Identification of Microorganisms Associated with the Quality Improvement of Dry-Aged Beef Through Microbiome Analysis and DNA Sequencing, and Evaluation of Their Effects on Beef Quality

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Abstract: The objective of this study was to isolate and identify the microorganisms, especially yeasts and molds, related to the improvement of beef quality during dry-aging of beef through microbiome analysis, and to examine the possibility of using them as starter culture strains to improve the efficiency of dry-aging beef production. Beef sirloins were dry-aged for 28 days using different wind speeds (0, 2.5, and 5 m/s) at 1 to 3 °C and 75% relative humidity, and microbial compositions were confirmed by microbiome analysis. Mold and yeast samples were plated on potato dextrose agar supplemented with 10% tartaric acid, and the isolated colonies were identified by DNA sequencing. The isolates were subjected to microbial characterization (morphological characterization, growth condition, and enzyme activity). Microbiome analysis showed that the dominant microorganisms were molds and yeasts identified as *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707. *Pilaira anomala* SMFM201611 and *D. hansenii* SMFM201707 were inoculated into 24 sirloins of the lowest grade. All samples were dry-aged for 0, 14, 21, and 28 days and analyzed for microbial growth, pH, shear force, ultrastructure, and flavor compounds (free amino acids and free fatty acids). Inoculation with *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 improved tenderness and cause the breakdown of myofibrils by proteolysis. Both microorganisms also produced free amino acids and fatty acids through proteolytic and lipolytic activities. These results indicate that *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 isolated and identified from dry-aged beef can improve the quality of low-grade beef during dry-aging.

Keywords: beef microflora, beef quality, debaryomyces hansenii, dry-aging beef, *Pilaira anomala*

Practical Application: During dry-aging, mold and yeast improve the quality of dry-aged beef. *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707 isolated from dry-aged beef can improve tenderness by breaking down myofibrils. Both microorganisms improve flavor by producing free fatty acids and amino acids, and the taste and aroma characteristics of low-grade beef may be improved during the dry-aging process.

Introduction

Most consumers prefer high-grade beef, which has good tenderness, and low-grade beef and tough cuts of beef are not preferred. Aging improves the quality of both high- and low-grade beef (Kerry, 2009; Novakofski & Brewer, 2006; Ryu et al., 2018). In particular, dry-aging improved the quality of low-grade beef, because this method can impart unique flavors and improved meat tenderness (Lee, Lee, Min, Jo, & Jung, 2015; Warren et al., 1992). In addition, consumers prefer dry-aged to wet-aged beef because of the former has a deeper flavor. This has led to a rapid growth of the dry-aging industry despite a lack of understanding of the process. Thus, the beef dry-aging process needs to be elucidated.

Previous studies showed that the factors affecting dry-aging are drying temperature, relative humidity, air, and duration (Perry, 2012; Šulcerová, Sýkora, Nedomová, & Mihok, 2017). However, microorganisms grow on the beef surface during aging, even at low temperatures. Hence, microorganisms may also play an important role in dry-aging. Although there have been limited studies on the effect of microorganisms on beef dry-aging, we can speculate that microorganisms may affect dry-aged beef quality based on various studies conducted on fermented meat products (Bolumar et al., 2006; Hugas & Monfort, 1997; Sørensen & Jakobsen, 1996). In addition, Dashdorj et al. (2016) showed that *Thamnidium elegans* can enhance meat flavor and taste.

During dry-aging, various microorganisms, especially yeasts and molds, can grow on the beef surface (Ryu et al., 2018; Tapp, 2016). The role of yeasts and molds in flavor development is well known, and is associated with their proteolytic and/or lipolytic activities (Lee et al., 2019). Microorganisms such as *Mucor*, *Aspergillus*, and *Penicillium* may vary with geolocations and are affected by seasons.

Hence, beef quality may be inconsistent. Moreover, pathogenic microorganisms may also grow on beef.

The objective of this study was to isolate and identify the microorganisms related to the improvement of beef quality during dry-aging through microbiome analysis and examine the possibility of using them as starter culture strains to affect the taste and aroma of dry-aged beef.

Materials and Methods

Microbiome analysis

To investigate the microbial compositions in dry-aged beef, dry-aging was carried out with 18 beef top sirloins at different wind speeds (0, 2.5, and 5 m/s) at 1 to 3 °C and 75% relative humidity. Wet aging was also conducted for the control group at the same conditions. To analyze the microbiome, DNA was extracted using the PowerSoil® DNA Isolation Kit (Cat. No. 12888, MO BIO, Hilden, NW, Germany). The ITS region of the extracted DNA was amplified using the primers ITS3 (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA TGC-3′). The final products were normalized and pooled using the PicoGreen kit (Thermo Fisher Scientific, Waltham, MA, USA), and the size of the libraries was verified using the LabChip GX HT DNA High Sensitivity kit (PerkinElmer, Waltham, MA, USA). They were sequenced using the MiSeq™ platform (Illumina, San Diego, CA, USA). Sequence data were quality-trimmed and aligned using the CD-HIT-EST-based operational taxonomic units (OTU) analysis program (Li, Fu, Niu, Wu, & Wooley, 2012) to remove low-quality, ambiguous, and chimera sequences. Clustering between sequences with over 97% similarity was performed to form species-level OTUs. A taxonomic assignment was performed on the representative sequences of each OTU with the organism information of the subject with the highest similarity by carrying out UCLUST on the reference DB (UNITE 6.0 2014-07-04). Using the OTU information, various microbial community comparisons were performed. Shannon's and Simpson's indexes were obtained to confirm species diversity and microbial community homogeneity in the dry-aged samples. Alpha diversity information was confirmed by the Chao1 value.

Isolation and identification of microorganisms

To isolate the dominant molds and yeasts observed in the microbiome analysis, dry-aging was conducted at 1 to 3 °C, 75% relative humidity, and 2.5 m/s wind speed for 28 days. The sample (25 g) was then placed in a sample bag (3M™; Maplewood, MN, USA) containing 50 mL 1% peptone water (PW; Becton Dickinson and Company, Sparks, MD, USA), followed by blending the dry-aged beef samples in a stomacher (BagMixer®; Interscience, St. Nom, Yvelines, France) for 1 min. The homogenate (1 mL) was serially diluted with 9 mL PW, 100 μL of the diluent was spread-plated on potato dextrose agar (PDA; Becton Dickinson and Company), and the plates were incubated at 20 °C for 7 days. The morphologically distinct mold and yeast colonies were transferred to different PDA plates using a sterile loop and further incubated at 20 °C for 4 days, and then stored at 4 °C before identification. The genomic DNA of the mold and yeast colonies on the PDA plates was extracted using the Chelex® 100 Molecular Biology Grade Resin (BIO-RAD, Hercules, CA, USA). The primers NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and NS8 (5′-TCC GCA GGT TCA CCT ACG GA-3′)

were used for polymerase chain reaction (PCR; DNA Engine Tetrad 2 Peltier Thermal Cycler; Bio-Rad). PCR amplification was conducted in a final volume of 30 μL containing *EF-Taq* polymerase (Solgent, Daejeon, Korea) as follows: initial stage of 95 °C for 2 min, 35 cycles with denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. A final 10-min extension was performed at 72 °C. The PCR products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA), and the sequences were analyzed using an ABI PRISM 3730XL Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with the PRISM BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems).

Microbial characterization

Morphological characterization. To investigate the morphological characterization of *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707, the isolates were incubated at 20 °C for 4 days. A small piece of *P. anomala* SMFM201611 mycelium was cut from the edge of the colony with a sterile scalpel and placed onto a slide. One drop of PW was added to the piece of mold mycelium, and a cover slip was placed at a 45° angle over the piece of mycelium and water to minimize air bubbles. The mycelium was dispersed using a sterile spreader (SPL, Lifesciences Co. Ltd, Gyeonggi, Korea) for observation under a light microscope (IX71 DVSF, Olympus, Shinjuku, Tokyo, Japan) at 400× magnification. *Debaryomyces hansenii* SMFM201707 culture in potato dextrose broth (PDB; Becton Dickinson and Company) (10 μL) was transferred onto a slide and covered with a cover slip for microscopic observation. To obtain spore and colony microstructures for observation with a scanning electron microscope (SEM), *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 colonies were grown on slide slip surfaces placed in PDB at 20 °C for 4 days. Slide slips with colonies were fixed in 1.8% glutaraldehyde solution (Sigma–Aldrich, St. Louis, MO, USA) for 30 min and post-fixed in 2% osmium tetroxide (Sigma–Aldrich) for 20 min at 25 °C. The samples were dehydrated using 25%, 50%, 75%, 90%, and 100% ethanol sequentially (5 min in each stage) at 25 °C. After treatment with 100% ethanol, the samples were treated with 40 μL hexamethyldisilazane (Sigma–Aldrich). After drying, the isolated samples were platinum coated using a 108 auto sputter coater (Cressington Scientific Instruments Ltd., Watford, England) and observed under field emission SEM (JSM-7600F; JEOL Ltd., Tokyo, Japan). SEM was also used to study the *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 morphologies at 3,000× magnification.

Growth conditions. To investigate the appropriate growth temperature, 150 μL each of *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 supernatants was inoculated in the center of PDA plates, and the plates were incubated at 4, 7, 10, 15, 20, 25, 30, and 37 °C for 4 days. To investigate the optimal pH range for growth, 150 μL each of *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 supernatants was inoculated in the center of PDA plates adjusted to pH levels 2.2, 3.9, 5.1, 6.9, and 10.1, and the plates were incubated at 20 °C for 4 days. After incubation, the zone of microbial growth was measured.

Extracellular enzyme activity. The extracellular protein and lipid degrading activities of *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 isolates were examined according to the procedures described by Ramos, Branquinha, and Santos (2017) and Atanassova et al. (2016). The colonies of *D. hansenii* SMFM201707 and *P. anomala* SMFM201611 were inoculated in 10 mL PD Band incubated at 20 °C for 4 days. Next, 100 μL

culture was inoculated into 10 mL PDB and incubated again at 20 °C for 4 days. The subcultures were centrifuged at 1,912 × g at 4 °C for 15 min, and the supernatants were collected. For the protease activity assay, PDA was supplemented with heated 1%, 7%, or 10% skim milk (Becton Dickinson and Company) at 110 °C for 15 min. Then, 10 mL media was poured into plates and solidified at 25 °C, and holes of 1 cm diameter were punched into the agar. Aliquots (150 μL) of the supernatants were inoculated in the holes and incubated at 20 °C for 4 days. The mold and yeast growth and the zone of hydrolysis around the hole were observed. For the lipase activity assay, PDA was supplemented with 1%, 7%, or 10% tributyrin. Next, 10 mL media was poured in plates and solidified at 25 °C, and holes of 1 cm diameter were punched into the agar. Aliquots (150 μL) of the supernatants were inoculated in the holes punched in the tributyrin medium and incubated at 20 °C for 4 days. The zone of hydrolysis around the hole indicated lipase production.

Inoculum preparation for dry-aging

Pilaira anomala SMFM201611 isolated from dry-aged beef was grown in PDA at 20 °C for 4 days. Phosphate-buffered saline (PBS; 5 mL, pH 7.4; KH_2PO_4 0.2 g, Na_2HPO_4 1.5 g, NaCl 8.0 g, KCl 0.2 g, and distilled water 1 L) was added to PDA, and the mold spores and colonies were scraped with a sterile spreader to obtain microbial cell suspensions. The cell suspensions were left aside for a while to allow the solids to precipitate, and the upper homogeneous suspensions were used as inoculum. The suspensions were diluted with PBS to obtain a concentration of 6 Log CFU/mL. A colony of *D. hansenii* SMFM201707 was inoculated into 10 mL PDB and incubated at 20 °C for 4 days. Then, 100 μL culture was inoculated into 10 mL PDB and incubated at 20 °C for 4 days. The subculture was centrifuged at 1,912 × g at 4 °C for 15 min. The pellet was washed twice with PBS and resuspended in PBS to obtain a concentration of 6 Log CFU/mL. Both the suspensions were used as inoculum.

Inoculation and dry-aging process

A total of 24 beef sirloins (*longissimus lumborum*) from different carcasses (Holstein steer, quality grade 3, initial pH 5.54 ± 0.01) were obtained and transferred to a meat processing plant (Seoul, Korea). Each side of sirloin of the lowest grade was irradiated with UV for 15 min in a laminar flow cabinet to remove the indigenous microflora. The inoculum was spread evenly on the surface of the beef samples by spraying with a sprayer in a laminar flow cabinet, and the control group was inoculated with the same volume of PBS. The beef samples were left for 15 min at 25 °C to allow cell attachment in the laminar flow cabinet, and the samples were aged at 0 to 4 °C and 75% relative humidity for 0, 14, 21, and 28 days.

Microbial analysis

The sample (25 g) of dry-aged beef was aseptically removed with a sterile knife, transferred into a sample bag containing 50 mL PW, and pummeled for 1 min; the homogenates were then serially diluted with 9 mL PW. Next, 100 μL diluent was spread-plated on PDA and incubated at 20 °C for 4 days; the colonies were counted manually.

Physicochemical analysis

pH. Dry-aged beef samples (1 g) were placed in filter bags (3M, St. Paul, MN, USA). Deionized distilled water (9 mL) was added to the filter bags, and the samples were homogenized for 30 s (T10 basic; Ika Works, Staufen, Germany). The suspensions were centrifuged at 2,265 × g for 10 min and filtered to obtain the supernatants. The pH of the dry-aged beef samples was measured using a pH meter (SevenGo; Mettler-Toledo Inc., Schwerzenbach, Switzerland). Before the measurement, the pH meter was calibrated with standard buffers (Mettler-Toledo Intl., Inc., Schwerzenbach, Switzerland).

Shear force. Dry-aged beef samples were vacuum-packaged (HFV-600L; Hankook Fujee Co., Ltd., Gyeonggi Do, Korea) and heated in a water bath until the core temperature reached 72 °C. The cooked samples were cut parallel to the muscle fiber into rounded cores (1.67-cm diameter) and placed under a Warner–Bratzler shear probe, perpendicular to the muscle fiber. The shear force (N) was measured using a texture analyzer (CT3 10K; Lloyd Instruments Ltd., Fareham, UK) with a cell load of 0.1 N and 200 mm/min cross-head speed.

Transmission electron microscopy (TEM). For transmission electron microscopy (TEM) analysis (LIBRA® 120; Carl Zeiss, Oberkochen, Germany), $1 × 4$ cm^2 specimens were collected from the dry-aged samples and fixed in Karnovsky's fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer [SCB]), washed three times with 0.05 M SCB, and fixed in 1% osmium tetroxide in 0.05 M SCB at 4 °C for 2 hr. The fixed specimens were washed with deionized distilled water and stained with 0.5% uranyl acetate at 4 °C for 30 min. After staining, the specimens were dehydrated with an increasing concentration of ethanol, followed by transition and infiltration with propylene oxide and/or Spurr resin. The specimens were polymerized in the Spurr resin for 24 hr and sectioned (MT-X, RMC, Tucson, AZ, USA) prior to TEM analysis. The ultrastructures of dry-aged beef inoculated with *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 were observed at 80 kV and 20,000× magnification.

Flavor compounds

Free fatty acids. Lipids were extracted from dry-aged beef samples according to the method described by Folch, Lees, and Sloane Stanley (1957). The extracted lipid (0.1 g) was weighed in a 15-mL test tube with 1 mL chloroform and internal standard (1 mg triundecanoate in 1 mL iso-octane; Supelco 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). Triglycerides were removed from the samples, and the free fatty acids (FFAs) extracted using the solid phase extraction kit (Supelclean™ LC-NH₂ SPE Tubes; Supelco). The extract was evaporated using nitrogen gas and heated at 85 °C for 10 min. For methylation, 2 mL 14% BF_3-methanol was added into the sample and heated at 85 °C for 10 min. After cooling, 2 mL iso-octane and 1 mL saturated sodium chloride were added to the samples and centrifuged at 1,573 × g for 3 min (Continent 512R; Hanil Co., Ltd., Incheon, Korea). The upper layer containing the fatty acid methyl ester (FAME) was dehydrated with anhydrous sodium sulfate, transferred to a vial, and analyzed using a gas chromatograph (HP 7890; Agilent Technologies, Santa Clara, CA, USA) with a split ratio (10:1). A capillary column (DB-23; 60 m × 250 μm × 0.25 μm, Agilent Technologies) was used. The injector and detector temperatures were maintained at 250 and 280 °C, respectively. The column oven temperatures were as follows: 50 °C for 1 min, increased to 130 °C at 25 °C/min, 170 °C at 8 °C/min, and held at 215 °C at 1.5 °C/min. Nitrogen was used as a carrier gas at a linear flow of 4 mL/min. Individual FAMEs were identified by comparing the relative retention times of the sample peaks with those of the external standards (37 FAME mix and CLA mix, Supelco)

Table 1–Values of richness and diversity of the dry-aged beef according to different air flow velocities.

Treatment	Total number of OTUs	Chao1 richness estimator	Shannon diversity index	Simpson diversity index
Dry 0 m/s	4.5 ± 0.7	4.5 ± 0.7	0.6 ± 0.2	0.23 ± 0.1
Dry 2.5 m/s	8.5 ± 0.7	10.0 ± 1.4	1.2 ± 0.8	0.44 ± 0.3
Dry 5 m/s	7.0 ± 0.0	7.00 ± 0.0	1.0 ± 0.1	0.31 ± 0.1
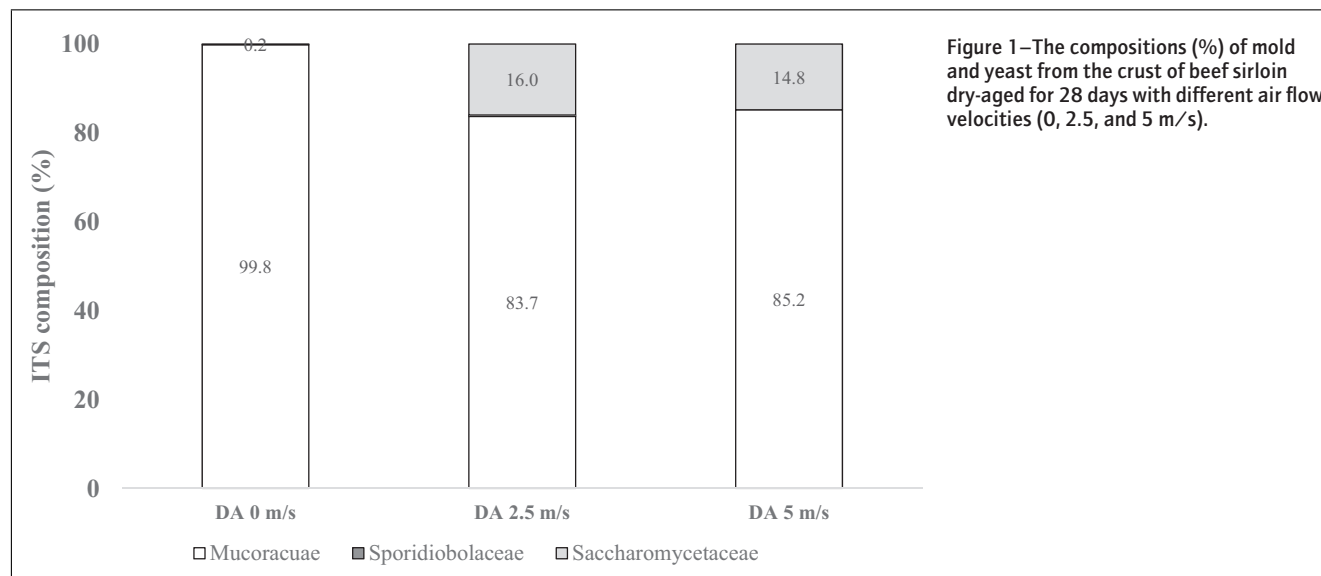
Figure 1–The compositions (%) of mold and yeast from the crust of beef sirloin dry-aged for 28 days with different air flow velocities (0, 2.5, and 5 m/s).

calculated according to the AOAC method (Satchithanandam, Fritsche, & Rader, 2001).

Free amino acids. The samples for the free amino acid (FAA) identification were prepared using the method described by Schwarz, Roberts, and Pasquali (2005) and injected into the high-performance liquid chromatography system (HPLC; Ultimate 3000; Thermo Fisher Scientific Inc.) with precolumn derivatization. The analytical conditions were as follows: mobile phase, 40 mM sodium phosphate dibasic buffer (pH 7.8) and distilled water (DW)/acetonitrile/methanol (10:45:45, v/v/v); flow rate and time, 1.5 mL/min for 35 min; column, VDSpher 100 C18-E (4.6 × 150 mm^2, 3.5 µm particles, VDS optilab Chromatographie Technik GmbH, Berlin, Germany); and detector, UV/Vis detector at 266 and 340 nm. The standard (Agilent Technologies) was used to generate a standard curve for calculating the peak area.

Statistical analysis

All data were analyzed using the general linear model procedure of SAS® (version 9.2; SAS Inst. Inc., Cary, NC, USA). Significant differences in least square means were compared using a pairwise t-test at $\alpha = 0.05$.

Results and Discussion

Microbial composition of dry-aged beef

A total of 376,008 reads were obtained from dry-aged beef samples after filtering the initial reads and carrying out quality trimming. The library size of each sample was normalized to the smallest number of sequences from the dry-aged beef samples (86,112 sequences) to minimize bias due to the difference in the sample sequence number. The number of OTUs observed at a 97% taxonomic cutoff ranged from 4.5 to 8.5, depending on air flow. Moreover, Shannon's and Simpson's diversity indices

and Chao1 richness estimators revealed that the presence of air flow increases microbial diversity and richness in dry-aged beef (Table 1). The result also showed that 2.5 m/s of air flow showed more microbial diversity than those dry-aged at 0 and 5 m/s of air flow because 2.5 m/s of air flow was appropriate to spread out mold and yeast and provide oxygen to them. After dry-aging for 28 days, microbiome analysis results showed that molds and yeasts were mostly predominant in dry-aged beef. The molds belonged to the Mucoraceae family, and the yeast was identified as *Debaryomyces* spp. of Saccharomycetaceae. The prevalence of Mucoraceae decreased in the presence of air flow, but the prevalence of *Debaryomyces* spp. (14.8% to 16.0%) dramatically increased with air flow speed. This result shows that the ratio of yeasts increased in the microbial composition of beef dry-aged at 2.5 and 5 m/s air flow. *Candida* spp. (0.1%) was found in beef dry-aged at 2.5 and 5 m/s air flow. *Rhodotorula* (0.3%) was observed only in beef dry-aged at 2.5 m/s air flow (Figure 1). Because other studies (Dave & Ghaly, 2011; Davies et al., 1998) showed the presence of *Candida* and *Rhodotorula* in beef samples at 0 to 7 °C, they might be observed in dry-aged beef.

Microbiological identification

***Pilaira anomala* SMFM201611.** Molds were selected from PDA based on morphological differences such as differences in color and diameter of mycelia. The mold colonies were further analyzed to identify their genus and species using 18S rRNA analysis, and the identified molds were *Penicillium oxalicum*, *Penicillium echinulatum*, *Mucor circinelloides*, and *P. anomala* (data not shown). Among these molds, *P. anomala* showed a morphology and flavor most similar to the predominant mold on the surface of the dry-aged beef (Figure 2A). An NCBI nucleotide BLAST search showed that the *P. anomala* sequence had 96% agreement and 2% difference with
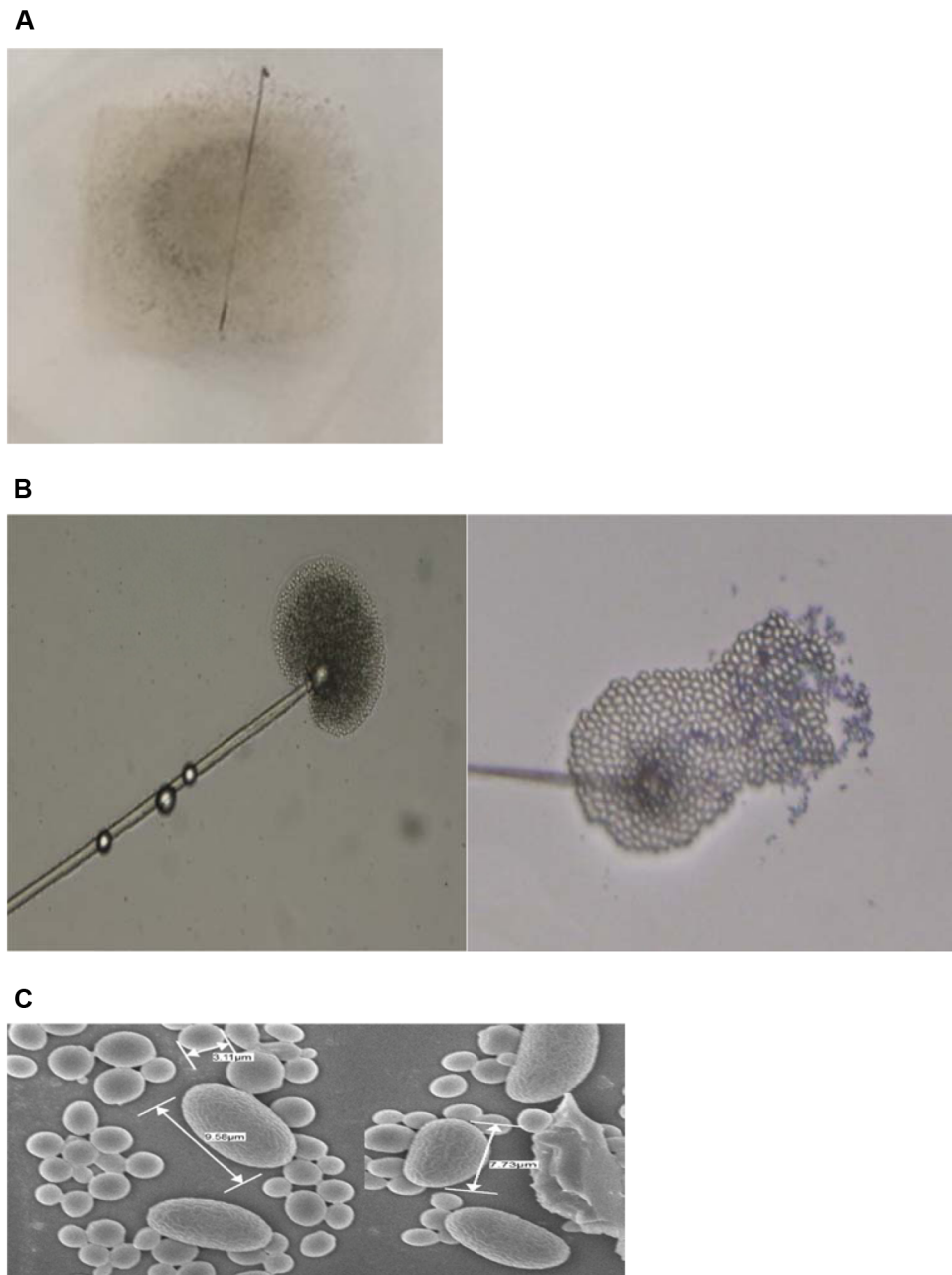
Figure 2–Morphology of *Pilaira anomala* SMFM201611 isolated from dry-aged beef. (A) Mold grown on PDA plate (B) light microscope at 400× magnification; (C) scanning electron microscope at 3,000× magnification.

the partial sequence of the 18S rRNA gene of *P. anomala* strain IUE 573 and was named *P. anomala* SMFM201611. It was observed to have a branched sporangiophore and no septa separating the cells. The matured spores of *P. anomala* SMFM201611 were spread out of the tip of the sporangium (Figure 2B). The spores were yellowish-brown and had an elliptical shape (Figure 2C). Schröt (1886) also described *P. anomala* sporangia as yellowish-brown spores with a size of 7 to 12.5 × 5.5 to 8 μm². The mature spores were 3.5 to 5 μm in width and 7.5 to 9.6 μm in length, and diffusion of the sporangial spores was observed when the mold was cultured for 6 to 7 days (Figure 2C).

Pilaira anomala SMFM201611 was able to grow in a wide range of temperatures, from 4 to 25 °C, and the optimal growth temperature range was 10 to 20 °C. However, its growth was not observed at temperatures above 30 °C (Table 2). The Mucoraceae family, to which *P. anomala* SMFM201611 belongs, has a minimum growth temperature of 5 °C and an optimal growth temperature of 20 °C (Kwon, Ahn, & Park, 2004). Sørensen and Jakobsen (1997) also reported that the optimum growth temperature range for Mucoraceae is 10 to 25 °C and that growth is possible up to 30 °C. It was difficult for *P. anomala* SMFM201611 to grow in the highly acidic environment, at pH

Table 2–Growth of *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707 on the PDA medium at 4 to 37 °C and pH 2.2 to 10.1.

		Colony diameter (mm)	
Treatment		*P. anomala* SMFM201611	*D. hansenii* SMFM201707
Temperature (°C)	4	15.4 ± 0.0[de]	12.1 ± 0.8[c]
	7	15.9 ± 0.8[cd]	12.4 ± 1.1[bc]
	10	21.5 ± 0.8[a]	13.5 ± 0.8[ab]
	15	17.3 ± 0.8[c]	14.0 ± 0.0[a]
	20	19.1 ± 0.8[b]	12.1 ± 0.8[c]
	25	14.0 ± 1.4[e]	13.1 ± 0.8[abc]
	30	0.0 ± 0.0[f]	0.0 ± 0.0[d]
	37	0.0 ± 0.0[f]	0.0 ± 0.0[d]
pH	2.2	8.9 ± 0.8[d]	1.4 ± 0.0[d]
	3.9	41.1 ± 0.8[c]	14.9 ± 0.8[c]
	5.1	59.7 ± 3.5[b]	16.8 ± 0.0[b]
	6.9	67.7 ± 0.8[a]	16.8 ± 1.4[b]
	10.1	66.3 ± 2.1[a]	19.1 ± 0.8[a]

The letters within the same column were significantly different ($P < 0.05$), and pH and temperature data analyzed, respectively.
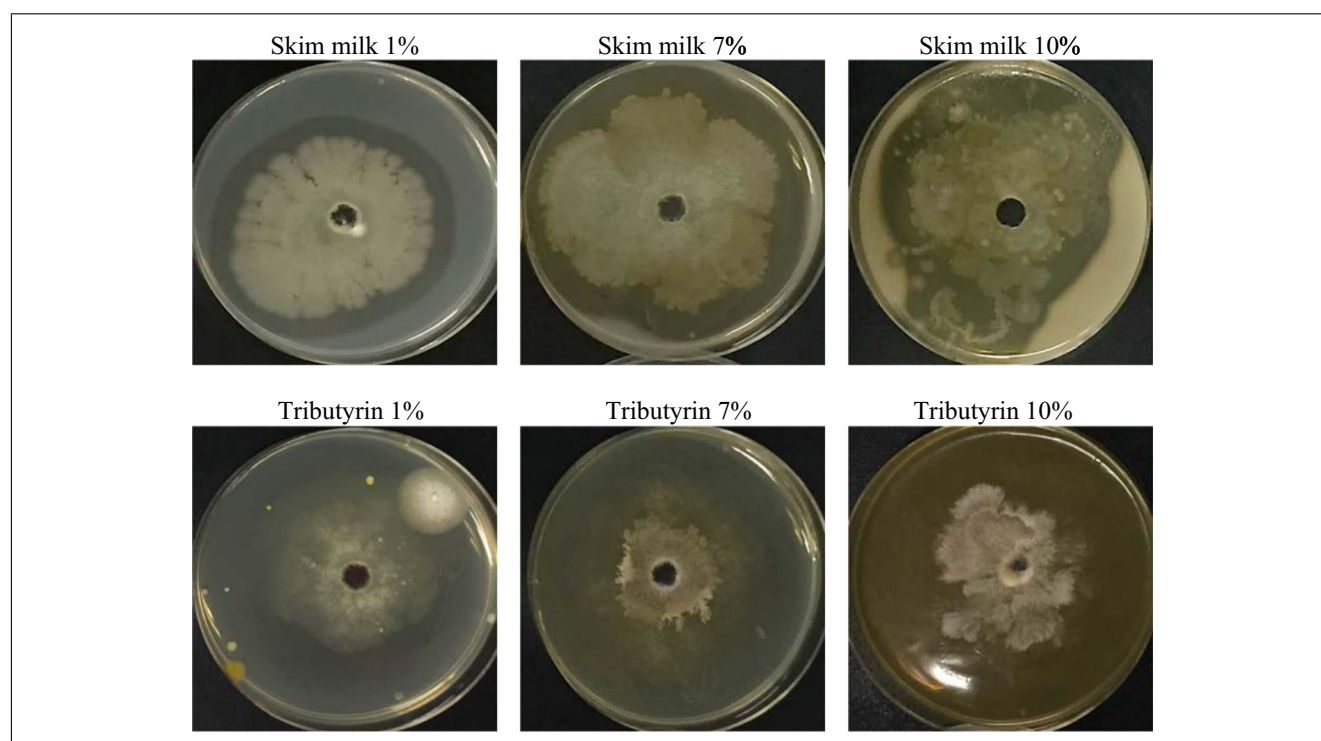

Figure 3–Protease and lipase activities of *Pilaira anomala* SMFM201611 on potato dextran medium containing skim milk (1%, 7%, and 10%) and tributyrin (1%, 7%, and 10%), respectively.

2.2, but it grew well at pH 5 to 10. In particular, its growth improved between pH 5 and 7, which is similar to the pH of beef (Table 2).

To evaluate if *P. anomala* SMFM201611 could degrade proteins and lipids, it was cultured on PDA containing skim milk and tributyrin, respectively. The results showed that as skim milk and tributyrin concentrations increased, its growth increased, forming a transparent ring around the inoculated area of the culture (Figure 3). This indicates that it has protein and lipid degradation activities, and it may improve the sensory characteristics of dry-aged beef during ripening. Hence, the mold was subjected to an inoculation study on beef to evaluate if it could affect beef quality during dry-aging.

***Debaryomyces hansenii* SMFM201707.** The yeast colonies isolated on PDA were further analyzed to identify the genus and species using 26S rRNA analysis, and *Candida* spp., *Scheffersomyces stipitis*, *Rhodotorula mucilaginosa*, and *D. hansenii* (data not shown) were identified. *Debaryomyces hansenii* can induce cheese and sausage fermentation and is known to promote flavor through fat degradation (Martorell, Fernández-Espinar, & Querol, 2005). Therefore, *D. hansenii* isolates were selected to investigate their possible use in the dry-aging process. An NCBI nucleotide BLAST search showed that the *D. hansenii* strain sequence had 98% agreement and 2% difference with a 1,064-bp partial sequence of the large subunit rRNA gene of *D. hansenii* isolate XS8. Thus, it was named *D. hansenii* SMFM201707. It has a small round
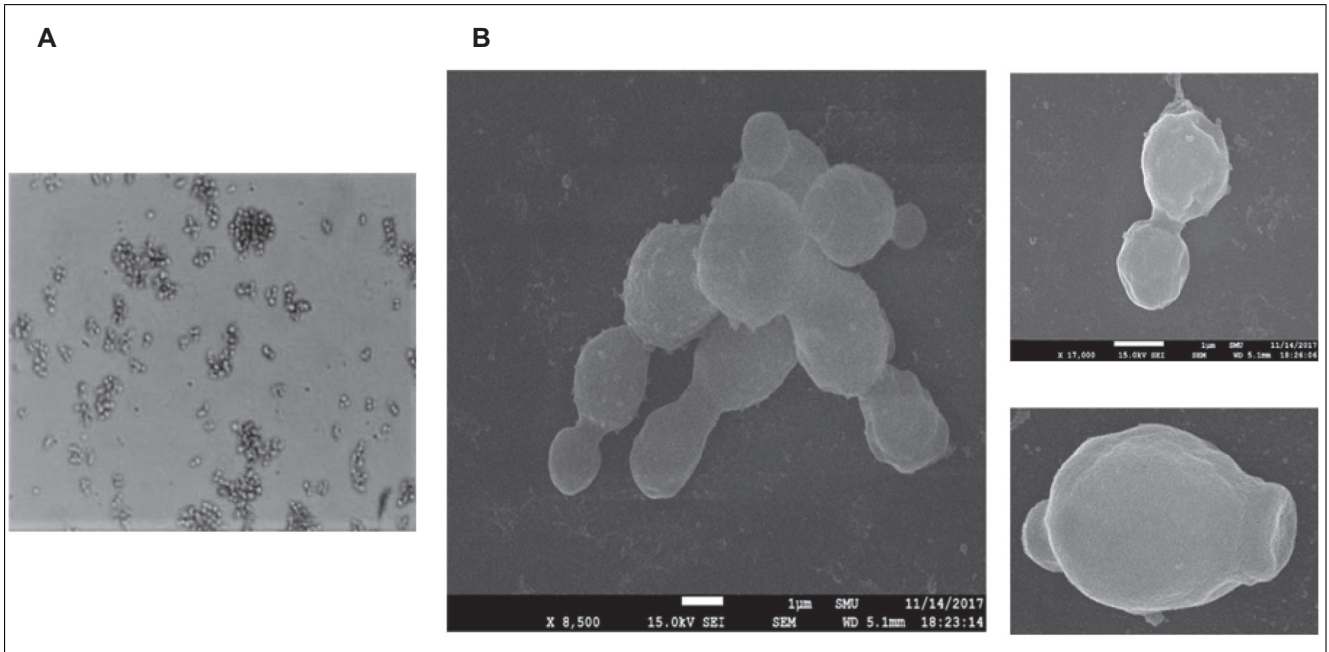
Figure 4–Morphology of *Debaryomyces hansenii* SMFM201707 isolated from dry-aged beef. (A) Light microscope at 400× magnification; (B) scanning electron microscope at 3,000× magnification.
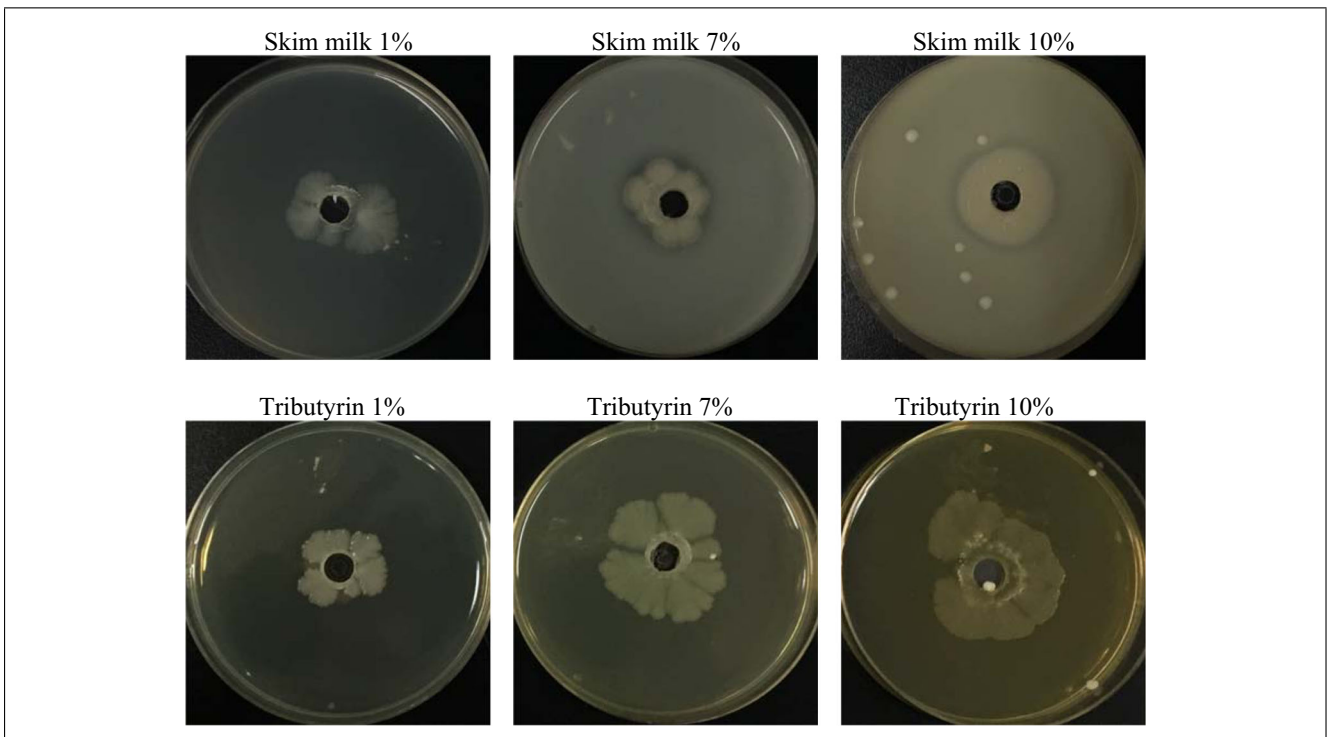

Figure 5–Protease and lipase activities of *Debaryomyces hansenii* SMFM201707 on potato dextran medium containing skim milk (1%, 7%, and 10 %) and tributyrin (1%, 7%, and 10%), respectively.

shape, and the cell size is 1.24 to 2.11 μm. This was consistent with the morphology of *D. hansenii* described in another study (Saluja & Prasad, 2007). Moreover, the appearance of budding was also observed when the yeast was cultured for 6 to 7 days (Figure 4). It grew at a wide range of temperatures from 4 to 25 °C, and the appropriate growth temperature range was 10 to 15 °C (Table 2). Its growth was not observed above 30 °C

(Table 2). It was difficult for *D. hansenii* SMFM201707 to grow in a highly acidic environment, at pH 2.2, but it grew well at pH 3.5 to 10 (Table 2). To evaluate if it could degrade proteins and lipids, the strain was subjected to a protease and lipase activity analysis, and the results showed that its growth increased with the increase in skim milk and tributyrin concentrations (Figure 5). These results indicate that it has protease and lipase activities,
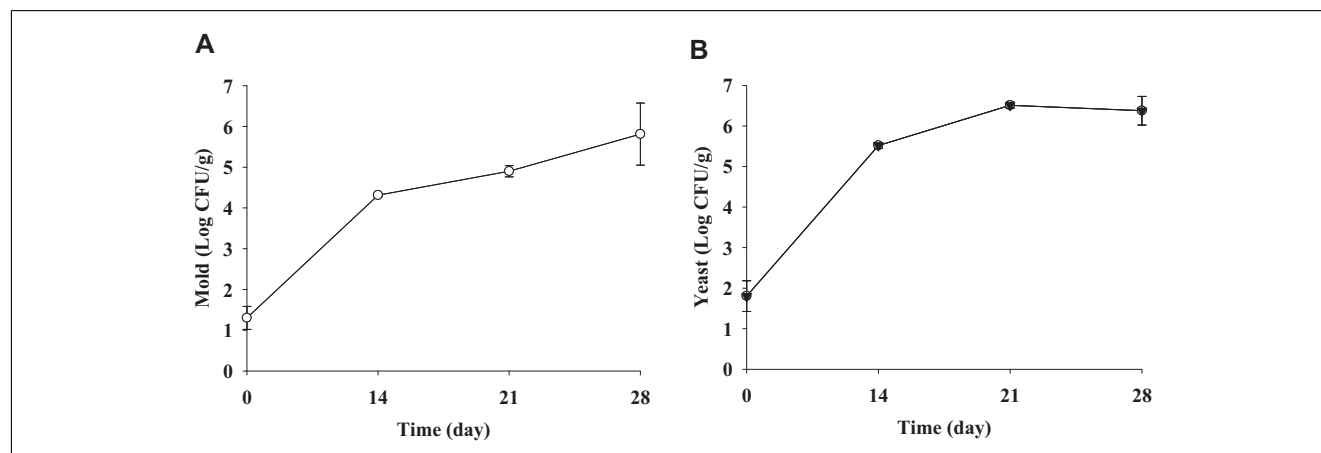
Figure 6–Cell counts of mold (A) and yeast (B) in dry-aged beef during aging at 1 to 4 °C for 28 days ($P < 0.05$).

Table 3–pH and shear force (mean ± SD) of dry-aged beef inoculated with *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707.

	Treatment	Dry-aging period (day)			
		0	14	21	28
pH	Control	5.54 ± 0.01[b]	5.54 ± 0.01[by]	5.69 ± 0.0[ay]	5.60 ± 0.01[by]
	P. anomala	5.54 ± 0.01[c]	5.57 ± 0.02[cx]	5.84 ± 0.06[ax]	5.70 ± 0.02[bx]
	D. hansenii	5.54 ± 0.01[c]	5.51 ± 0.01[dy]	5.65 ± 0.02[ay]	5.58 ± 0.02[by]
Shear force (N)	Control	37.00 ± 7.38[a]	19.61 ± 2.12[cy]	15.89 ± 1.40[dy]	25.19 ± 1.50[bx]
	P. anomala	37.00 ± 7.38[a]	19.66 ± 3.14[cy]	13.04 ± 5.51[dz]	27.44 ± 5.69[bx]
	D. hansenii	37.00 ± 7.38[a]	23.46 ± 3.93[bx]	23.83 ± 1.97[bx]	16.73 ± 1.56[cy]

The letters (a to d) within the same row were significantly different ($P < 0.05$), and pH and shear force data analyzed, respectively.
The letters (x to z) within the same column were significantly different ($P < 0.05$), and pH and shear force data analyzed, respectively.

which could improve beef quality and flavor by degrading beef proteins and lipids. Hence, it was inoculated on beef, and the beef samples were dry-aged to evaluate if it could affect their quality.

Changes in factors related to beef quality and flavor

Microbiological analysis. In dry-aged beef, the number of mold cell counts significantly increased ($P < 0.05$) in *P. anomala* SMFM201611-inoculated samples (Figure 6A). The number of yeast cell counts also significantly increased ($P < 0.05$) in the *D. hansenii* SMFM201707-inoculated samples (Figure 6B). The growth rate of yeast (5 Log CFU/g on day 14) was faster than that of mold (5 Log CFU/g on day 21) during the dry-aging periods (Figure 6).

Physicochemical traits. *pH.* There were no dramatic changes in the pH values of *P. anomala* SMFM201611- and *D. hansenii* SMFM201707-inoculated beef samples (Table 3). This result indicates that the microbial growth did not affect the pH of beef.

Shear force and ultrastructure of beef muscle. The proteolytic activities of both *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 can influence the tenderness of dry-aged beef through the microstructure breakdowns of animal muscle (Toldra, 2006). In this study, *P. anomala* SMFM201611 inoculation significantly decreased ($P < 0.05$) the shear force of dry-aged beef as the dry-aging period increased up to 21 days, and the values were lower ($P < 0.05$) than those of the control and *D. hansenii* SMFM201707-inoculated groups ($P < 0.05$; Table 3). However, the shear force in the

D. hansenii SMFM201707-inoculated group decreased steadily until 28 days, and the values were lower ($P < 0.05$) than those of the control and *P. anomala* SMFM201611 groups (Table 3), whereas during dry-aging, water content may be decreased, and it may cause the increased shear force on day 28 in control and *P. anomala* SMFM201611-inoculated samples. This result may be caused by higher *D. hansenii* prevalence than *P. anomala* during dry-aging as shown in Figure 6.

The myofibril ultrastructure image by TEM analysis illustrated that *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 broke down the myofibril ultrastructure, and *P. anomala* SMFM201611 caused more myogibrill breakdowns (Figure 7). Patrignani et al. (2007) showed that *D. hansenii* contributes to proteolysis in fermented sausage, and other studies reported its important activities on myosin, actin, as well as sarcoplasmic proteins in fermented sausage (Martín et al., 2002; Rodriguez, Núñez, Cordoba, Bermúdez, & Asensio, 1998). This result indicates that *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 may improve the tenderness of dry-aged beef by breaking down myofibrils through proteolytic activity.

Flavor compounds. *Free fatty acids.* During the dry-aging process, both *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 generated FFAs because of their high lipolytic activity (Table 4), which hydrolyzes triglycerides or phospholipids to FFAs (Alapont, Martínez-Culebras, & Lopez-Mendoza, 2015; Chizzolini, Novelli, & Zanardi, 1998). For the concentration of FFAs, *P. anomala* SMFM201611-inoculated samples had increased FFA concentration for all FFAs, and *D. hansenii* SMFM201707-inoculated samples had increased FFA concentrations in six of
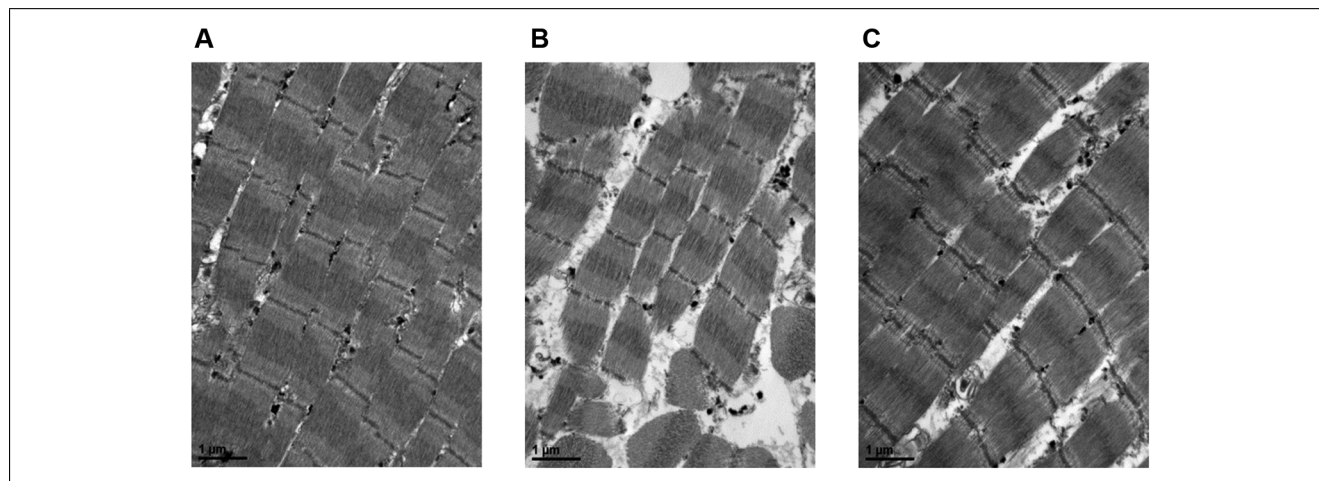
Figure 7–The ultrastructure of beef sirloin inoculated with (A) PBS (control), (B) *Pilaira anomala* **SMFM201611, or (C)** *Debaryomyces hansenii* **SMFM201707 after 28 days of dry-aging process.**

Table 4–Free fatty acid content (mg/g) of dry-aged beef sirloin inoculated with *Pilaira anomala* **SMFM201611 or** *Debaryomyces hansenii* **SMFM201707 for 28 days.**

	Treatment	Dry-aging period (day)			
		0	**14**	**21**	**28**
Lauric acid (C12:0)	Control	0.03 ± 0.00^c	0.03 ± 0.00^c	0.04 ± 0.01^{by}	0.05 ± 0.00^{ax}
	P. anomala	0.03 ± 0.00^b	0.04 ± 0.00^b	0.17 ± 0.01^{ax}	0.04 ± 0.00^{by}
	D. hansenii	0.03 ± 0.00^b	0.03 ± 0.00^b	0.04 ± 0.00^{ay}	0.03 ± 0.00^{bz}
Myristic acid (C14:0)	Control	0.18 ± 0.04^b	0.19 ± 0.01^{by}	0.59 ± 0.14^{ay}	0.75 ± 0.02^{ax}
	P. anomala	0.18 ± 0.04^c	0.52 ± 0.04^{bcx}	3.47 ± 0.20^{ax}	0.63 ± 0.00^{by}
	D. hansenii	0.18 ± 0.04^b	0.23 ± 0.10^{by}	0.59 ± 0.10^{ay}	0.24 ± 0.02^{bz}
Myristoleic acid (C14:1)	Control	0.05 ± 0.01^c	0.06 ± 0.00^{bcy}	0.12 ± 0.04^{aby}	0.17 ± 0.00^{ax}
	P. anomala	0.05 ± 0.01^d	0.12 ± 0.00^{cx}	0.43 ± 0.02^{ax}	0.17 ± 0.00^{bx}
	D. hansenii	0.05 ± 0.01^b	0.06 ± 0.04^{by}	0.18 ± 0.02^{ay}	0.09 ± 0.01^{by}
Palmitic acid (C16:0)	Control	4.25 ± 0.54^c	4.12 ± 0.19^{cy}	9.75 ± 1.75^{by}	12.64 ± 0.24^{ax}
	P. anomala	4.25 ± 0.54^c	7.85 ± 0.62^{bx}	43.48 ± 1.65^{ax}	9.68 ± 0.19^{by}
	D. hansenii	4.25 ± 0.54^b	5.08 ± 1.22^{by}	9.06 ± 1.43^{ay}	4.73 ± 0.17^{bz}
Palmitoleic acid (C16:1)	Control	0.19 ± 0.04^c	0.26 ± 0.02^{cy}	0.77 ± 0.17^{by}	1.07 ± 0.02^{ax}
	P. anomala	0.19 ± 0.04^c	0.75 ± 0.05^{bx}	3.84 ± 0.15^{ax}	0.93 ± 0.02^{by}
	D. hansenii	0.19 ± 0.04^b	0.25 ± 0.11^{by}	0.86 ± 0.12^{ay}	0.35 ± 0.02^{bz}
Stearic acid (C18:0)	Control	2.90 ± 0.36^b	2.58 ± 0.11^{by}	4.87 ± 0.88^{ay}	5.44 ± 0.08^{ax}
	P. anomala	2.90 ± 0.36^c	3.70 ± 0.30^{bcx}	16.34 ± 0.65^{ax}	4.43 ± 0.10^{by}
	D. hansenii	2.90 ± 0.36^b	3.12 ± 0.66^{bxy}	4.49 ± 0.79^{ay}	2.73 ± 0.04^{bz}
Oleic acid (C18:1)	Control	1.85 ± 0.36^c	2.30 ± 0.10^{cy}	7.11 ± 1.41^{by}	9.14 ± 0.27^{ax}
	P. anomala	1.85 ± 0.36^d	5.62 ± 0.39^{cx}	31.77 ± 1.45^{ax}	7.71 ± 0.14^{by}
	D. hansenii	1.85 ± 0.36^b	2.52 ± 0.88^{by}	6.81 ± 0.94^{ay}	2.76 ± 0.22^{bz}
Linoleic acid (C18:2)	Control	0.23 ± 0.01^c	0.35 ± 0.02^c	1.08 ± 0.07^{ay}	0.90 ± 0.07^{bx}
	P. anomala	0.23 ± 0.01^c	0.56 ± 0.13^b	2.82 ± 0.11^{ax}	0.72 ± 0.01^{by}
	D. hansenii	0.23 ± 0.01^b	0.49 ± 0.08^a	0.48 ± 0.04^{az}	0.38 ± 0.02^{az}
Linolenic acid (C20:3)	Control	0.05 ± 0.00^d	0.07 ± 0.01^c	0.13 ± 0.01^{by}	0.15 ± 0.01^{ax}
	P. anomala	0.05 ± 0.00^d	0.09 ± 0.03^c	0.21 ± 0.01^{ax}	0.12 ± 0.00^{by}
	D. hansenii	0.05 ± 0.00^c	0.07 ± 0.03^{bc}	0.09 ± 0.01^{abz}	0.11 ± 0.01^{ay}
Arachidonic acid (C20:4)	Control	0.09 ± 0.01^b	0.12 ± 0.02^b	0.31 ± 0.02^{ay}	0.31 ± 0.04^{ax}
	P. anomala	0.09 ± 0.01^c	0.16 ± 0.07^c	0.64 ± 0.03^{ax}	0.26 ± 0.02^{bx}
	D. hansenii	0.09 ± 0.01^b	0.11 ± 0.07^b	0.17 ± 0.01^{abz}	0.20 ± 0.01^{ay}

The letters (a to d) within the same row were significantly different ($P < 0.05$), and data analyzed each free fatty acid, respectively.
The letters (x to z) within the same column were significantly different ($P < 0.05$), and data analyzed each free fatty acid, respectively.

10 FFAs on day 21 of dry-aging (Table 4). Especially, oleic, palmitic, and stearic acids were the most abundant fatty acids in the *P. anomala* SMFM201611-inoculated samples on day 21 of dry-aging (Table 4). A high proportion of oleic acid improves beef quality (Hwang & Joo, 2017). FFAs play a considerable role in the formation of aroma volatiles in meat and meat products (Mottram, 1998; Toldra, 1998). These results suggest that *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 produce aroma volatiles in beef from FFAs through lipolysis activity and the aroma volatiles can be more abundant in *P. anomala* SMFM201611-inoculated samples than in *D. hansenii* SMFM201707-inoculated samples. This may be caused by the higher lipolytic activity of *P. anomala* SMFM201611 compared to *D. hansenii* SMFM201707, as shown in Figure 3 and 5.

Table 5–Free amino acid content (mg/100 g; mean ± SD) of dry-aged beef sirloin inoculated with *Pilaira anomala* SMFM201611 and *Debaryomyces hansenii* SMFM201707 for 28 days.

	Treatment	0	14	21	28
			Dry-aging period (day)		
Glutamic acid	Control	3.53 ± 0.16[d]	9.42 ± 1.49[cy]	17.28 ± 1.05[by]	32.63 ± 2.21[ax]
	P. anomala	3.53 ± 0.16[c]	13.90 ± 4.25[bxy]	22.65 ± 1.48[ax]	23.00 ± 0.86[ay]
	D. hansenii	3.53 ± 0.16[c]	14.95 ± 1.23[bx]	15.54 ± 1.34[by]	19.78 ± 0.47[az]
Methionine	Control	2.46 ± 0.21[d]	5.86 ± 0.19[cy]	10.61 ± 0.55[by]	14.52 ± 0.42[ax]
	P. anomala	2.46 ± 0.21[d]	6.98 ± 0.14[cy]	13.46 ± 0.47[ax]	11.01 ± 0.11[by]
	D. hansenii	2.46 ± 0.21[d]	7.32 ± 0.21[cx]	10.62 ± 0.30[by]	11.54 ± 0.36[ay]
Valine	Control	3.94 ± 0.14[d]	9.80 ± 0.36[cy]	18.24 ± 0.79[by]	25.85 ± 0.46[ax]
	P. anomala	3.94 ± 0.14[d]	10.06 ± 0.17[cy]	22.69 ± 0.99[ax]	18.28 ± 0.06[bz]
	D. hansenii	3.94 ± 0.14[d]	11.83 ± 0.23[cx]	16.92 ± 0.80[by]	20.19 ± 0.92[ay]
Phenylalanine	Control	4.38 ± 0.03[d]	8.31 ± 0.39[c]	14.33 ± 0.80[by]	20.77 ± 0.42[ax]
	P. anomala	4.38 ± 0.03[c]	10.78 ± 3.34[b]	18.44 ± 0.72[ax]	15.34 ± 0.20[ay]
	D. hansenii	4.38 ± 0.03[d]	9.38 ± 0.38[c]	13.95 ± 0.30[by]	15.24 ± 0.53[ay]
Isoleucine	Control	3.10 ± 0.05[d]	6.84 ± 0.28[cy]	13.11 ± 0.59[by]	18.76 ± 0.28[ax]
	P. anomala	3.10 ± 0.05[d]	7.23 ± 0.19[cy]	16.35 ± 0.79[ax]	13.73 ± 0.16[by]
	D. hansenii	3.10 ± 0.05[d]	8.15 ± 0.21[cx]	12.12 ± 0.32[by]	14.00 ± 0.70[ay]
Leucine	Control	5.91 ± 0.24[d]	12.87 ± 0.37[cz]	25.05 ± 1.16[by]	35.15 ± 0.47[ax]
	P. anomala	5.91 ± 0.24[d]	13.62 ± 0.30[cy]	29.61 ± 1.27[bx]	23.61 ± 0.15[az]
	D. hansenii	5.91 ± 0.24[d]	15.55 ± 0.37[cx]	21.84 ± 0.84[bz]	25.56 ± 1.09[ay]
Total	Control	23.33 ± 0.43[d]	53.10 ± 2.92[cy]	98.62 ± 4.81[by]	147.69 ± 4.13[ax]
	P. anomala	23.33 ± 0.43[d]	62.57 ± 5.81[cx]	123.19 ± 5.47[ax]	104.97 ± 1.09[by]
	D. hansenii	23.33 ± 0.43[d]	67.19 ± 0.92[cx]	90.98 ± 3.74[by]	106.31 ± 3.78[ay]

The letters (a to d) within the same row were significantly different ($P < 0.05$), and data analyzed each free amino acid, respectively.
The letters (x to z) within the same column were significantly different ($P < 0.05$), and data analyzed each free amino acid, respectively.

Free amino acids. FAAs are one of the main taste-active compounds in meat and meat products, and they generate several aroma volatiles through the Maillard reaction with reducing sugar (Mottram, 1998). The changes in isoleucine, leucine, phenylalanine, and valine contents were analyzed in this study (Table 5). FAAs contribute to the sweet, bitter, and umami tastes, influence saltiness and sourness, together with acids and inorganic/sodium salts, and possess a cooked beef aroma (Shahidi, 1994; Toldra, Aristoy, & Flores, 2000). In addition, glutamic acid is one of the most important components of umami taste in meat (Zhao, Schieber, & Gänzle, 2016). Differences in FAAs may cause different aromas in aged beef. For example, methionine can generate a cooked beef aroma at a low threshold value, whereas isoleucine, leucine, phenylalanine, serine, and valine produce Strecker aldehydes and heterocyclic compounds (Mottram, 1998; Toldra et al., 2000). In our experiment, levels of most FAAs generally increased ($P < 0.05$) in both *P. anomala* SMFM201611- and *D. hansenii* SMFM201707-inoculated samples. On day 14, *D. hansenii* SMFM201707-inoculated samples had higher ($P < 0.05$) FAA (glutamic acid, methionine, valine, isoleucine, and leucine) content than the control, and *P. anomala* SMFM201611-inoculated samples had higher ($P < 0.05$) leucine content than the control (Table 5). On day 21, the contents of all tested FAA were higher ($P < 0.05$) in *P. anomala* SMFM201611 than in control (Table 5). The FAA contents were lower ($P < 0.05$) in *D. hansenii* SMFM201707-inoculated samples than in *P. anomala* SMFM201611-inoculated samples. However, on day 28, the *D. hansenii* SMFM201707-inoculated group had a higher ($P < 0.05$) FAA content than the *P. anomala* SMFM201707-inoculated group, with the exception of phenylalanine, possibly because of the more pronounced proteolysis activity of *D. hansenii* SMFM201707 at the later stage of the dry-aging process. According to previous studies, differences in FAAs can cause a different aroma development in dry-aged beef (Koutsidis et al., 2008; Shahidi, 1994). This result indicates that *P. anomala* SMFM201611 and *D. hansenii*

SMFM201707 inoculation may produce FAAs because of their proteolysis activities, as shown in Figure 3 and 5, and the change in FAA content may influence the development of taste and aroma in dry-aged beef (Mottram, 1998).

Conclusions

In conclusion, *P. anomala* SMFM201611 and *D. hansenii* SMFM201707 isolated from dry-aged beef decreased the dry-aging period of beef inoculated with them compared to the non-inoculated beef. *Pilaira anomala* SMFM201611-inoculated samples generally had higher FFA and FAA contents than the others on day 21, and *D. hansenii* SMFM201707-inoculated samples had higher FAA (glutamic acid, methionine, valine, isoleucine, and leucine) than the control on day 14. In addition, the tenderness of *D. hansenii* SMFM201707-inoculated samples gradually decreased during dry-aging, and the value was the lowest on day 28. The improved tenderness of low-grade beef may be caused by the breakdown of myofibrils through their protease activity. They also have potential to improve the taste and aroma characteristics of dry-aged beef by increasing FFA and FAA concentration through lipolysis and proteolysis activities.

Acknowledgments

Author Contributions

H. Oh contributed to the study design, conducted the experiments, and wrote the manuscript draft. H. Lee contributed to the study design, conducted the experiments, and wrote the interpretation of the results. J. Lee conducted the experiments and

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collected the data. C. Jo designed the study and revised the data information. Y. Yoon had overall responsibility for the study design and contributed to revise the manuscript.

Conflict of Interest

The authors declare no competing financial interest.

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