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Abstract

This study aimed to isolate, identify, and evaluate novel Korean starter cultures for use in fermented sausages. A total of 72 strains were isolated from various indigenous sources, including traditional Korean fermentation starters such as Nuruk, Jeotgal, and mud flats. Among them, two strains were identified as *Penicillium nalgiovense*, a traditional strain for making the fermented sausages; they were designated *P. nalgiovense* SD01 and *P. nalgiovense* SJ02. A comparative analysis of SD01 and SJ02 was performed using Mold600 commercial starter culture. Proteolytic and lipolytic assays, and assessments of growth and mycotoxin production were performed. Strain SJ02 exhibited superior lipolytic and proteolytic activities, as well as an enhanced growth rate at the optimal salinity level of 2% NaCl compared to Mold600. Dry-fermented sausages prepared with SJ02 and the commercial strain Mold600 exhibited similar qualities. Sausages fermented with SJ02 tended to have lower thiobarbituric acid reactive substance levels (TBARS) and significantly increased adhesiveness compared to M600. In addition, TPA and color results showed no significant difference in quality from dry-fermented sausages made with the commercial strain. Profiles of mycotoxin-related genes was similar for both strains. The findings indicate that *P. nalgiovense* SJ02 is a promising domestic starter culture for the production of dry fermented sausages and application of SJ02 could potentially enhance the local meat processing industry.

Keywords: *Penicillium nalgiovense*, Korean mudflat, dry fermented sausage, fungal starter, sensory quality

Introduction

Mold fermentation, particularly for meat, is a global tradition where diverse foods are crafted using various fungal species (Leistner, 1990). In Europe, molds play a pivotal role in the culinary landscape, especially in the production of dry-cured meats and cheeses. Southern European countries like Italy, Spain, and Southern France are renowned for their extensive use of molds in meat processing, showcasing a tradition that highlights the region's gastronomic heritage(Spotti et al., 2008). These countries employed the unique properties of molds to enhance flavor, texture, and preservation of their traditional meat and cheese products, exemplifying the widespread appreciation and application of mold fermentation in enhancing food quality and safety (El-banna et al., 1987; Geisen et al., 1992). For meat fermentation, employing specific mold strains, such as *Penicillium nalgiovense* for salami and salchichon not only imparts distinctive tastes and aromas but also inhibits the growth of harmful bacteria, ensuring the meat is safe for consumption (Bernáldez et al., 2013; Magistà et al., 2016).

Starters, including bacteria (notably lactic acid bacteria and coagulase-negative staphylococci), yeasts, and molds, are crucial for fermenting meat products, enhancing safety through rapid acidification and antimicrobial substance production. These starters not only help standardize product properties and reduce ripening times but also mitigate microbiological risks from foodborne pathogens (*Salmonella spp., Listeria spp.,* etc) and chemical hazards like biogenic amines, nitrosamines, polycyclic aromatic hydrocarbons, and mycotoxins. Specifically, the lactic acid bacteria reflects their extensive involvement in metabolic activities during *fermentation* (Radulović et al., 2011). They create an acidic environment by lowering the pH to 4.6~5.9, thereby influencing meat color, texture, and fostering both fermentation and drying.

The careful selection of non-toxigenic mold strains is essential, as it guarantees the health benefits of fermented meats without compromising safety. Thus, mold fermentation stands as a pivotal practice in the food industry, marrying tradition with modern safety and quality standards (Laranjo et al., 2019). Strains, such as *Penicillium nalgiovense* and *P. chrysogenum*, are used to protect dried fermented sausages in the meat processing industry (Bernáldez et al., 2013). Fungal starters have antioxidant effects, prevent spoilage-induced discoloration, enhance texture, generate unique flavors through fat and protein degradation, and influence the appearance of sausages (Philipp and Pedersen, 1988). Moreover, fungal starters protective against pathogenic and spoilage microorganisms (Geisen et al., 1992; Leistner, 1994; López-Díaz et al., 2001; Singh and Dincho, 1994).

Recently the domestic market for fermented meat products, including fermented sausages, increased in South Korea. Consequently, the demand for fungal starters continues to increase. *P. nalgiovense* is the only domestically approved fungal starter strain. Importation of foreign strains is necessary for the utilization of starter products in fermented meat products. However, research on fermented sausages has predominantly focused on investigating the impact of additives on sensory quality, sodium-alternatives, and storage function (Yim et al., 2020a; Yim et al., 2020b; Yoon et al., 2018). Consequently, the capability and benefits of starter strains derived from local sources remain significantly underexplored (Administration, 2018; Chung et al., 2017). In accordance with the Nagoya Protocol on Access to Generic Resources and the Fair and Equitable Sharing Arising from their Utilization to the Convention on Biological Diversity, the production of fermented foods using domestic bacteria is very important and essential for the fermented food industry. This study aimed to identify domestically

derived starter strains suitable for fermented sausages to address the need for homegrown starter development to advance domestic fermented meat production.

Materials and Methods

Isolation and preparation of fungal strains

The fungal starter strains were isolated from traditional fermented foods in South Korea, including Nuruk and Jeotgal, and from mudflats located in Seondo-ri, Hwawangsan, Geumjeong, Soyulgok, Biin-myeon, Seocheon-gun, and Chungcheongnam-do. Isolates were inoculated on potato dextrose agar (PDA) and incubated at 25℃ for 7 days. A total of 72 separate strains were isolated. Two mudflat isolates were identified as *P. nalgiovense* and were designated SD01 and SJ02 (*P. nalgiovense* SD01 and *P. nalgiovense* SJ02). To prepare spore suspensions, spores were harvested and mixed with 10 mL of 0.1% (w/v) sterile peptone water. Mold600 (M600; Chr. Hansen, Hoersholm, Denmark) was used as the control. All strains were inoculated at a concentration of 8 log colony-forming units (CFUs). Each strain was stored in 20% glycerol solution at -80℃ until required.

Identification of isolates

For strain identification, lactophenol cotton blue staining at the genus level, internal transcribed spacer (ITS) sequencing, and phylogenetic analysis confirmed *P. nalgiovense* species. The ITS sequences of the isolates were amplified by PCR and analyzed at Macrogen Ltd. (Seoul, South Korea). PCR was performed as described previously (Frisvad et al., 2013). β-tubulin (BenA) and the ITS sequences were generated and combined with sequences used in Table 1. DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) as

106 previously described (Ojo-Okunola et al., 2020). PCR was performed using a total
107 volume of 20 μL. The reaction mixture included 200 ng/μL of DNA solution, 10 μL of
108 pre-mixture (AccuPower® Taq PCR Master Mix; Bioneer, Daejeon, South Korea), 1 μL
109 of each primer, and RNase-free water. PCR for β-tubulin involved an initial
110 denaturation step at 94℃ for 5 min, followed by 40 cycles of denaturation at 94℃ for
111 45 s, annealing at 55℃ for 45 s, extension at 72℃ for 1 min, and a final extension cycle
112 at 72℃ for 7 min. The PCR sequences were detected using BLAST in the GenBank
113 database located at the National Center for Biotechnology Information and compared
114 with ITS sequences of previously reported strains. Phylogenetic analysis was performed
115 to determine the genetic relationship of SJ02 within the *P. nalgiovense* using MEGA
116 software (version 4.0; http://www.megasoftware.net). The neighbor-joining method was
117 used to construct a phylogenetic tree using the maximum composite likelihood model
118 (Kimura, 1980; Saitou and Nei, 1987).
119
120 PCR detection of mycotoxin genes
121 Positive control strains were procured from the Korean Collection for Type Cultures
122 (KCTC, Jeongeup, South Korea). They included *P. roquefortii* Thom (KCTC 6080) and
123 *P. chrysogenum* (KCTC 6933). Aflatoxin, Ochratoxin A, Patulin, Sterigmatocystin,
124 Cyclopiazonic acid, Penicillin (pcbAB), Penicillin (penDE), Mycophenolic acid,
125 Roqueforine C, and Penicillin (pcbC) β-tubulin were analyzed by PCR using the
126 primers listed in Table 1 (Bernáldez et al., 2013; Färber and Geisen, 1994; López-Díaz
127 et al., 2001; Moavro et al., 2019; Rodríguez et al., 2012). PCR for Aflatoxin (omt-1),
128 Ochratoxin A (otanpsPN), Patulin (idh), Sterigmatocystin (fluG), Cyclopiazonic acid
129 (dmaT), Penicillin (pcbAB), Penicillin (penDE), and Penicillin (pcbC) involved an
130 initial denaturation step at 95℃ for 2 min, followed by 30 cycles of denaturation at

95℃ for 30 s, annealing at 60℃ for 1 min, extension at 72℃ for 1 min, and a final extension cycle at 72℃ for 5 min. For Mycophenolic acid (mpaC) and Roqueforine C (rds/roqA), PCR was performed as just described with annealing temperatures of 54℃ and 50℃, respectively. Fluorescencee amplification was performed to verify the PCR products.

Crude cell free extract enzymatic activity (CCFA) assay

Extracellular proteolytic and lipolytic enzymes were prepared from the proteolytic broth (Proteo M), as previously described (Biaggio et al., 2016). Proteo M contained (per L) 6.392 g of KH_2PO_4, 0.522 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 g of yeast extract, 5 g of NaCl, and 5 g of skim milk powder. In addition, extracellular lipolytic enzymes were prepared from the lipolytic broth (CHO lipo) as previously described. CHO lipo contained (per L) 6.0 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of $(NH_4)_2SO_4$, 4.0 g of peptone, and 10 mL of Tween 20. The medium was adjusted to pH 6.0 and autoclaved for 15 min at 121℃. Extracellular proteolytic and lipolytic enzyme production for each *Penicillium* species was performed in 50 mL of Proteo M or CHO lipo inoculated with 1 mL (10^6 spores/mL) of each *Penicillium* species. They were incubated at 25℃ and 150 rpm for 14 days. After incubation, each production broth was centrifuged at 10,000 rpm for 5 min. Each supernatant was filtered through a 0.22 μm syringe filter to obtain the crude cell free extract (CCFE), which was assayed. The enzymatic activity of CCFE was assessed on substrate agar (SA) and B20 plates. A central well was created in the agar plates using a 17 mm cork-borer. CCFE (500 μL) was added to each well. The plate assays were performed in triplicate. The plates were incubated at 25℃ overnight and observed for the presence of a clearance halo for proteolytic activity or a precipitation zone for lipolytic activity.

Proteolytic activity assay

The proteolytic activity was determined as previously described (Magistà et al., 2016). Media were prepared to measure extracellular protease activity, as shown in Table 2. To ascertain the best temperature for maximizing proteolytic activity and growth, the strain was inoculated on skim milk agar plates and maintained at 18℃ and 12℃ for 9 days. The strain suspension was inoculated at two spots with 5 μL of a suspension containing 10^4 spores/mL. The enzymatic activity indices for the proteolytic plate assays were determined following the previously described method (Lester and SL, 1975). The semiquantitative enzymatic index (EI) was calculated as EI = H/C, where H represents the diameter of the degradation halo and C is the diameter of the fungal colony. The halo zone around the colony was measured (mm) daily over a span of 9 days, using a ruler along two diametrically opposed directions (Lumi Abe et al., 2015).

Lipolytic activity assay

The lipolytic activity was determined as previously described (Magistà et al., 2016). To measure the extracellular lipase activity, media were prepared as shown in Table 2. Each strain was inoculated onto plates containing 1% Tween 20 (B20) or 1% Tween 80 (B80) and incubated at 18℃ and 12℃ for 9 days. The strain suspension was inoculated at two spots with 5 μL of a 10^4/mL spore suspension. The enzymatic activity indices for the lipolytic plate assays were determined as previously described (Lester and SL, 1975). The semiquantitative EI was calculated as EI = H/C, where H represents the diameter of the visible precipitation zone produced by the formation of calcium salt crystals from the enzymatic hydrolysis of the ester bond in the presence of lauric acid

(Tween 20) or oleic acid (Tween 80), and C is the diameter of the fungal colony. The precipitation zone around the colony was measured (mm) daily over a span of 9 days using a ruler along two diametrically opposed directions (Lumi Abe et al., 2015).

Optimization of growth rate

To determine optimal growth conditions, the strains were inoculated onto meat agar prepared using fermented sausage recipes and NaCl agar prepared by adding different concentrations of NaCl (1, 2, 4, and 6% w/v) to malt extract agar (MEA). Each isolate was inoculated with 2 μL (10^8 spores/mL) at three spots on both the meat and NaCl plates. Subsequently, the plates were incubated at 25℃ for 5-7 days and the growth size were determined using Image J software.

Manufacture of dry fermented sausages

To evaluate the fermented sausages manufactured with the identified SJ02 strain, we prepared dry fermented sausages. A mixture of minced pork hindlegs (85%) and fat (15%) was seasoned with salt (2.5%), sodium nitrite (0.015%), pepper (0.3%), red wine (0.4%), dextrose (0.5%), and coriander (0.05%). Additionally, a 0.0125% LAB starter (TRADI 302, Chr. Hansen) was integrated into the mixture. The meat batter was then carefully packed into fibrous casings with a diameter of 40 mm, and the surfaces of the casings were punctured using a sausage pricker. Suspensions of fungal starter cultures (M600 and SJ02) were meticulously adjusted to a concentration of 10^8 spores/mL and evenly applied to hanging sausages using liquid mold spray (approximately 150 mL). The sausages were incubated for 48 h at 25℃ with a relative humidity of approximately 90%. This was followed by a subsequent 4-day drying phase at 18℃ and 75% relative humidity. The curing process was approximately 27 days at 12℃ and 70% relative

206 humidity. Throughout the manufacturing process, the sausages were continuously
207 monitored for weight loss, which was tracked until they reached 40% w/w, signifying
208 successful completion of the fermentation process. Dry fermented sausages were
209 sampled from each batch to evaluate their physical, chemical, microbiological, and
210 sensory characteristics. All assessments were performed three times for each batch.
211
212 Food born pathogen analysis from fermented sausage
213 Detection evaluation of Bacillus cereus, Clostridium perfrigens, Staphylococcus
214 aureus, Listeria monocytogenes and Escherichia coli O157:H7 was conducted in
215 manufactured sausages. To evaluate microbial safety, 25 g of each sausage sample was
216 aseptically collected, transferred to a stomacher sample bag, and homogenized in 225
217 mL of the respective suspension media using a model WES-400 Stomacher WiseMix
218 (Daihan Scientific, Wonju, South Korea). Appropriate dilutions were spread onto the
219 selective media listed in Table 3 (Kim et al., 2008).
220
221 Color analysis
222 Sausage color was measured in the samples (six 2-cm thick cubic shapes without
223 casing) using a model CR-400 colorimeter (Minolta Camera Co., Ltd., Tokyo, Japan)
224 calibrated with the manufacturer's white calibration plate (L*=93.7, a*=0.3158, and
225 b*=0.3322). The mean values of triplicate measurements for each sample represented
226 the Commission Internationale de l'Eclairage L* (lightness), a* (redness), and b*
227 (yellowness) (Essid and Hassouna, 2013).
228
229 Texture profile analysis (TPA)

To test the texture properties, a model TX-700 texture analyzer (Lamy Rheology, Champagne-au-Mont-d'Or, France) was used as previously described (Essid and Hassouna, 2013). More than six cubic samples (2×2×2 cm) of each sample were evaluated. The test was performed by compressing with a cylinder probe (2 cm diameter) at following conditions: a speed of 1 mm/s, distance of 16 mm, force start of 1N, wait position at 0.1 mm, up speed of 5 mm/s, and delay for 2s, while employing the two-bite test methodology, consistent with the sampling technique used for color analysis. Hardness (N), adhesiveness (mJ), cohesiveness (N), gumminess (N), and chewiness (N) of the samples were calculated using TPA curves.

Thiobarbituric acid reactive substance (TBARS) analysis

To evaluate the rancidity of the fermented sausages, TBARS were measured as previously described (Woo et al., 2023). Approximately 3.5 g of the sample was mixed with 500 μL of 0.01% butylated hydroxytoluene and an extraction solvent composed of 7% trichloroacetic acid, 4N HCl, and 4% $HClO_4$. The homogenate was filtered through the Whatman No. 41 filter paper (Whatman Inc., Little Chalfont, UK). Next, 2 mL of the filtered solution was combined with 2 mL of 20 mM TBA and heated at 80℃ for 60 min. After cooling in cold water for 10 min, the absorbance of the solution was measured at 534 nm by using a Multiskan Go spectrophotometer (Multiskan Go Thermo Fisher Scientific, Waltham, MA, USA). The TBARS values were determined using a standard curve.

Electronic nose (E-nose)

Volatile compounds present in the samples were analyzed using the Heracles II E-nose system (Alpha MOS, Toulouse, France). Approximately 1 g of each sample

without casing was placed in a 20 mL headspace vial. The headspace vial was then incubated at 60℃ for 20 min while being agitated at 500 rpm to promote the generation of volatile compounds. The collected volatile compounds were coupled to the E-nose system using an automatic sampler. For the gas chromatography analysis, 3 mL of volatile compounds were injected at a speed of 125 μL/s and a temperature of 200℃ into the gas injection port connected to the E-nose. The analysis was conducted using MXT-5 and MXT-1701 columns, with an incubation temperature of 50℃ and a total analysis time of 10 min. Separated peaks were identified and confirmed using AlphaSoft version 14.2 (Alpha MOS, Toulouse, France) integrated into the E-nose system (Hong et al., 2021).

Electronic tongue (E-tongue)

An Astree electronic tongue system (Alpha MOS) was used to examine the taste of the samples. The samples were diluted 1:100 in distilled water, homogenized, and filtered prior to analysis. The E-tongue sensor includes five taste component sensors (AHS-sourness, CTS-saltiness, NMS-umami, PKS-sweetness, and ANS-bitterness) and two index sensors (CPS and SCS) that simulate human sensory responses. The sensor values of CPS and SCS were employed for calibration. For analysis, the E-tongue sensor was exposed to the sample extract for 2 min, and the strength of the sensor response to individual taste components was measured through contact. To ensure accuracy and prevent cross-contamination, the individual taste component sensors were washed with purified water for each analysis. The results were verified using AlphaSoft version 14.2 (Hong et al., 2021; Tian et al., 2020).

Statistical analysis

All experiments were conducted in at least three replicates. Enzyme activity assays, growth assays, color analyses, and sensory evaluations were performed by one-way analysis of variance using the GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). TPA and TBARS assays were performed using independent t-tests. Significant differences between data were determined using Dunnett's tests ($P < 0.05$).

Results and Discussion

Isolation and identification of *P. nalgiovense*

Of the 72 strains obtained from Nuruk, Jeotgal, and the mudflats, 24 were chosen based on their colony morphology. They all belonged to the genus *Penicillium* (Table 4). The two strains were definitively identified as *P. nalgiovense* by ITS sequencing. The resulting phylogenetic tree is presented in Fig 1. A for *Penicillium nalgiovense* OBF SD01 and Fig 1. B for *Penicillium nalgiovense* OBF SJ02. Microscopic analysis of the conidial morphology of the two selected strains confirmed the characteristic *Penicillium* conidial shape (Fig 2). Under the microscope, the combination of septate hyphae, branching at acute angles, erect conidiophores with radiate conidial heads, and chains of conidia give *Penicillium* its unique and recognizable appearance. These strains were designated *P. nalgiovense* OBF SD01 (KACC83057BP) and *P. nalgiovense* OBF SJ02 (KACC83058BP) and were formally deposited in the Korean Agricultural Culture Collection (KACC).

Mycotoxin production by the fungal starter is shown in Fig 3. Consistent with previous studies, the toxin production capabilities of the isolates were evaluated alongside *P. roquefortii* Thom (KCTC 6080) and *P. chrysogenum* (KCTC 6933), which are mycotoxin-producing strains as positive strains (Andersen and Frisvad, 1994; El-

banna et al., 1987; Laich et al., 1999; Lopez-Diaz and Flannigan, 1997; López-Díaz et al., 2001; Ludemann et al., 2009; Papagianni et al., 2007).

DNA extracted from both *P. roquefortii* Thom (KCTC 6080) and *P. chrysogenum* (KCTC 6933) as positive controls and selected strains was identified as belonging to the genus *Penicillium*. This identification was based on the amplified sequences of the ITS and β-tubulin housekeeping genes specific to *P. nalgiovense* (data not shown). The expression of two PC strains producing Mycophenolic acid and Penicillin was also confirmed. Upon evaluation of the isolated strains SD01 and SJ02, it was observed that the major mycotoxins producing genes including Aflatoxin, Ochratoxin A, Patulin, and Cyclopiazonic acid, were not detected. However, Sterigmatocystin (fluG), Penicillin (pcbAB), Penicillin (penDE), Mycophenolic acid (mpaC), Roqueforine C (rds/roqA), and Penicillin (pcbC) was detected. Profiles of genes exhibited patterns similar to those of the M600 commercial strain. Despite *P. nalgiovense* being the most frequently used starter culture for aged and fermented meat products, the fact that this fungus can secrete penicillin into the meat product underscores the importance of obtaining strains that are incapable of synthesizing this antibiotic (Andersen and Frisvad, 1994). It appears that the M600 strain can also secrete antibiotics, suggesting the importance of employing gene disruption techniques to further ensure the safety of our strains for application in the food industry(Laich et al., 2003). However, it has been reported that significant amounts of penicillin were found in the casing and the outer layer of salami meat during the early stages of the curing process, coinciding with fungal colonization, but no penicillin was detected in the cured salami (Laich et al., 1999). Therefore, even though the penicillin synthesis genes exist, the potential for them to be eliminated by penicillinase during the ripening of fermented sausages suggests that our strains might be necessary for faster application in the food industry.

Enzymatic activity assay

The enzymatic activity of protease and lipase plays a crucial role in the manufacturing of dry fermented sausages, significantly influencing both the flavor and texture of the final product. Therefore, evaluating the enzymatic activity is considered essential for discovering new fungal starters (Magistà et al., 2016). To observe the proteolytic and lipolytic enzyme activities of three *Penicillium nalgiovense* strains (SD01, SJ02, and M600), spores were cultured on SA and SAP media for measuring proteolytic enzyme activity and B20 and B80 media for lipolytic enzyme activity. The clearance halo areas around the colonies were measured from day 2 to day 9. Figure 4, A displays colony corresponding to days 2, 5, and 10, and quantitative values for the EI (Enzyme Index) were presented (Fig 4, B). SJ02 exhibited the earliest clearance halo on SA media, followed by M600 showing similar activity, while SD01 showed the latest activity on day 9. However, in SAP media supplemented with PDA, which contained rich nutrition to fungal growth, M600 and SD01 showed similar levels of activity, with SJ02 displaying slightly delayed activity. The strain not significantly affected by the nutrient environment in terms of proteolytic activity are suggested to be the control fungi M600 and SJ02. Moreover, lipolytic enzyme activity of SJ02 was shown to be the highest in both B20 and B80 media, particularly showing a continuous increase trend in B20.

In the CCFE activity assay, precipitation zone reflects lipolytic enzyme activity. The precipitation zone diameter of SJ02 (2.184 mm) was significantly larger than that of M600 (1.635 mm) at the first day. On the second day, the lipolytic enzyme activity of M600 was 2.168 mm, whereas that of SJ02 was 2.668 mm, confirming that the lipolytic enzyme activity of SJ02 had substantially increased (Fig 5). These findings suggest that the two isolated strains of *P. nalgiovense* we identified could potentially contribute

more effectively to the breakdown of fat and protein in meat and to the traditional flavor development process than the M600 strain (Toldrá, 2010).

The findings suggest that SJ02, in particular, might be a superior candidate due to its high proteolytic and lipolytic activities, which are crucial for the development of flavor and texture in dry fermented sausages (Galvalisi et al., 2012). The use of strains like SJ02 could contribute to enhancing the quality of meat products by promoting more efficient breakdown of proteins and fats, thus potentially improving the sensory characteristics of the final product. Recently, there has been active research into utilizing lactic acid bacteria starters, such as *Staphylococcus* or *Lactobacillus*, to further enhance the flavor of fermented sausages (Kieliszek et al., 2021; Uppada et al., 2017; Wang et al., 2022). It is necessary to discover Korean fermented food-derived lactic acid bacteria that can provide optimal sensory qualities with SJ02 traits.

Optimization of growth rate

Because *P. nalgiovense* is widely recognized for its beneficial role in the early colonization of fermented sausages, contributing significantly to the flavor development, prevention of undesirable molds, appearance and safety of the final product, it is well known that it is very important for candidate fungi to proliferate rapidly in the early stages (Laranjo et al., 2019; Magistà et al., 2016). To compare the growth of fungi without directly manufacturing sausages, we prepared culture dishes under the seasoning conditions as those used for sausage production (Manufacture of dry fermented sausages in Materials and Methods) and inoculated these plates with spores from each strain to observe their growth. The assessment of growth rates on meat plates yielded similar growth patterns for the three strains, establishing strain adaptability and growth performance (Fig 6). To determine the optimal salinity

conditions, the growth rates were compared at different concentrations of NaCl. The growth rates of all three strains were higher at 2% NaCl than at other concentrations. Notably, SJ02 exhibited a significantly higher growth rate (16.47 mm) on day 3 than SD01 (15.48 mm) and M600 (13.19 mm). These results indicate that SJ02 is a promising strain for producing industrially fermented meat products.

Microbiological safety of dry fermented sausage

To ensure the safety against undesirable bacteria, specifically pathogenic bacteria, when making fermented sausages using SJ02 and M600, both quantitative and qualitative analyses of *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella spp*. were examined. Additionally, the mycotoxins Aflatoxin and Ochratoxin A were also analyzed. The microbiological safety and toxin safety of fermented sausages using the SJ02 strain were verified to be as safe as those with the M600 strain. No quantitative changes in the lactic acid bacteria starter named Bactoferm TRADI 302, contains *Lactobacillus sakei*, *Staphylococcus carnosus*, and *Staphylococcus xylosus* were observed (Table 5). The application of SJ02 in dry fermented sausage production can ensure food safety by preventing the proliferation of harmful foodborne pathogens (Bernaldez et al., 2017; Büchter et al., 2020; Lester and L., 2018).

Quality assay of dry fermented sausages

As fatty acid oxidation progresses, levels of malondialdehyde (MDA) and acetal compounds increase. These compounds are correlated with food deterioration and thus serve as parameters of meat product freshness. The reaction of 2-TBA with these compounds produces a colored complex, which is a widely accepted evaluating method

of the extent of fatty acid degradation and oxidation. Unsaturated fatty acids are susceptible to oxidation, generating peroxides such as hydroperoxides that can damage proteins and DNA, induce mutations and carcinogenesis, and compromise food quality. A TBARS value exceeding 1 MDA ppm (μg/mL) is considered high rancidity and renders the product inedible (Administration, 2009). The TBARS value for dry fermented sausages with SJ02 was <1 MDA ppm (μg/mL); it is considered safe for fatty acid deterioration in meat products (Table 6).

The dry fermented sausages made with SJ02, identified from domestic source in this study, have consequently similar color and texture characteristics compared with that made with the commercial starter M600. Specifically, no significant differences were observed between M600 and SJ02 sausages. In addition, textural indicators, such as hardness, cohesiveness, gumminess, and chewiness, were not significantly different between sausages made with M600 and SJ02 when analyzed using a texture analyzer. Only adhesiveness value in SJ02 sausages was higher ($P<0.05$) than that in M600 sausages (Table 6). Notably, one of main role of fungal starters have been recognized influencing the flavor and texture of dry fermented sausages (Casaburi et al., 2008). These factors significantly shape consumer preferences and have been actively investigated to enhance the quality of dry fermented sausages (Bourdichon et al., 2012; Fadda et al., 2010). Similar quality characteristics with the current research can be observed in previous studies (Chinaglia et al., 2014; Fadda et al., 2001; Seong et al., 2008; Tian et al., 2020; Witte et al., 1970; Yang et al., 2018; Zhao et al., 2011).

E-nose and E-tongue analyses

The E-nose technology have been used to determine aroma and taste profiles in targeted food products, serving as an indicator of the olfactory and gustatory

perceptions experienced by consumers during the consumption of food. The present study used the E-nose technique to systemically evaluate the distinct flavor and taste of dry fermented sausages influenced by each strain (Jeong et al., 2022).

In the E-nose analysis, M600 sample exhibited elevated levels of ethanol and propanal, which are associated with pungent odors, in comparison to SJ02 sample. Conversely, the concentrations of propan-2-one and butyl butanoate, which represent fruity characteristics, were higher in SJ02 than in M600 (Fig 7). Notably, E-nose analysis revealed that the different utilization of the fungal starters, M600 and SJ02, in fermented dry sausages led to variation in the composition of the final products. These observations suggest the potential for future investigations to identify difference in flavor and taste between sausages manufactured using M600 and SJ02 strains, thus confirming the distinct charateristics of SJ02-isolated fermented sausages in South Korea.

The results of the E-tongue analysis are presented as a radar graph, representing taste profiles measured by the E-tongue in dry fermented sausages using the fungal starters M600 and SJ02 (Fig 7). Each taste parameter, along with representative taste qualities, was determined using five taste sensors (AHS, PKS, CTS, NMS, and ANS) (Ju et al., 2016).

The AHS sensor, which measures acidity and bitterness, primarily captures the acidity in fermented dried sausages. The AHS values were recorded as 7.3 ± 0.2 for M600 and 4.7 ± 2.0 for SJ02, indicating lower level of bitterness in SJ02 and a higher level of acidity in M600. The CTS sensor, which evaluates saltiness, yielded values of 5.1 ± 0.0 for M600 and 7.0 ± 0.9 for SJ02, highlighting a higher level of saltiness in the SJ02 sample. With regard to NMS sensor responsible for savory and salty tastes, M600 recorded 6.3 ± 0.0, while SJ02 displayed 5.8 ± 0.2, indicating a greater savory taste in

M600. Finally, the ANS sensor, which detects bitterness, recorded 5.7 ± 0.2 for M600 and 6.3 ± 0.2 for SJ02, signifying elevated bitterness in SJ02.

When comparing the taste characteristics of fermented dried sausages produced using strains M600 and SJ02 through E-tongue analysis, no statistically significant differences were observed in the measured values between the two strains. The findings imply that fermented dried sausages crafted using the two strains had similar taste characteristics. Therefore, the potential utilization of the SJ02 strain for adjusting the taste attributes of fermented dried sausages in accordance with the microbial strain is suggested. However, further studies are necessary including sensory evaluations conducted by trained panels that consider a range of diverse taste perceptions (Van Ba et al., 2018).

Conclusions

This study identified two *Penicillium nalgiovense* strains, SD01 and SJ02, as potential starter cultures for fermented sausages from Korean sources. SJ02, in particular, showed enhanced enzymatic activities and growth in high-salinity conditions compared to a commercial starter, Mold600. Fermented sausages applied with SJ02 exhibited improved enzymatic activity and safety, and we found that *P. nalgiovense* SJ02 exhibited similar qualities to *P. nalgiovense* M600, confirming the potential of our isolate SJ02 as a domestic commercial strain to replace M600.

Acknowledgment

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References

Administration RD. 2009. Development of strarter for the production of korean style fermented sausage. Suwon: Rural Development Administration.

Administration RD. 2018. Screening of starter cultures and development of utilization technology for korean fermented sausage. Jeonju: Rural Development Administration.

Andersen S, Frisvad J. 1994. Penicillin production by penicillium nalgiovense. Letters in Applied Microbiology 19:486-488.

Bernaldez V, Cordoba JJ, Andrade MJ, Alia A, Rodriguez A. 2017. Selection of reference genes to quantify relative expression of ochratoxin a-related genes by penicillium nordicum in dry-cured ham. Food Microbiol 68:104-111.

Bernáldez V, Córdoba JJ, Rodríguez M, Cordero M, Polo L, Rodríguez A. 2013. Effect of penicillium nalgiovense as protective culture in processing of dry-fermented sausage “salchichón”. Food Control 32:69-76.

Biaggio RT, Silva RRD, Rosa NGD, Leite RSR, Arantes EC, Cabral TPDF, Juliano MA, Juliano L, Cabral H. 2016. Purification and biochemical characterization of an extracellular serine peptidase from aspergillus terreus. Preparative Biochemistry and Biotechnology 46:298-304.

Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, Harnett J, Huys G, Laulund S, Ouwehand A. 2012. Food fermentations: Microorganisms with technological beneficial use. International journal of food microbiology 154:87-97.

Büchter C, Koch K, Freyer M, Baier S, Saier C, Honnen S, Wätjen W. 2020. The mycotoxin beauvericin impairs development, fertility and life span in the nematode caenorhabditis elegans accompanied by increased germ cell apoptosis and lipofuscin accumulation. Toxicology Letters 334:102-109.

Casaburi A, Di Monaco R, Cavella S, Toldra F, Ercolini D, Villani F. 2008. Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. Food Microbiol 25:335-347.

Chinaglia S, Chiarelli LR, Maggi M, Rodolfi M, Valentini G, Picco AM. 2014. Biochemistry of lipolytic enzymes secreted by penicillium solitum and cladosporium cladosporioides. Biosci Biotechnol Biochem 78:245-254.

Chung K-Y, Lee M-S, Yim D-G. 2017. The effects of different curing agents on vbn, tba and microbial changes of fermented sausages during aging. Journal of Agriculture & Life Science 51:141-147.

El-Banna AA, Pitt JI, Leistner L. 1987. Production of mycotoxins by penicillium species.

ACCEPTED

512 Systematic and Applied Microbiology 10:42-46.
513 Essid I, Hassouna M. 2013. Effect of inoculation of selected staphylococcus xylosus and
514 lactobacillus plantarum strains on biochemical, microbiological and textural
515 characteristics of a tunisian dry fermented sausage. Food Control 32:707-714.
516 Fadda S, López C, Vignolo G. 2010. Role of lactic acid bacteria during meat conditioning
517 and fermentation: Peptides generated as sensorial and hygienic biomarkers. Meat
518 science 86:66-79.
519 Fadda S, Vignolo G, Aristoy MC, Oliver G, Toldrá F. 2001. Effect of curing conditions
520 and lactobacillus casei crl705 on the hydrolysis of meat proteins. Journal of Applied
521 Microbiology 91:478-487.
522 Färber P, Geisen R. 1994. Antagonistic activity of the food-related filamentous fungus
523 penicillium nalgiovense by the production of penicillin. Applied Environmental
524 Microbiology 60:3401-3404.
525 Frisvad JC, Houbraken J, Popma S, Samson RA. 2013. Two new penicillium species
526 penicillium buchwaldii and penicillium spathulatum, producing the anticancer
527 compound asperphenamate. FEMS Microbiol Lett 339:77-92.
528 Galvalisi U, Lupo S, Piccini J, Bettucci L. 2012. Penicillium species present in uruguayan
529 salami. Rev Argent Microbiol 44:36-42.
530 Geisen R, Lücke F-K, Kröckel L. 1992. Starter and protective cultures for meat and meat
531 products. Fleischwirtschaft (Frankfurt) 72:894-898.
532 Hong SJ, Boo CG, Heo SU, Jo SM, Jeong H, Yoon S, Lee Y, Park S-S, Shin E-C. 2021.
533 Investigation of taste and flavor properties of radish varieties harvested in korea using
534 electronic tongue and electronic nose. Korean Journal of Food Science and Technology
535 53:375-381.
536 Jeong C-H, Lee S-H, Kim H-Y. 2022. Microbiological composition and sensory
537 characterization analysis of fermented sausage using strains isolated from korean
538 fermented foods. Food Science of Animal Resources 42:928.
539 Ju M-G, Kim J-H, Jang H-J, Yeon S-J, Hong G-E, Park W, Seo HG, Lee C-H. 2016.
540 Changes of physicochemical and sensory properties of fermented sausage from sulfur-
541 fed pork. Korean Journal for Food Science of Animal Resources 36:729.
542 Jung Y-S, Yoon H-H. 2019. Quality characteristics of commercial dry fermented sausages.
543 J East Asian Soc Diet Life 29:319-325.
544 Kieliszek M, Pobiega K, Piwowarek K, Kot AM. 2021. Characteristics of the proteolytic

ACCEPTED

545 enzymes produced by lactic acid bacteria. Molecules 26.
546 Kim M-G, Oh M-H, Lee G-Y, Hwang I-G, Kwak H-S, Kang Y-S, Koh Y-H, Jun H-K,
547 Kwon K-S. 2008. Analysis of major foodborne pathogens in various foods in korea. J
548 Food Science Biotechnology 17:483-488.
549 Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions
550 through comparative studies of nucleotide sequences. Journal of molecular evolution
551 16:111-120.
552 Laich F, Fierro F, Cardoza RE, Martin JF. 1999. Organization of the gene cluster for
553 biosynthesis of penicillin in penicillium nalgiovense and antibiotic production in cured
554 dry sausages. Applied Environmental Microbiology 65:1236-1240.
555 Laich F, Fierro F, Martin JF. 2003. Isolation of penicillium nalgiovense strains impaired
556 in penicillin production by disruption of the pcbab gene and application as starters on
557 cured meat products. Mycol Res 107:717-726.
558 Laranjo M, Potes ME, Elias M. 2019. Role of starter cultures on the safety of fermented
559 meat products. Front Microbiol 10:853.
560 Leistner L. 1990. Mould-fermented foods: Recent developments. Food Biotechnology
561 4:433-441.
562 Leistner L. Starter and protective cultures for foods in europe. Microbes for better living,
563 Micon 94 and 35 th AMI Conference. p^pp 417-423.
564 Lester H, L. AS. 2018. The use of solid media for detection of enzyme production by
565 fungi. Mycologia 67:597-607.
566 Lester H, Sl A. 1975. The use of solid media for detection of enzyme production by fungi.
567 Mycologia 67:597-607.
568 Lopez-Diaz T-M, Flannigan B. 1997. Mycotoxins of aspergillus clavatus: Toxicity of
569 cytochalasin e, patulin, and extracts of contaminated barley malt. Journal of food
570 protection 60:1381-1385.
571 López-DíAz T, Santos J, García-López M, Otero A. 2001. Surface mycoflora of a spanish
572 fermented meat sausage and toxigenicity of penicillium isolates. International Journal
573 of Food Microbiology 68:69-74.
574 Ludemann V, Pose G, Moavro A, Maliaviabarrena MG, Fandiño R, Ripoll G, Basílico JC,
575 Pardo AG. 2009. Toxicological assessment of penicillium nalgiovense strains for use
576 as starter cultures in the manufacture of dry fermented sausages. Journal of food
577 protection 72:1666-1670.

ACCEPTED

Lumi Abe CA, Bertechini Faria C, Fernandes De Castro F, De Souza SR, Dos Santos FC, Novais Da Silva C, Tessmann DJ, Barbosa-Tessmann IP. 2015. Fungi isolated from maize (zea mays l.) grains and production of associated enzyme activities. International journal of molecular sciences 16:15328-15346.

Magistà D, Ferrara M, Del Nobile M, Gammariello D, Conte A, Perrone GJIJOFM. 2016. Penicillium salamii strain item 15302: A new promising fungal starter for salami production. 231:33-41.

Moavro A, Stenglein S, Delfederico L, Wagner J, Ludemann V. 2019. Novel use of penicillium nalgiovense on stuffed semi–hard and hard cheeses. Lwt 110:255-261.

Ojo-Okunola A, Claassen-Weitz S, Mwaikono KS, Gardner-Lubbe S, Zar HJ, Nicol MP, Du Toit E. 2020. The influence of DNA extraction and lipid removal on human milk bacterial profiles. Methods and protocols 3:39.

Papagianni M, Ambrosiadis I, Filiousis G. 2007. Mould growth on traditional greek sausages and penicillin production by penicillium isolates. Meat Sci 76:653-657.

Philipp S, Pedersen P. 1988. Mould cultures for the food industry: A short review with special reference to the cheese-and sausage production. Danish Dairy and Food Industry (Denmark).

Radulović Z, Živković D, Mirković N, Petrušić M, Stajić S, Perunović M, Paunović D. 2011. Effect of probiotic bacteria on chemical composition and sensory quality of fermented sausages. Procedia Food Science 1:1516-1522.

Rodríguez A, Rodríguez M, Luque MI, Justesen AF, Córdoba JJ. 2012. A comparative study of DNA extraction methods to be used in real-time pcr based quantification of ochratoxin a-producing molds in food products. Food Control 25:666-672.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular biology and evolution 4:406-425.

Seong P-N. 2013. Technology development prospects of fermented meat products. Korean J Food Sci Ind Anim

Resour 2:19-26.

Seong P-N, Kim J-H, Cho S-H, Lee C-H, Kang D-W, Hah K-H, Lim D-G, Park B-Y, Kim D-H, Lee J-M. 2008. The effects of salt and nano 2 on physico-chemical characteristics of dry-cured ham. Food Science of Animal Resources 28:493-498.

Singh B, Dincho D. 1994. Molds as protective cultures for raw dry sausages. Journal of food protection 57:928-930.

ACCEPTED

611 Spotti E, Berni E, Cacchioli C. 2008. Characteristics and applications of molds. In Meat
612 biotechnology. Toldrá F (ed.). Springer New York, New York, NY.
613 Tian X, Li ZJ, Chao YZ, Wu ZQ, Zhou MX, Xiao ST, Zeng J, Zhe J. 2020. Evaluation by
614 electronic tongue and headspace-gc-ims analyses of the flavor compounds in dry-cured
615 pork with different salt content. Food Research International 137:109456.
616 Toldrá F. 2010. Handbook of meat processing. John Wiley & Sons.
617 Uppada SR, Akula M, Bhattacharya A, Dutta JR. 2017. Immobilized lipase from
618 lactobacillus plantarum in meat degradation and synthesis of flavor esters. J Genet Eng
619 Biotechnol 15:331-334.
620 Van Ba H, Seo H-W, Seong P-N, Kang S-M, Kim Y-S, Cho S-H, Park B-Y, Ham J-S, Kim
621 J-H. 2018. Lactobacillus plantarum (kacc 92189) as a potential probiotic starter culture
622 for quality improvement of fermented sausages. Korean Journal for Food Science of
623 Animal Resources 38:189.
624 Wang J, Hou J, Zhang X, Hu J, Yu Z, Zhu Y. 2022. Improving the flavor of fermented
625 sausage by increasing its bacterial quality via inoculation with lactobacillus plantarum
626 msz2 and staphylococcus xylosus ycc3. Foods 11.
627 Witte V, Krause G, Bailey M. 1970. A new extraction method for determining 2-
628 thiobarbituric acid values of pork and beef during storage. Journal of food Science
629 35:582-585.
630 Woo S-H, Sung J-M, Park H, Kim J, Kim Y-J, Kim T-K, Lee H, Choi Y-S. 2023. Inhibitory
631 effect of natural extract mixtures on microbial growth and lipid oxidation of sausages
632 during storage. Journal of Animal Science and Technology 65:225-243.
633 Yang Z, Xue KS, Sun X, Williams PL, Wang JS, Tang L. 2018. Toxicogenomic responses
634 to zearalenone in caenorhabditis elegans reveal possible molecular mechanisms of
635 reproductive toxicity. Food Chem Toxicol 122:49-58.
636 Yim D-G, Ali M, Nam K-C. 2020a. Comparison of meat quality traits in salami added by
637 nitrate-free salts or nitrate pickling salt during ripening. Food Science of Animal
638 Resources 40:11-20.
639 Yim D-G, Shin D-J, Jo C, Nam K-C. 2020b. Effect of sodium-alternative curing salts on
640 physicochemical properties during salami manufacture. Food Science of Animal
641 Resources 40:946-956.
642 Yoon JY, Kim D, Kim E-B, Lee S-K, Lee M, Jang A. 2018. Quality and lactic acid bacteria
643 diversity of pork salami containing kimchi powder. Korean journal for food science of

ACCEPTED

644 animal resources 38:912-926.
645 Zhao G, Lin X, Dou W, Tian S, Deng S, Shi J. 2011. Use of the smart tongue to monitor
646 mold growth and discriminate between four mold species grown in liquid media. Anal
647 Chim Acta 690:240-247.
648
649
650 **Figure Legends**
651
652 **Fig 1. Phylogenetic analysis of ITS1, 4 sequence of *Penicillium nalgiovense* SD01**
653 **(A) and SJ02 (B)**. The number in each branch indicates bootstrap values obtained after
654 a bootstrap test with 1000 replications. The neighbor-joining tree displays values of
655 divergence between ITS region sequences.
656
657 **Fig 2. Microscopic image of *Penicillium nalgiovense* isolates stained using**
658 **lacophenol cotton blue stain.**
659 Conidiophores of *P. nalgiovense* isolates and M600 (100×). M600, commercial starter
660 culture; SD01, *P. nalgiovense* SD01; SJ02, *P. nalgiovense* SJ02.
661
662 **Fig 3. PCR screening of mycotoxin target gene from *Penicillium* strains.** (A) M600,
663 commercial starter culture. (B) SD01, *Penicillium nalgiovense* SD01. (C) SJ02, *P.*
664 *nalgiovense* SJ02. L, 100 bp Plus DNA Ladder; line 1, β-tubulin (BenA); line 2,
665 Aflatoxin (omt-1); line 3, Ochratoxin A (otanpsPN); line 4, Patulin (idh); line 5,
666 Sterigmatocystin (fluG); line 6, Cyclopiazonic acid (dmaT); line 7, Penicillin (pcbAB);
667 line 8, Penicillin (penDE); line 9, Mycophenolic acid (mpaC); line 10, Roqueforine C
668 (rds/roqA); and line 11, Penicillin (pcbC).
669

Fig 4. Enzymatic Index (EI) calculated activity of the isolates. Indices of activity determined in proteolytic (SA and SAP) and lipolytic (B20 and B80) plate assays. M600, commercial starter culture; SD01, *Penicillium nalgiovense* SD01; SJ02, *P. nalgiovense* SJ02. SA and SAP were detected at 2 ~9 days. B20 and B80 plates were detected at 5 ~9 days.

Fig 5. Extracellular enzymatic activities confirmed in assays of crude cell free extract (CCFE). M600, commercial starter culture; SD01, *Penicillium nalgiovense* SD01; SJ02, *P. nalgiovense* SJ02. All strains were cultured in Proteo M broth and CHO lipo broth to generate CCFE. These extracts were used for the enzymatic assays, with clearance halos on SKMA and precipitation zones on BMT20. The results strongly indicate the extracellular localization of enzymatic activity. Statistical significance is indicated as *P < 0.001 compared to M600.

Fig 6. Growth optimization. (A) Growth optimization of fungal starters on meat plates for dry fermented sausage. M600, commercial starter culture; SD01, *Penicillium nalgiovense* SD01; SJ02, *P. nalgiovense* SJ02. (B) Optimization of growth rate of the isolates evaluated on solid media using different concentrations of NaCl. In comparison to 1% NaCl, significant differences in growth rates are denoted as *P < 0.05, **P < 0.01, and ***P < 0.001. A graph comparing the growth rates of fungal starters at the optimized salinity level (2% NaCl) with M600 as the reference showed significant differences (*P < 0.05 and **P < 0.01).

Fig 7. Sensory compounds of dry fermented sausage analyzed by E-nose and E-tongue. (A) Volatile compounds of dry fermented sausage using fungal starters. Peaks

695 are reported in order of elution: 1, ethanol; 2, propan-2-one; 3, 2-methylpropanal; 4,
696 butan-2-one; 5, methyl propanoate; 6, 3-methyl-1-butanol; 7, (E)-2-hexenal; 8, butyl
697 butanoate; 9, 1, 8-cineole; 10, propanal; 11, propan-2-one; 12, butanal; 13, butan-2-one;
698 14, 3-methyl-1-butanol; and 15, 4-methylhexan-1-ol. M600, manufactured fermented
699 sausage with commercial starter culture; SJ02, manufactured fermented sausage with *P.*
700 *nalgiovense* SJ02. **** $P < 0.0001$ compared to M600. (B) E-tongue analysis results of
701 dry fermented sausage using fungal starters. AHS (Sourness), Sourness, Astringency,
702 Bitterness; PKS (Sweetness), Sweetness; CTS, Saltness; NMS (Umami), Umami,
703 Saltness, Astringency; and ANS, Bitterness. M600, manufactured fermented sausage
704 with commercial starter culture; SJ02, manufactured fermented sausage with *P.*
705 *nalgiovense* SJ02.
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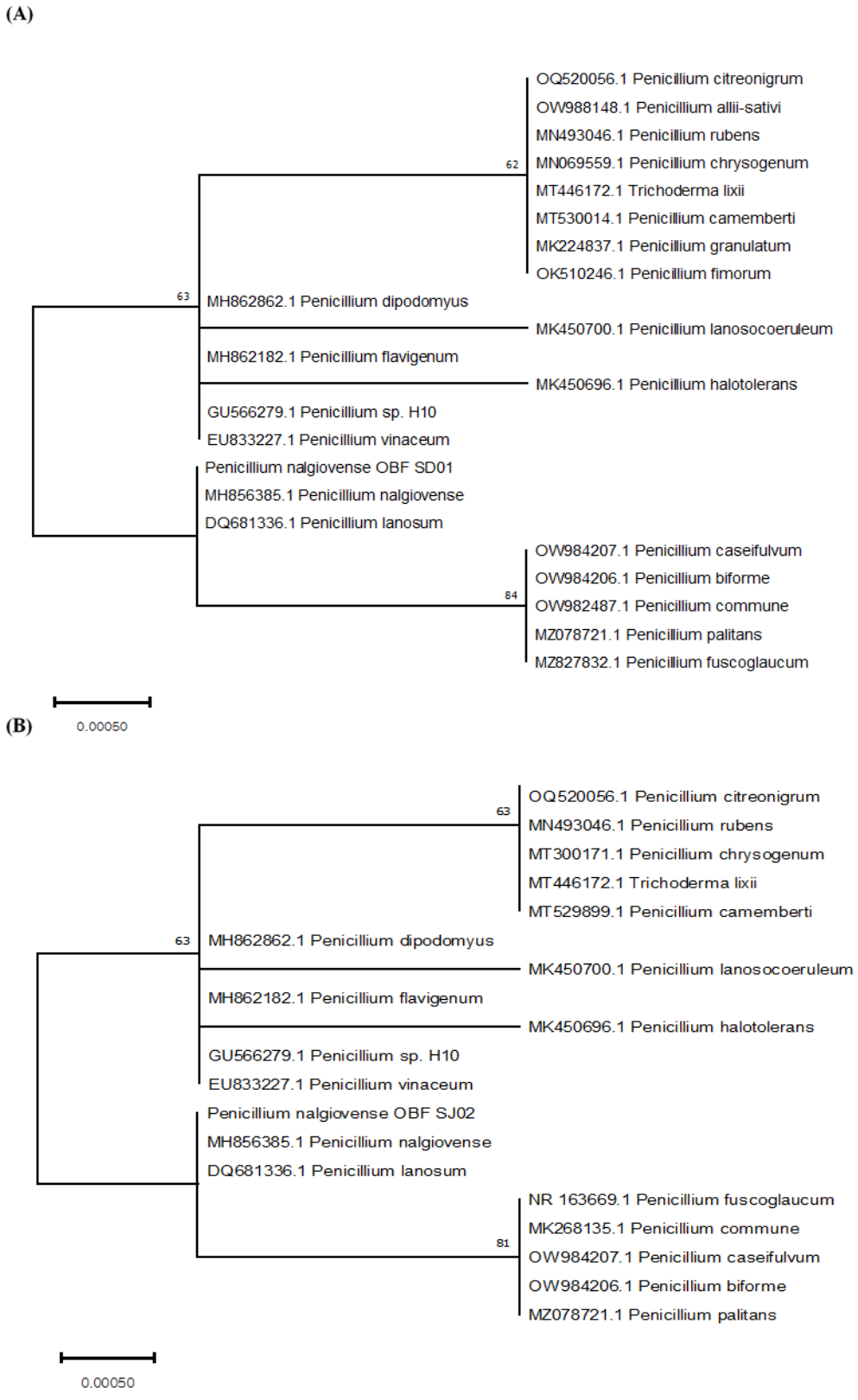
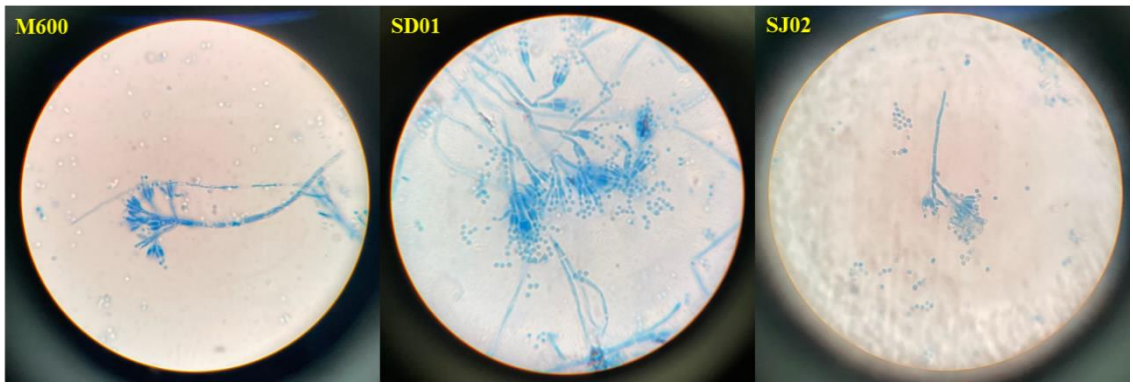
Tables and Figures

Figure 1



712 **Figure 2**

713 

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715 **Figure 3**

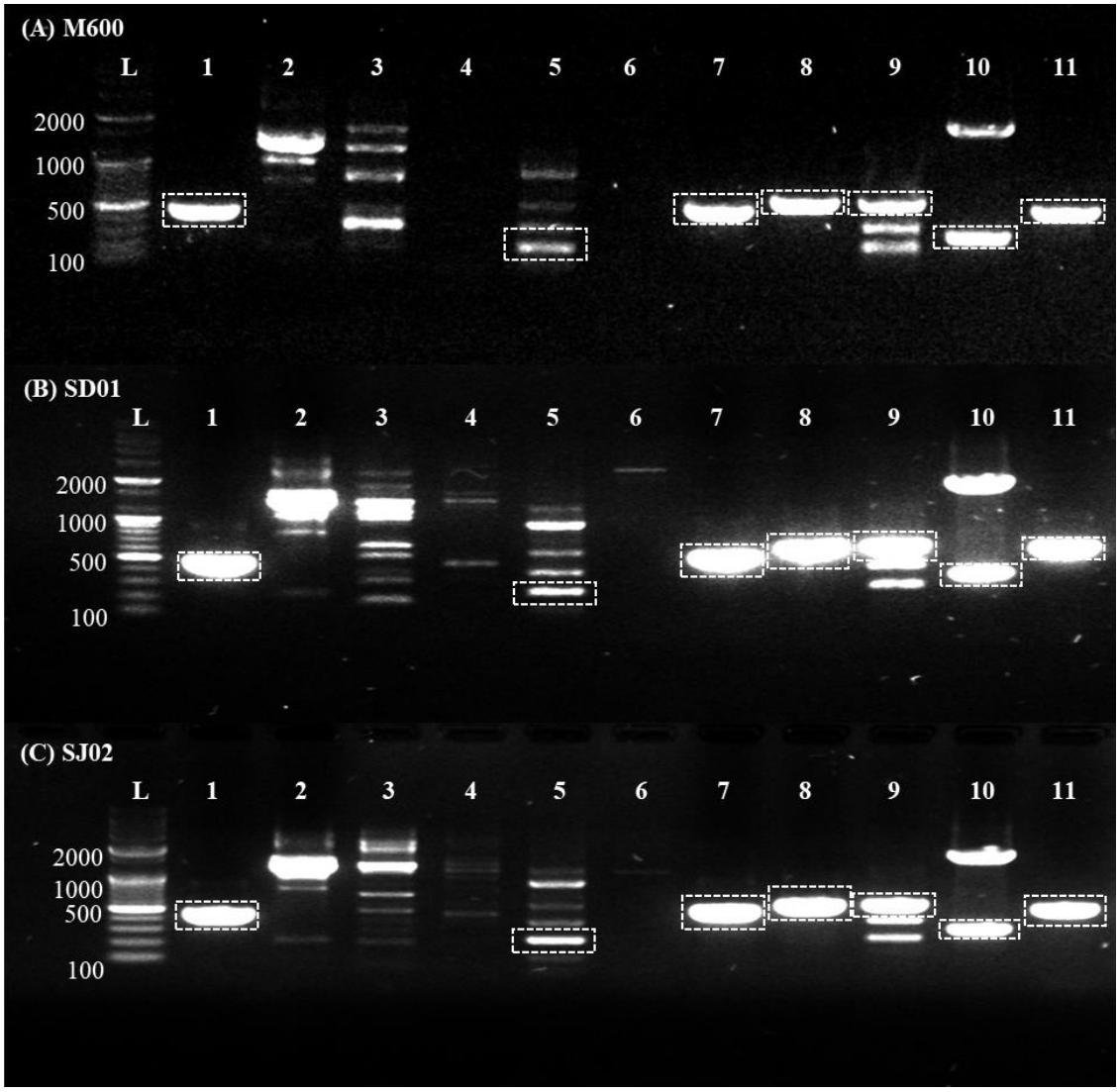

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Figure 4

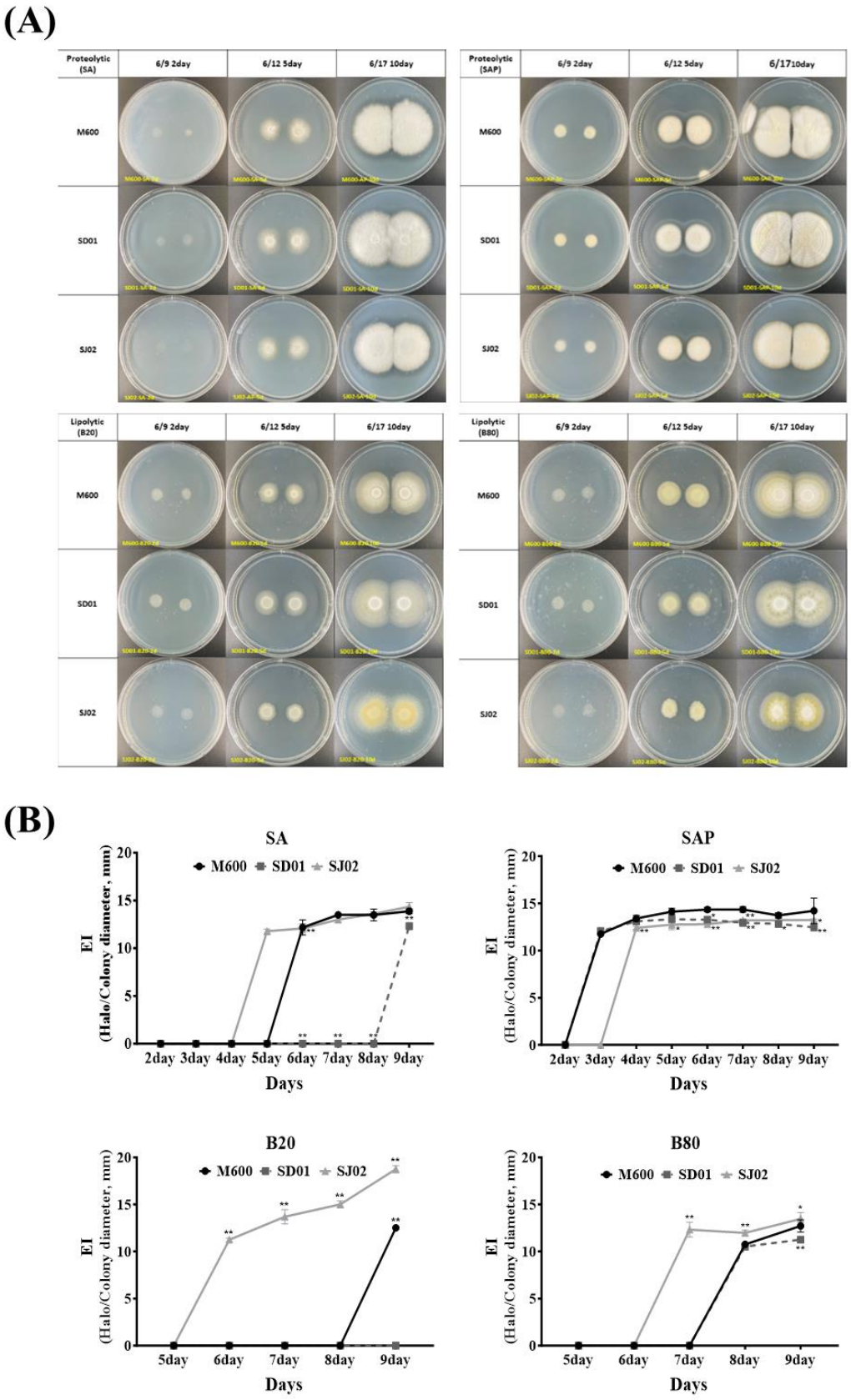

(A)
Proteolytic (SA)
6/9 2day
6/12 5day
6/17 10day
M600
SD01
SJ02
Proteolytic (SAP)
6/9 2day
6/12 5day
6/17 10day
M600
SD01
SJ02
Lipolytic (B20)
6/9 2day
6/12 5day
6/17 10day
M600
SD01
SJ02
Lipolytic (B80)
6/9 2day
6/12 5day
6/17 10day
M600
SD01
SJ02
(B)
SA
M600 SD01 SJ02
EI (Halo/Colony diameter, mm)
2day 3day 4day 5day 6day 7day 8day 9day
Days
SAP
M600 SD01 SJ02
EI (Halo/Colony diameter, mm)
2day 3day 4day 5day 6day 7day 8day 9day
Days
B20
M600 SD01 SJ02
EI (Halo/Colony diameter, mm)
5day 6day 7day 8day 9day
Days
B80
M600 SD01 SJ02
EI (Halo/Colony diameter, mm)
5day 6day 7day 8day 9day
Days

Figure 5

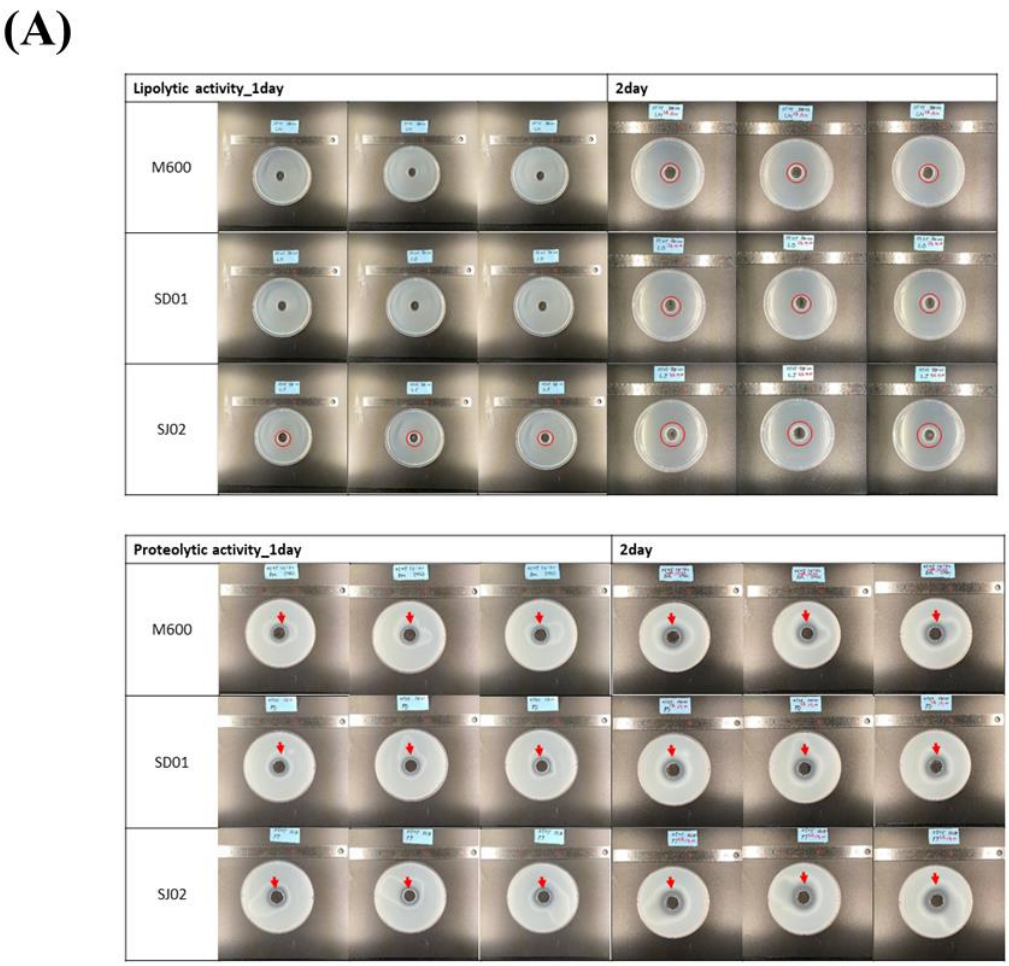
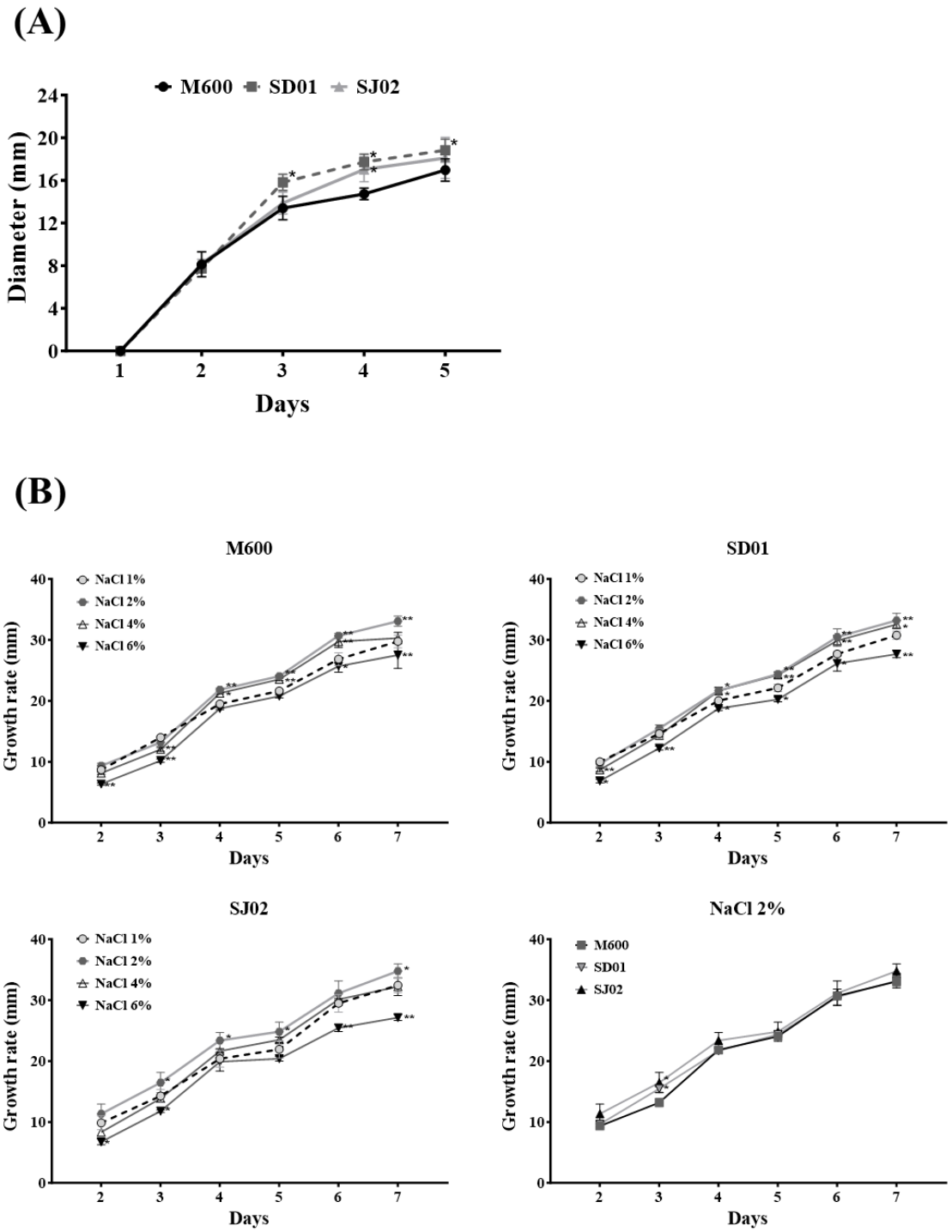
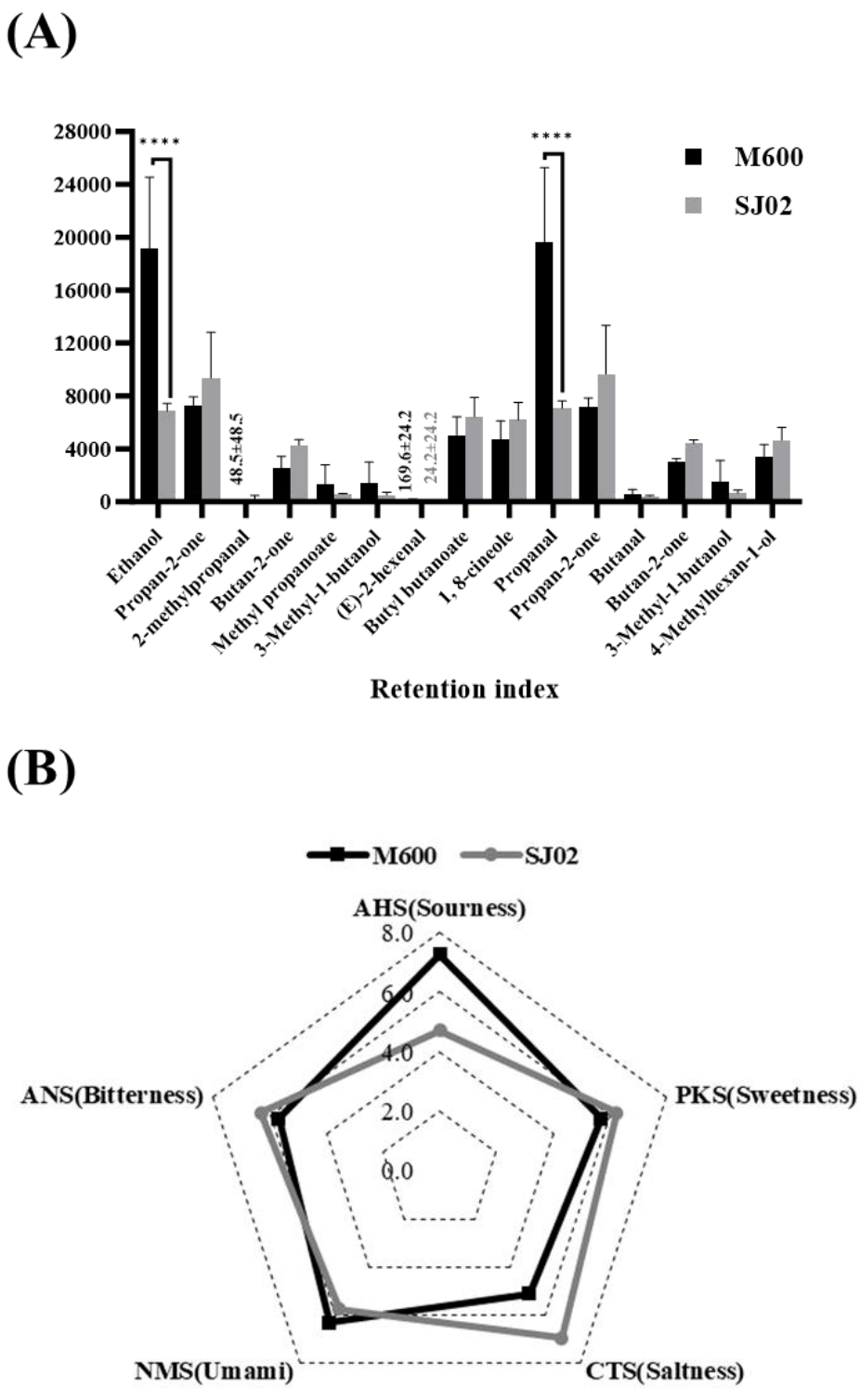

(A)
Lipolytic activity_1day
2day
M600
SD01
SJ02
Proteolytic activity_1day
2day
M600
SD01
SJ02
(B)
Proteolytic assay
Halo zone diameter(mm)
3.5
3.0
2.5
2.0
1.5
*
*
M600 SD01 SJ02 M600 SD01 SJ02
1day
2day
Lipolytic assay
Precipitation zone diameter(mm)
3.0
2.5
2.0
1.5
1.0
*
*
M600 SD01 SJ02 M600 SD01 SJ02
1day
2day

Figure 6


(A)
M600
SD01
SJ02
Diameter (mm)
Days
(B)
M600
SD01
SJ02
NaCl 2%
NaCl 1%
NaCl 2%
NaCl 4%
NaCl 6%
Growth rate (mm)
Days

733 **Figure 7**



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736 **Table 1. PCR primers used in this study**

No.	Target	Primer	Sequence	Size (bp)
1	Aflatoxin	omt-1-F	GGCCGCCGCTTTGATCTAGG	123
2		omt-1-R	ACCACGACCGCCGCC	
3	Ochratoxin A	otanpsPN-F	GCCGCCCTCTGTCATTCCAAG	117
4		otanpsPN-R	GCCATCTCCAAACTCAAGCGTG	
5	Patulin	idh-F	GGCATCCATCATCGT	229
6		idh-R	CTGTTCCTCCACCCA	
7	Sterigmatocystin	fluG-F	GAGTGCCACCGTGATGACC	172
8		fluG-R	TGATGGGTCGGTGGTTGG	
9	Cyclopiazonic acid	dmaT-F	TTCACGCTCGTGGAACTTCT	64
10		dmaT-R	GGGTCACAAAGATCGCAAGAT	
11	Mycophenolic acid	mpaC-F	TCTGTCAAGGCAGACTGGTG	587
12		mpaC-R	TCGTCCGATAGCTCAGTGTG	
13	Roqueforine C	rds/roqA-F	ACTACACCGCCATTGACTCC	360
14		rds/roqA-R	CTCAATCTCGTGCACCTCAA	
15	β-tubulin	BenA-F	GGTAACCAAATCGGTGCTGCTTTC	291
16		BenA-R	ACCCTCAGTGTAGTGACCCTTGGC	
17	pcbAB	pcbAB-F	TCGTGCTGGATGACACCAAGGCACG	551
18		pcbAB-R	CACCAGGATTATCCGATTCAGTGAT	
19	pcbC	pcbC-F	TGTGGCCGGACGAGAAGAAGCATCC	600
20		pcbC-R	TCTTGTTGATTAGACTAACTAATCC	
21	penDE	penDE-F	ACCAAAGAGAACCTGATCCGGTTAA	650
22		penDE-R	ATGACAAACATCTCATCAGGGTTGG	
23	ITS	ITS-F	TCCGTAGGTGAACCTGCGG	83
24		ITS-R	TCCTCCGCTTATTGATATGC	

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738 **Table 2. Recipes of the media used to determine enzymatic activity**

Enzymatic acitivity	Media	Ingredient	Total/1L
Protease	**SA**	Skim milk powder	10 g/100 ml
		1.5% agar	13.5 g/900 ml
		NaCl	10 g/L
	SAP	Skim milk powder	10 g/100 ml
		1.5% agar	13.5 g/900 ml
		NaCl	10 g/L
		PDA	39 g/L
Lipase	**B20**	peptone	10 g
		NaCl	5 g
		Calcium chloride dihydrate	0.1 g
		1.5% agar	15 g/L
		Tween 20	10 ml/L
	B80	peptone	10 g
		NaCl	5 g
		Calcium chloride dihydrate	0.1 g
		1.5% agar	15 g/L
		Tween 80	10 mL/L

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Table 3. Selective media for foodborne pathogens

Foodborne pathogen	Enrichment	Separation
Bacillus cereus	Sample 25 g/0.1% Peptone water 225 mL, 121℃, 15 min	MYP Agar Base 21.5 g/450 mL, 121℃, 15 min, Polymyxin B supplement 1 vial, Egg Yolk Emulsion 50 mL
Clostridium perfrigens	Cooked meat 1 g/10 mL, 121℃, 15 min, Sample solution (0.1% PW) 1 mL/10 mL	TSC agar (Perfringens Agar Base (TSC/SFP) 23 g/500 ml, 121℃, 10 min, Perfringens (TSC) Supplement 1 vial, Egg Yolk Emulsion 25 mL
Staphylococcus aureus	Sample 25 g/225 mL, Tryptone Soya Broth (CM0129, Oxoid) 30 g/L, NaCl 10 g/L, 121℃, 15 min	Baird-Parker Agar Base (CM0275, Oxoid) 31.5 g/500 ml, Egg Yolk Tellurite Emulsion (SR0054C, Oxoid) 25 mL, 121℃, 15 min
Listeria monocytogenes	Sample 25 g/225 mL, Listeria Enrichment Broth(CM0862, Oxoid) 36 g/L, Listeria Selective Enrichment Supplement (SR0141E, Oxoid) 2 vial/L, 121℃, 15 min Fraser broth 0.1 mL/10 mL, Fraser Broth Base (CM0895, Oxoid) 28.7 g/500 mL, Fraser Broth Supplement (SR0156, Oxoid) 1 vial, 121℃, 15 min	PALCAM Agar Base (CM0877B, Oxoid) 34.5 g/500 ml, PALCAM Selective Supplement (SR0150E, Oxoid) 1 vial, 121℃, 15 min
***Escherichia coli* O157:H7**	Sample 25 g/225 mL, mTSB broth (Modified Tryptic Soy) 33 g/L, Norobiocin supplement, 121℃, 15 min	TC-SMAC (Sorbitol MacConkey Agar) 25.75 g/500 mL, 121℃, 15 min, Cefixime Tellurite Selective Supplement 1 vial
***Salmonella* spp.**	Buffered Peptone Water 20 g/L, 121℃, 15 min, Sample 25 g/225 mL RV(Rappaport-Vassiliadis (RV) Enrichment Broth) 15 g/500 mL, 115℃, 15 min, Sample solution 0.1 mL/10 mL	XLT4 agar (XLT4 agar) 2935 mg/500 mL, XLT4 supplement 2.3 mL, boil for 1 min

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Table 4. Isolates identified by ITS sequencing

No.	Origin[1)]	Morphology		Identification_ITS	Strain ID
		Sample ID	Color of colony Observe(O), Reverse(R)		
1	Mudflat (Seondori-1)	C1-1-3	(O) white , (R) yellow	*Penicillium lanosum*	
2	Mudflat (Seondori-2)	C2-1-3	(O) white , (R) yellow	*P. lanosum*	
3	Mudflat (Seondori-3)	C3-2-3	(O) white , (R) yellow	*P. nalgiovense*	*P. nalgiovense* SD01
4	Mudflat (Seondori-4)	S1-1-3	(O) white , (R) white	*P. lanosum*	
5	Mudflat (Seondori-5)	S2-1-3	(O) white , (R) yellow	*P. lanosum*	
6	Mudflat (Seondori-6)	S3-1-3	(O) white , (R) yellow	*P. lanosum*	
7	Mudflat (Seondori-7)	S4-1-3	(O) white , (R) yellow	*P. camemberti*	
8	Nuruk-1	#2-2-3	(O) white , (R) white	*P. commune*	
9	Nuruk-2	#4-2-3	(O) white , (R) white	*P. camemberti*	
10	Nuruk-3	#4-3-3	(O) white , (R) yellow	*P. lanosum*	
11	Nuruk-4	#5-2-3	(O) white , (R) white	*P. camemberti*	
12	Mudflat (Seondori-1)	#7-1-3	(O) white , (R) yellow	*P. nalgiovense*	*P. nalgiovense* SJ02
13	Mudflat (Seondori-2)	#7-2-3	(O) white , (R) white	*P. camemberti*	
14	Mudflat (Seondori-3)	#7-3-3	(O) white , (R) yellow	*P. lanosum*	
15	Mudflat (Seondori-4)	#8-1-3	(O) white , (R) yellow	*P. lanosum*	
16	Mudflat (Seondori-5)	#8-2-3	(O) white , (R) white	*P. camemberti*	
17	Mudflat (Seondori-6)	#8-3-3	(O) white , (R) yellow	*P. lanosum*	
18	Mudflat (Seondori-7)	#9-2-3	(O) white , (R) white	*P. camemberti*	
19	Mudflat (Seondori-8)	#9-3-3	(O) white , (R) yellow	*P. lanosum*	
20	Mudflat (Seondori-9)	#10-1-3	(O) white , (R) yellow	*P. lanosum*	

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21	Mudflat (Seondori-10)	#10-2-3	(O) white , (R) white	*P. commune*
22	Mudflat (Seondori-11)	#10-3-3	(O) white , (R) yellow	*P. lanosum*
23	Mudflat (Seondori-12)	#10-4-3	(O) white , (R) yellow	*P. lanosum*
24	Mudflat (Seondori-13)	#10-5-3	(O) white , (R) yellow	*P. lanosum*

[1)] Mudflat (Seondori-1~13), It was isolated from Seondo-ri mud flats located in Biin-myeon, Seocheon-gun, Chungcheongnam-do, South Korea; Nuruk-1, Sanseong nuruk (Hwawangsan); Nuruk-2~3, Sanseong nuruk (Geumjeong); Nuruk-4, Songhakgokja (Soyulgok).

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Table 5. Microbiological safety assessment of dry fermented sausages

No.	Foodborne pathogen	Sample[1)]	
		M600	**SJ02**
1	*Staphylococcus aureus*	(-)	(-)
2	*Listeria monocytogenes*	(-)	(-)
3	*Salmonella spp.*	(-)	(-)
4	Total Aflatoxin, Ochratoxin A, (μg/kg)	(-)	(-)
5	Coliform, log (CFU/g)	0	0
6	Lactic acid bacteria, log (CFU/g)	9.74	9.79

[1)] M600, manufactured fermented sausage with commercial starter culture; SJ02, manufactured fermented sausage with *P. nalgiovense* SJ02; (-), negative.

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Table 6. Evaluation of quality parameters in dry fermented sausages using mold starters M600 and SJ02

Analysis	Parameter	M600[1)]	SJ02
TBARS[2)]	**MDA (μg/mL)**	0.75 ± 0.09	0.58 ± 0.09
Meat Color[3)]	**L* (lightness)**	41.82 ± 3.96	42.80 ± 4.69
	a* (redness)	9.56 ± 0.97	8.67 ± 1.11
	b* (yellowness)	6.56 ± 0.92	6.50 ± 0.85
TPA[4)]	**Hardness (N)**	2.80 ± 0.36	2.75 ± 0.50
	Cohesiveness (N)	0.23 ± 0.06	0.27 ± 0.09
	Adhesiveness (mJ)	0.68 ± 0.34	1.05 ± 0.39*
	Gumminess (N)	0.64 ± 0.20	0.73 ± 0.26
	Chewiness (N)	0.18 ± 0.16	0.20 ± 0.15

[1)] M600: manufactured fermented sausage with commercial starter culture; SJ02: manufactured fermented sausage with *P. nalgiovense* SJ02. [2)] TBARS (Thiobarbituric acid reactive substance), mean ± SD (n=3). [3)] Meat Color, mean±SD (n=6). [4)] Texture profile analysis, mean ± SD (n=6). The results are presented as mean±SD; * $P < 0.05$ indicates a significant difference in the measured values compared to M600.

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