) and

tone of color (hue angle) were calculated as chroma = $(a^{*2} + b^{*2})^{1/2}$ and hue angle = arctangent (b^*/a^*) (AMSA, 2012).

2.5 pH measurement

The pH levels of WAB and DAB were measured as described by Bendall (1975). Briefly, 5 g of sample was homogenized with 50 mL of 5 mM sodium iodoacetate in 150 mM potassium chloride. The homogenate was passed through a Milpap filter paper (Azumi Filter Paper Co. Ltd., Osaka, Japan). The pH of the filtrate was measured using a calibrated pH probe (F-51; Horiba, Ltd., Kyoto, Japan).

2.6 Meat quality measurements

Expressible drip loss was determined as described by Hamm (1986) with slight modifications. Briefly, 1 g of minced WAB and DAB meat was placed between two 100-mesh nylon filters that were sandwiched between four sheets of previously weighed No. 526 filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The assembly was subjected to 30 kg of pressure for 1 min. After pressing, the filter papers were reweighed. The expressible drip loss was calculated as the percentage of weight gain of the filter papers.

Cooking loss was measured by the method of Honikel (1998) with a slight modification. One-inch steak samples that had already undergone instrumental color measurement were weighed and placed inside a plastic bag and incubated for 60 min at 70 °C in a water bath (SP-12R, Taitec Corporation, Saitama, Japan). The samples were then cooled, patted dry with paper towels, and then reweighed. The difference between the weights before and after incubation was calculated and expressed as a ratio relative to the weight before incubation.

After determining cooking loss, the incubated steak was wrapped in polyvinylidene chloride film and stored at 4 °C. Then, >10 cores were taken from the steak using a 1.27-cm-diameter coring device situated to collect samples parallel to the longitudinal axis of the

muscle fiber. The cores were then sheared by a Warner-Bratzler meat shear (GR Manufacturing Company, Manhattan, KS, USA). The Warner-Bratzler shear force (WBSF) of the steak was calculated as the average maximum force (kg) required to shear through each sample.

2.7 Free amino acid contents

The FAA contents of WAB and DAB were measured as previously described (Aro Aro et al., 2010), with slight modifications. Then, 5 g of minced meat was homogenized with 50 mL ultrapure water using a Physcotron (Niti-On Co., Ltd., Chiba, Japan). The homogenate was centrifuged at 10,000 × *g* at 4 °C for 20 min, and the supernatant was filtered through a No. 5C filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Then, 4 g of filtrate was mixed with an equal volume of 4% trichloroacetic acid (TCA), incubated at 37 °C for 30 min, and filtered again. This filtrate served as the 2% TCA soluble fraction that was analyzed using a high-speed amino acid analyzer (LA-8080, Hitachi High-Tech Science Co., Tokyo, Japan) that operates based on the ninhydrin method (Moore & Stein, 1948). The individual and total FAA contents of WAB and DAB were calculated by interpolation from a standard curve generated using amino acid mixture type H (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and reported as mg/100 g meat.

2.8 Fatty acid analysis

Total lipids were extracted from 0.5 g of minced WAB and DAB samples using the Bligh and Dyer (1959) method. The samples were placed in a test tube, and the liquid was evaporated using nitrogen gas. The total lipids were methylated by heating with 1 mL of 5% hydrogen chloride methanol solution at 125 °C for 3 h. After cooling, 1 mL of chloroform and 0.9 mL of distilled water were added to samples, mixed for 1 min, and centrifuged at 2,000 rpm for 10 min. The upper layer was removed, added with 1 mL of methanol and 0.9 mL of

distilled water, mixed for 1 min, and centrifuged at 2,000 rpm for 10 min. These procedures were repeated once. After the upper layer was removed, the bottom layer was evaporated by nitrogen gas and fatty acid methyl esters (FAMEs) were separated from other compounds by thin-layer chromatography. FAMEs were analyzed by gas chromatography–flame ionization detector (SHIMADZU GC-2014) equipped with a Zebron ZB-FAME capillary column (30 m × 0.25 I.D. mm, 0.20 μm film thickness; Phenomenex Inc., USA, CA) using nitrogen as the carrier gas (1.3 mL/min). The injection temperature was 250 ℃, and the detector was set at 260 ℃. The column temperatures were increased from 140℃ to 240 ℃ at a rate of 4 ℃/min and held at 240 ℃ for 15 min. FAMEs were identified by comparing to Supelco 37 Component FAME standards (Merck KGaA, Darmstadt, Germany).

2.9 Volatile compound analysis

Approximately 1 g of WAB and DAB samples were minced, placed into 15 mL glass vials sealed with a PTFE/silicone septum, and stored at −80 ℃ before analysis. The volatile compounds were prepared according to the headspace solid-phase microextraction (HS-SPME) method (Yu, Sun, Tian, & Qu, 2008) using a divinylbenzene/carboxen/polydimethylsiloxane (50/30 μm thickness) SPME fiber (Supelco Co., Bellefonte, PA, USA). Then, the compounds were thermally desorbed in the injection port of a gas chromatograph-mass spectrometer (SHIMADZU GCMS-QP2010) by heating the fiber for 1 min at 260 ℃. The GC-MS was equipped with the nonpolar InertCap I column (30 m × 0.25 I.D. mm, 1.50 μm film thickness, GL Sciences Inc., Tokyo, Japan). Helium was used as carrier gas with a flow pressure of 78.9 kPa. Manual injection was carried out in splitless mode. The column temperature was first held at 40 ℃ for 10 min, then allowed to rise to 200 ℃ at a rate of 5 ℃/min and 250 ℃ at a rate of 20 ℃/min, and held at 250 ℃ for 5 min. The MS detector was operated in scan mode (30–550 *m/z*) using electron impact ionization (70eV). The interface and detector temperatures were kept at 250 ℃. The resulting mass

spectra were deconvoluted using AMDIS GC/MS Analysis version 2.73 and matched to Massbank of North America GC-MS Spectra. Additionally, to obtain similarity values higher than 98, the mass spectra were compared to commercial GC-MS libraries such as NIST05 and NIST05s using Shimadzu GCMS solution software.

2.10 Statistical analysis

Bacterial counts are expressed as $\log_{10}$ CFU/g and were statistically analyzed after log transformation. All continuous data are expressed as the mean ± SD. Means were compared by Student *t*-tests using JMP 13 (SAS Institute Inc., NC, USA) to identify differences between WAB (n = 3) and DAB (n = 3) groups. *P*-values < 0.05 are considered statistically significant.

3. Results and Discussion

3.1 Visual appearance and weight loss of aged beef rumps

We visually examined the changes in Holstein rump meat that was wet- and dry-aged for 35 days. In contrast to the WAB (Fig. 1A), the crust of the DAB was almost completely covered with white- and gray-colored mold (Fig. 1B). The WAB and DAB lost 1.5% and 13.4% of their weights, respectively, during aging. In the DAB, the weight loss from trimming and the combined weight loss were 9.1% and 21.5%, respectively. Overall, our values for the weight losses of the DAB due to aging, trimming, and their combination are lower than those of dry-aged strip loin reported by DeGeer et al. (2009) (19.1%, 34.4%, and 46.9%, respectively).

3.2 Microbial composition

3.2.1 Mold and yeast identification

Based on morphology, the predominant fungi observed on the DAB were Mucoraceae (Fig. 2). Two types of morphologically distinct Mucoraceae were distinguished in the isolated strains (Fig. 2A and D). We chose a representative strain for each type and named them KT1a (Fig. 2A, B, and C) and KT1b (Fig. 2D, E, and F). These strains grew at 4 °C but not at 25 °C, indicating that they are psychrophilic. Under a stereomicroscope, KT1a showed the typical sporangia of Mucoraceae (Fig. 2C, arrow). KT1b also formed sporangia (Fig. 2F, arrow), with the characteristic structures shown in Fig. 2F (arrowhead). Based on the ITS and D1/D2 sequences, KT1a and KT1b were identified as *M. flavus* and *H. pulchrum*, respectively. Moreover, the partial actin gene sequence of KT1b was identical to that from *H. pulchrum* FSU875 (GenBank ID: EU736234.1). These results are consistent with the morphological observations, and overall, the data suggest that *M. flavus* and *H. pulchrum* are major players in the dry aging process.

M. flavus has been reported to play a major role in the dry aging of beef (Hanagasaki & Asato, 2018). Because *M. flavus* is not monophyletic (Walther et al., 2013), we compared the ITS sequences of *M. flavus* KT1a to those of other known strains. The results indicated that *M. flavus* KT1a is closely related to *M. flavus* strains CBS 992.68 (GenBank ID: JN206067.1), PG268 (AB916510), and PG272 (AB916507). Strains CBS 992.68, PG268, and PG272 were isolated in Antarctica (Walther et al., 2013) and the Svalbard Arctic (Singh, Tsuji, Gawas-Sakhalker, Loonen, & Hoshino, 2016), suggesting that these *M. flavus* strains prefer a cold habitat, including refrigerators used for dry aging. A recent review (Morin-Sardin, Nodet, Coton, & Jany, 2017) described *Mucor* as a Janus-faced genus; however, various *Mucor* spp. are used in the food industry. Furthermore, *M. flavus* KT1a is a psychrophile that does not grow at 25 °C, suggesting that this strain is not pathogenic to humans and endothermic animals. These characteristics indicate that this fungal strain is safe for DAB production.

Helicostylum (also known as *Chaetostylum*) and *Thamnidium* are known to regularly occur on aging and tenderizing beef held in coolers and are associated with so-called

"whiskery beef" (Jensen, 1954; Hesseltine & Anderson, 1957). Sato et al. (2013) reported finding *H. pulchrum* on the cut surface of commercially available brie cheese, and the extracts of cheese inoculated with this mold were nontoxic to human neuroblastoma cells (Sato et al., 2013). This report supports the safety of this mold in foods; however, further research is needed to confirm the safety of *H. pulchrum* found on DAB. To the best of our knowledge, the combination of *M. flavus* and *H. pulchrum* has not been previously reported as part of the regular fungal community found on DAB.

Three morphologically distinct *Penicillium* sp. and one *Debaryomyces sp*. were isolated frequently from plates cultured at 25 °C. *Penicillium* sp. and *Debaryomyces* sp. have previously been reported to occur on the surface of DAB (Ryu et al., 2018). ITS sequencing identified the three *Penicillium* sp. as species belonging to series *Camembertiorum*; two ITS sequences were identical to those of several species, including *P. commune*, and the remaining sequence was closest to *P. echinulatum*. *Penicillium cavernicola* is another species belonging to series *Camembertiorum* and has been isolated from Divle Cave raw milk cheese (Ozturkoglu-Budak, Wiebenga, Bron, & de Vries, 2016). Members of *Penicillium* series *Camembertiorum* grow well at 15 °C and are typically found on proteinaceous and lipid-containing foods (Visagie et al., 2014). These characteristics are consistent with our results, suggesting that these species are advantageous under dry aging conditions. The yeast isolate was identified as a member of the *Debaryomyces hansenii* complex. The *D. hansenii* complex is often found in food products, including DAB (Oh, Lee, Lee, Jo, & Yoon, 2019) and cheese (Fröhlich-Wyder, Arias-Roth, & Jakob, 2019). Recently, members of this complex have been shown to play a role in inhibiting the growth of *Penicillium* spp., including *Penicillium* series *Camembertiorum* (Medina-Cordova, Rosales-Mendoza, Hernández-Montiel, & Angulo, 2018), suggesting that the *D. hansenii* complex competitively controls the overgrowth of *Penicillium* spp. that are sometimes associated with bad taste and smell.

Mycotoxin production by contaminated fungi on foods and feeds is a major safety

concern. Mucoraceae is not recognized as a major mycotoxin producer. On the other hand, although *Penicillium* series *Camembertiorum* is broadly used for food production, some *Penicillium* spp., such as *P. expansum* (a patulin producer), are recognized as major mycotoxin producers. Using mycotoxin analysis, aflatoxins (B_1, B_2, G_1, and G_2), patulin, deoxynivalenol, and zearalenone were not detected in the DAB (data not shown).

3.2.2 Bacterial abundance

Bacterial growth is an important parameter related to the production of safe aged beef. The bacterial abundance levels on the crust and inner parts of the DAB and WAB aged for 35 days are shown in Table 2. On the crust of the DAB, there were 4.64 ± 0.75 log CFU of total bacteria/cm^2 and less than 30 CFU of lactic acid bacteria/cm^2 (Table 1). Coagulase-negative staphylococci were found at 3.16 ± 0.50 log CFU/cm^2 (Table 1). Although there are no investigations reporting bacterial abundance per area of DAB crust aged for 35 days, Li et al. (2014) reported that the total and lactic acid bacteria reached 8.75 and 3.20 log CFU/cm^2, respectively, on meat surfaces that had been dry-aged for 19 days. Thus, the total and lactic acid bacteria were present at lower levels under our conditions.

In the inner part of the DAB, the abundance levels of the total and lactic acid bacteria were significantly higher and lower, respectively, than the corresponding levels in the WAB (Table 1). Similar results have been reported for DAB produced in a dry aging bag compared to WAB produced in a vacuum bag (Li, Babol, Wallby, & Lundström, 2013). It has been suggested that the anaerobic conditions of vacuum packing allow lactic acid bacteria to predominate in WAB (Ahnström, Seyfert, Hunt, & Johnson, 2006; Parrish, Boles, Rust, & Olsen, 1991). This finding is supported by the accelerated growth of lactic acid bacteria in WAB compared to DAB in our study. Staphylococci were not detected in the inner part of the WAB, while 2.20 ± 0.45 log CFU of coagulase-negative staphylococci/g (Table 1) was detected in the DAB. In addition, coliforms were significantly suppressed in the inner part of

the DAB (1.97 ± 0.97 log CFU/g DAB compared to 3.64 ± 0.00 log CFU/g WAB) (Table 1). Coliforms are indicators of hygiene during food processing. Although the DAB was exposed to air flow during the entire aging period, compared to wet aging, our results suggest that dry aging with fungi reduces the contamination of the inner part of the rump block by coliforms.

3.3 Proximate composition

The proximate composition of DAB and WAB are shown in Table 2, comparing the effects of aging method after 35 days. The moisture content of DAB for (69.6% ± 0.2%) was significantly lower than that of WAB (71.9% ± 0.2%) (p <0.001), which agrees with Lee et al. (2019), who reported that the moisture content of beef strip loin dry-aged for 28 days in Korea is lower than that of the wet-aged meat. Li et al. (2014) showed that the water content of naked DAB was lower than that of vacuum-aged beef, not only in the outer layer but also in the inner layer of the beef. Similarly, more water evaporated in the DAB manufactured in our study. Meanwhile, the crude fat content in DAB (7.55% ± 1.1%) was significantly higher than that in WAB (5.10% ± 0.7%) (p <0.05) (Table 2). Ueda et al. (2007) showed that moisture and crude fat contents are negatively correlated; moreover, moisture is replaced by fat as the amount of fat increases in meat. Our results are consistent with this report. Because of the higher crude fat content, the calorie content of DAB is also significantly higher than that of WAB (Table 2). Crude protein, ash, and carbohydrate contents did not differ between WAB and DAB (Table 2).

3.4 Instrumental color analysis

The surface appearance of steaks derived from WAB and DAB is shown in Fig. 3. Instrumental color measurements show that the L* values in DAB were significantly higher than those in WAB, while the values of a*, b*, chroma (vividness), and hue angle (tone of color) did not differ significantly between WAB and DAB (Table 3). Higher L* values are

expected, along with greater reflectance, as the moisture content of meat increases (Dikeman, Obuz, Gök, Akkaya, & Stroda, 2013). However, DAB, with lower moisture content, showed a brighter color than WAB in the current study. Our results are consistent with those of Kim et al. (2019a), who showed that, in Korean Hanwoo rump, the L* values are higher in DAB than in WAB, whereas the a* and b*values are not significantly affected by the aging method.

3.5 pH and meat quality

After 35 days of aging, the pH values of the DAB and WAB were 5.62 ± 0.01 and 5.56 ± 0.01, respectively. The pH of the DAB with Mucoraceae was significantly higher than that of the WAB (p=0.012) (Table 4), a result similar to that of Australian beef loin dry- and wet-aged for 35 days (5.62 and 5.44, respectively) (Ha et al., 2019). Table 1 shows lower lactic acid bacteria levels in the DAB than in the WAB after 35 days of aging. Lactic acid bacteria produce lactic acid and acetic acid in meat (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015); thus, the difference in the abundance of lactic acid bacteria in the DAB and WAB may explain their pH difference.

Water holding capacity is an important factor affecting meat quality, and it is reflected by parameters such as expressible drip loss and cooking loss (Irie, Izumo, & Mohri, 1995). The WAB and DAB did not differ significantly in their expressible drip loss values (41.0% ± 2.7% and 41.2% ± 3.6%, respectively) (Table 4). In contrast, the DAB yielded a higher cooking loss value (17.1% ± 0.6% vs. 14.9% ± 1.2%) (Table 4). This result is inconsistent with a previous report showing that the aging method (dry or wet aging) does not affect cooking loss in beef loins (Kim, Kemp, & Samuelsson, 2016). Additionally, the cooking loss value we obtained for DAB is considerably lower than that of beef dry-aged for 4 weeks with *M. flavus* (28.3%) (Hanagasaki & Asato, 2018). These discrepancies indicate that cooking loss is affected by factors other than the aging method and species of Mucoraceae.

Meat tenderness is an important characteristic that determines consumer acceptability and

preference for meat. A previous report showed a correlation between meat tenderness measured by WBSFs and consumer sensory ratings (Destefanis, Brugiapaglia, Barge, & Dal Molin, 2008). Thus, we evaluated the tenderness of the WAB and DAB based on shear force measurements and found values of 2.41 ± 0.31 and 2.06 ± 0.25 kg, respectively (Table 4), which were not significantly different. The Mucoraceae fungus *Thamnidium* is thought to release proteases that tenderize aging meat (PrimeSafe, Agency of the Government of the State of Victoria, Australia. Aging of Beef. PN:PO5:201709 https://www.primesafe.vic.gov.au/standards-and-guidelines/primenotes/ageing-of-beef/). However, our results, along with those of Hanagasaki (2018), suggest that beef achieves a level of tenderness with or without Mucoraceae. Furthermore, most consumers perceive beef steak with WBSF values <3.36 kg as "tender" and "very tender" (Destefanis et al., 2008). Therefore, our results suggest that 35 days of dry or wet aging produces meat tender enough to render any effect of Mucoraceae undetectable.

3.6 Free amino acids

FAA levels in meat increase via proteolysis through both endogenous and microbial enzymes during the aging process and are key components contributing to taste and flavor (Zhao, Schieber, & Gänzle, 2016). Among FAAs, glutamic acid is especially responsible for the "umami" taste in meat (Zhao, Schieber, & Gänzle, 2016). Unexpectedly, the glutamic acid content of the DAB was significantly lower than that of the WAB after 35 days of aging (Table 5). Moreover, the total amino acid content was also significantly lower in the DAB than in the WAB (Table 5). Although dry aging with molds and yeast should accelerate FAA production, this was not observed after 35 days of dry aging. This result agrees with that of Oh et al. (2019), who previously showed that 28 days of dry-aging sirloin with *P. anomala* (Mucoraceae) or *D. hansenii* resulted in significantly lower glutamic acid and total FAA levels than those in WAB, the noninoculated control. However, this report also showed that

the total amino acid content in DAB with *P. anomala* peaked at day 21 and decreased on day 28, while the corresponding content in WAB still gradually increased on day 28 (Oh et al., 2019). Therefore, it is possible that the FAA content of our DAB may have peaked before 35 days.

3.7 Fatty acid composition

Regardless of the aging method, the major fatty acids were C16:0 (palmitic acid), C18:0 (stearic acid), and C18:1 (oleic acid) after 35 days of aging (Table 6), which is consistent with previous reports on chill-aged rumps (Bermingham et al., 2018) and dry-aged sirloin with the Mucoraceae fungus *P. anomala* (Oh, Lee, Lee, Jo, & Yoon, 2019). Additionally, the fatty acid composition of the WAB and DAB did not differ significantly (Table 6). Therefore, the aging method and the presence of *M. flavus* and *H. pulchrum* did not affect the composition of predominant fatty acids. Among the minor fatty acids, we observed C15:0 (pentadecanoic acid) and C17:0 (margaric acid), which are unique to ruminant meat fat (Wu & Palmquist, 1991). The level of C17:0 in the DAB was significantly higher than that in the WAB (Table 6), and previous reports indicate that some microorganisms, including molds and yeast, produce C17:0 from carbon sources such as amino acids, sugars, and organic acids via propionyl-CoA (Řezanka, Kolouchová, & Sigler, 2015; Bhatia et al., 2019; Zhang, Liang, Zong, Yang, & Lou, 2020). The higher levels of molds, yeast, and bacteria in the crust and inner part of the DAB may explain the higher level of C17:0 in the DAB than in the WAB. Additionally, we detected lower levels of C15:1 (*cis*-pentadecenoic acid) and C18:2 (linoleic acid) in the DAB; however, these levels did not differ significantly from those in the WAB (Table 6).

3.8 Volatile aroma compounds

HS-SPME and GC-MS identified 25 volatile aroma compounds in the WAB and DAB

after 35 days of aging, which included 4 acids, 7 alcohols, 9 aldehydes, 4 ketones, and 1 pyridine. The identities of the volatile compounds and the peak areas of their signals are presented in Figure 4. The predominant volatile compound was hexanal, which occurred at similar levels in both the WAB and DAB (Figure 4). The WAB produced more acids (acetic, butanoic, and pentanoic acids), which are responsible for the sour taste of WAB. Acetic and pentanoic acids were not detected in the DAB (Figure 4). Casaburi et al. (2015) previously reported that acetic and butanoic acids are produced in spoiling meat by lactic acid bacteria, which can be related to the higher level of lactic acid bacteria in WAB (Table 1) and the suppression of these acids in DAB. Among the alcohols, six compounds differed between the WAB and DAB. The levels of 1-butanol and 3-methyl-1-butanol in the DAB were significantly lower than those in the WAB, whereas those of 1-pentanol, 1-hexanol, 1-heptanol, and 1-octen-3-ol in the DAB were significantly higher than those in the WAB (Figure 4). Some of the differences in the levels of these alcohols in the WAB and DAB are remarkable and suggest that these volatile compounds may characterize the DAB manufactured under our conditions. According to a previous report, some volatile alcohols are produced by fungi via fatty acids (Schnürer, Olsson, & Börjesson, 1999). In particular, 1-octen-3-ol (mushroom-like aroma) is formed by the oxygenation of C18:2 linoleic acid and cleavage of its hydroperoxide (Matsui, Sasahara, Akakabe, & Kajiwara, 2003) and has been found in some molds, such as *Penicillium* (Larsen & Frisvad, 1995). The lower levels of linoleic acid in DAB (Table 6) may mean that this fatty acid had been used for 1-octen-3-ol production by a fungus growing on DAB. Altogether, our results suggest that dry aging significantly enhances the levels of 1-hexanol and 3-methylbutanal (nutty flavor), 1-octen-3-ol (mushroom-like or earthy flavor), and 3-hydroxy-2-butanone (buttery flavor). These flavors have often been used to describe DAB: beefy, buttery, nutty, and earthy (Dashdorj, Tripathi, Cho, Kim, & Hwang, 2016). Among the compounds detected in the current study, 1-heptanol, 3-methylbutanal, 2-butanone, and pyridine were found only in the DAB. 3-Methylbutanal is

described as nutty, and 2-butanone is described as sweet and fruity (Figure 4). Previous reports describe *M. flavus* as releasing a nutlike odor (Hanagasaki & Asato, 2018), while *H. pulchrum* produces a sweet, pleasant, and somewhat apple odor (Hesseltine & Anderson, 1957). Therefore, the favorable odor associated with DAB is derived from volatile compounds detected in this study, and *M. flavus* and *H. pulchrum* might contribute to their production.

4. Conclusion

In this study, we showed the distribution of microorganisms and characteristics of meat produced by the dry aging of Holstein steer rump for 35 days in Hokkaido, Japan. We identified the furry molds that predominate on the crust of DAB as *M. flavus* and *H. pulchrum*. To our knowledge, this is the first time the combination of *M. flavus* and *H. pulchrum* has been reported on DAB. We evaluated the effects of the molds on the meat characteristics and found that they played no role in differentiating the water holding capacity, tenderness, and FAA content of DAB from those of WAB. However, volatile compounds responsible for mushroom-like, nutty, sweet, and fruity flavors are enhanced in DAB. These results suggest that *M. flavus* and *H. pulchrum* may contribute to the unique aromas and flavors used to describe DAB. However, further research is needed to determine the safety and effects of *M. flavus* and *H. pulchrum* on DAB.

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Declaration of interest

None.

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Tables

Table 1

Bacterial abundance of beef rump wet- and dry-aged for 35 days.

Bacterial abundance	Crust (log_{10} CFU/cm^2)	Inner part (log_{10} CFU/g)	
	Dry	Wet	Dry
Total bacteria	4.64 ± 0.75	4.42 ± 0.15	5.32 ± 0.52 *
Lactic acid bacteria	<30 †	4.08 ± 0.20	2.03 ± 0.45 **
Staphylococci	3.16 ± 0.50	ND	2.20 ± 0.45
Coliform	3.10 ± 1.63	3.64 ± <0.01	1.97 ± 0.97 *

Values are expressed as the mean ± standard deviation of log_{10} CFU/cm^2 in the crust of dry-aged beef (n=4) and log_{10} CFU/g in the inner part of dry- and wet-aged beef (n = 3). *Escherichia coli* was not detected among the coliforms isolated from either the crust or inner parts (wet- and dry-aged). †The abundance was less than 30 CFU/cm^2 even in an undiluted solution. ND, not detected.

**p<0.01 vs. inner part of wet-aged beef; * p<0.05 vs. inner part of wet-aged beef

Table 2

Proximate composition and calorie contents of beef rump wet- and dry-aged for 35 days.

Proximate composition	Wet			Dry			*p* value
Moisture (%)	71.9	±	0.2	69.6	±	0.2	<0.001
Crude protein (%)	19.7	±	0.2	19.6	±	0.2	0.890
Crude fat (%)	5.10	±	0.7	7.55	±	1.1	0.029
Ash (%)	1.07	±	0.01	1.08	±	0.02	0.545
Carbohydrate (%)	2.23	±	0.50	2.13	±	0.44	0.800
Calorie (kcal/100 g)	134	±	4	155	±	6	0.007

Values are expressed as mean ± standard deviation (n = 3).

Table 3

Instrumental color of beef rump wet- and dry-aged for 35 days.

	Wet			Dry			*p* value
CIE L*	29.6	±	0.8	31.6	±	0.0	0.0134
CIE a*	11.6	±	1.0	11.9	±	0.5	0.6377
CIE b*	11.9	±	2.5	9.9	±	0.4	0.2536
Chroma	16.6	±	2.4	15.5	±	0.7	0.4813
Hue angle	45.4	±	3.9	39.9	±	0.1	0.0705

CIE L*, lightness; CIE a*, redness, CIE b*, yellowness; chroma, vividness; hue angle, tone of color.

Values are expressed as mean ± standard deviation (n = 3).

Table 4

pH and instrumental meat quality of beef rump wet- and dry-aged for 35 days.

	Wet			Dry			*p* value
pH	5.56	±	0.01	5.62	±	0.01	0.012
Expressible drip loss (%)	41.0	±	2.7	41.2	±	3.6	0.949
Cooking loss (%)	14.9	±	1.2	17.1	±	0.6	0.049
Shear force (kg)	2.41	±	0.31	2.06	±	0.25	0.205

Values are expressed as mean ± standard deviation (n = 3).

Table 5

Free amino contents in beef rump wet- and dry-aged for 35 days.

Free amino acid (mg/100 g)	Wet		Dry		*p* value
Glu	103.55	± 0.34	92.62	± 0.77	<0.0001
Total	934.95	± 3.07	830.98	± 5.27	<0.0001

Glu, glutamic acid; total free amino acid, total of threonine, serine, glycine, alanine, proline, glutamine, lysine, valine, methionine, isoleucine, leucine, phenylalanine, arginine, histidine, aspartic acid, glutamic acid, cysteine, and tyrosine.

Values are expressed as mean ± standard deviation (n = 3).

Table 6

Fatty acid compositions of beef rump wet- and dry-aged for 35 days.

	Fatty acid (%)	Wet			Dry			*p* value
C14:0	Myristic acid	3.65	±	0.47	3.59	±	0.98	0.927
C14:1	Myristoleic acid	1.33	±	0.25	1.12	±	0.26	0.365
C15:0	Pentadecanoic acid	0.71	±	0.24	0.61	±	0.08	0.520
C15:1	*cis*-10-Pentadecanoic acid	0.96	±	0.13	0.75	±	0.06	0.056
C16:0	Palmitic acid	26.13	±	1.05	27.32	±	2.32	0.465
C16:1	Palmitoleic acid	4.96	±	0.33	4.43	±	0.67	0.284
C17:0	Margaric acid	1.26	±	0.05	1.45	±	0.10	0.041
C17:1	*cis*-10-Heptadecenoic acid	1.84	±	0.22	1.47	±	0.23	0.114
C18:0	Stearic acid	8.05	±	1.09	8.50	±	0.87	0.607
C18:1	Elaidic acid	4.09	±	0.42	4.64	±	0.35	0.156
C18:1	Oleic acid	37.49	±	2.38	38.72	±	3.10	0.612
C18:2	Linoleic acid	3.93	±	0.16	3.18	±	0.54	0.081
	Others	5.58	±	2.14	4.22	±	0.48	0.344

Values are expressed as mean ± standard deviation (n = 3).

Figure legends

Figure 1

Visual appearance of rump beef blocks (A) wet-aged (vacuum-packed in plastic bag) and (B) dry-aged (naked) for 35 days in an aging room of a meat company in Japan.

Figure 2

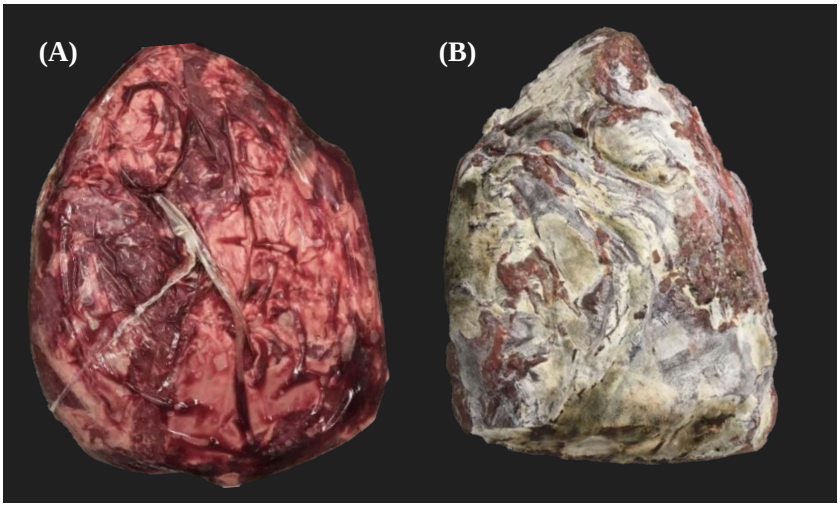
Morphological characteristics of *Mucor flavus* and *Helicostylum pulchrum* isolates. Colony appearance (A) and microscopic images (B and C) of *M. flavus*. Colony appearance (D) and microscopic images (E and F) of *H. pulchrum*. The arrows and arrowhead indicate sporangia and branchlets that have globose sporangiola with spinelike terminations. The scale bars are 1 mm in B and E. The scale bars are 500 μm in C and F.

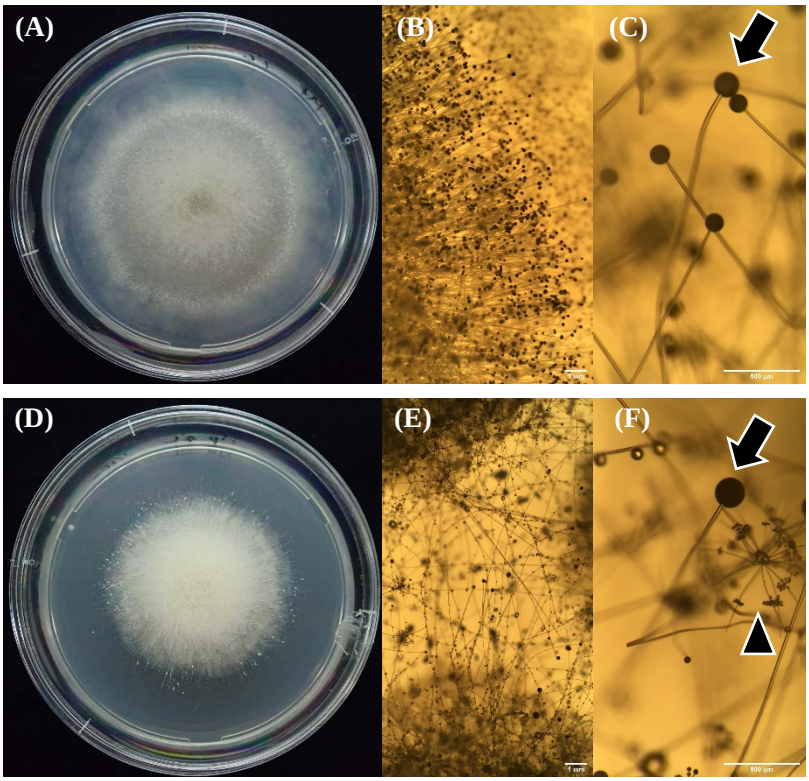
Figure 3

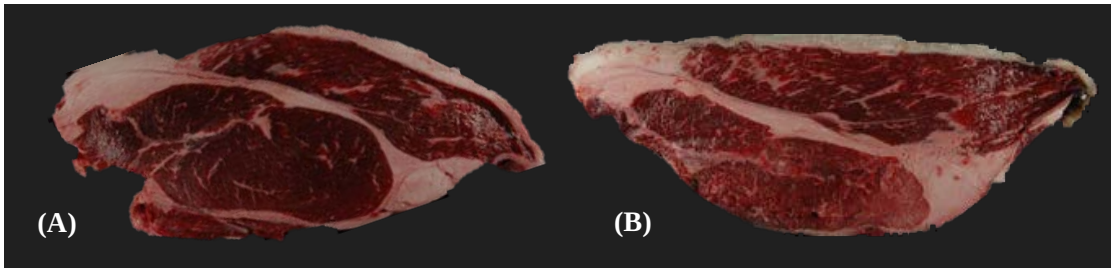
The surface appearance of 1-inch-thick steak cut from beef aged for 35 days. (A) wet-aged beef and (B) dry-aged beef. These images were taken after 1 h of blooming at 4 °C.

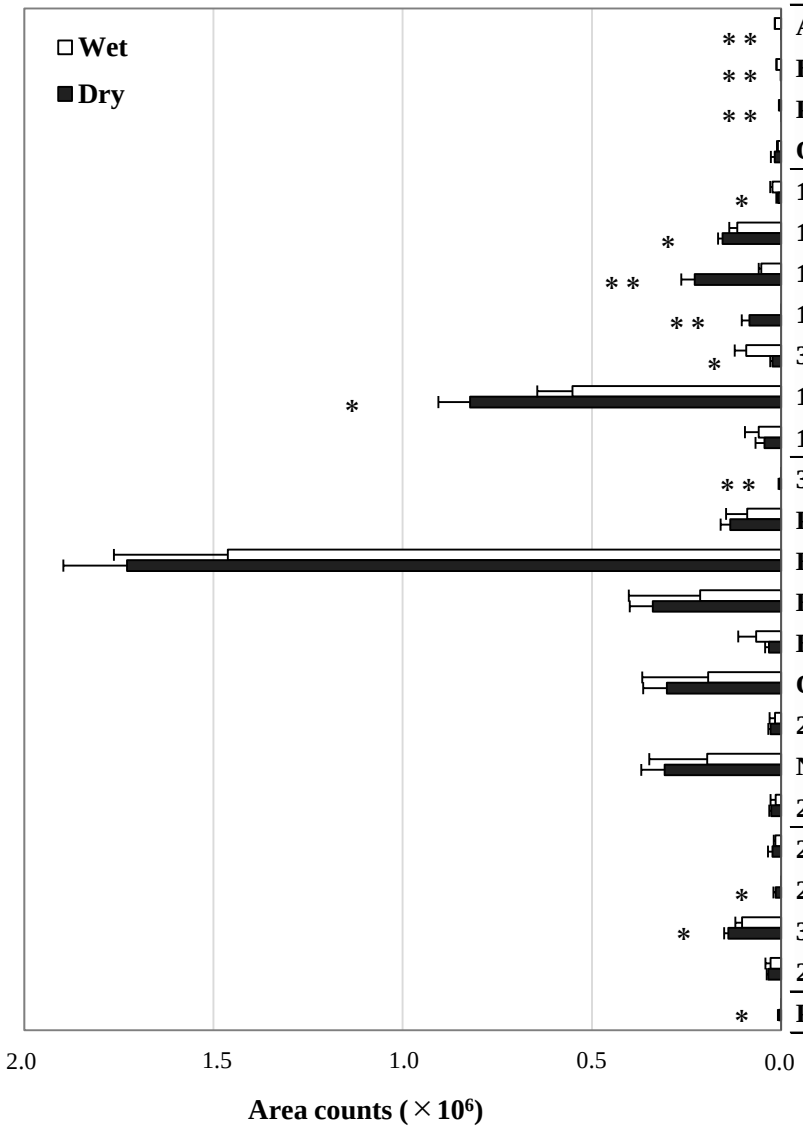
Figure 4

Volatile aroma compounds extracted by SPME in beef wet- and dry-aged for 35 days. Data are expressed as peak area count of compounds in WAB and DAB. Aroma descriptions were obtained from the following publications: [a] Frauendorfer & Schieberle, 2008; [b] Majcher et al., 2019; [c] Yu et al., 2019; [d] Liang et al., 2016; [e] Wrona et al., 2017; [f] Niu et al., 2011; [g] Migita et al., 2017; [h] Migita et al., 2017; [i] Feng et al., 2019; [j] Bi et al., 2020; [k] Liu et al., 2019; [l] Vera et al., 2020; [m] Yang et al., 2008; and [n] Fukami et al., 2002.


(A)
(B)


(A)
(B)
(C)
(D)
(E)
(F)

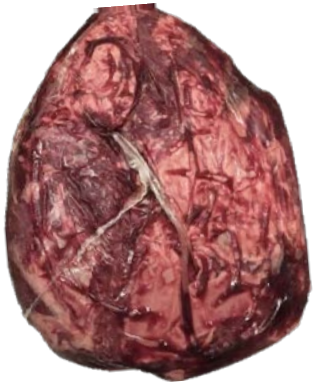

(A)
(B)



Compound	Chemical group	Aroma description
Acetic acid	Acids	sour, pungent, vinegar [a,b,c]
Butanoic acid		rancid, cheesy [d]
Pentanoic acid		sour, cheesy [d]
Octanoic acid		sweet, chessy [e]
1-Butanol	Alcohols	spicy [f]
1-Pentanol		green tea, crop-like [g]
1-Hexanol		nutty, popcorn-like [g]
1-Heptanol		woody, oily, corn-like [g,h]
3-Methyl-1-butanol		malty, fruity [a,b]
1-Octen-3-ol		mushroom-like [a]
1-Octanol		waxy, green, citrus [i]
3-Methylbutanal	Aldehydes	nutty, malty, cocoa-like, light apple [a,c,i]
Pentanal		vegetable, green [i]
Hexanal		grass-like, green, fruity, sweaty [i,j]
Heptanal		fatty, green [k]
Benzaldehyde		almond, burnt sugar [c]
Octanal		fatty, soapy [l]
2-Octenal		fatty, green [j]
Nonanal		fatty, soapy, citrus, green [j,l]
2-Nonenal		green, cucumber, fatty [i]
2,3-Butanedione	Ketones	sweet, caramel, buttery [b,f]
2-Butanone		sweet, fruity [d]
3-Hydroxy-2-butanone		buttery [d]
2-Heptanone		fruity, sweet [m]
Pyridine	Pyridines	rancid [n]

Beef rump blocks were aged for 35 days in same aging room (2.9 ℃ and 90% RH).

Wet-aged beef (WAB)



Vacuum-packaged

Dry-aged beef (DAB)

Exposed to air flow

Identification of fungal species on DAB crust

Mucor flavus **(KT1a)**

Helicostylum pulchrum **(KT1b)**

& three *Penicillium* spp.

Meat characteristics of DAB

- Lactic acid bacteria and coliforms were suppressed in its inner part.
- Tenderness and FAA contents of DAB and WAB did not differ.
- Mushroom-like, nutty, sweet, and fruity flavors were enhanced.