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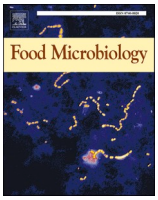

Genomic features, aroma profiles, and probiotic potential of the *Debaryomyces hansenii* species complex strains isolated from Korean soybean fermented food

Da Min Jeong, Su Jin Yoo, Min-Seung Jeon, Byung Hee Chun, Dong Min Han, Che Ok Jeon, Seong-il Eyun, Young-Jin Seo, Hyun Ah Kang *

Department of Life Science, Chung-Ang University, Seoul, 06974, South Korea

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ABSTRACT

Fermented soybean products are gaining attention in the food industry owing to their nutritive value and health benefits. In this study, we performed genomic analysis and physiological characterization of two *Debaryomyces* spp. yeast isolates obtained from a Korean traditional fermented soy sauce "ganjang". Both *Debaryomyces hansenii* ganjang isolates KD2 and C11 showed halotolerance to concentrations of up to 15% NaCl and improved growth in the presence of salt. Ploidy and whole-genome sequencing analyses indicated that the KD2 genome is haploid, whereas the C11 genome is heterozygous diploid with two distinctive subgenomes. Interestingly, phylogenetic analysis using intron sequences indicated that the C11 strain was generated via hybridization between *D. hansenii* and *D. tyrocola* ancestor strains. The *D. hansenii* KD2 and *D. hansenii*-hybrid C11 produced various volatile flavor compounds associated with butter, caramel, cheese, and fruits, and showed high bioconversion activity from ferulic acid to 4-vinylguaiacol, a characteristic flavor compound of soybean products. Both KD2 and C11 exhibited viability in the presence of bile salts and at low pH and showed immunomodulatory activity to induce high levels of the anti-inflammatory cytokine IL-10. The safety of the yeast isolates was confirmed by analyzing virulence and acute oral toxicity. Together, the *D. hansenii* ganjang isolates possess physiological properties beneficial for improving the flavor and nutritional value of fermented products.

1. Introduction

Debaryomyces hansenii, a non-conventional yeast species belonging to the class Hemiascomycetes, has been isolated from saline water, foods, fruits, and even the human gut (Breuer and Harms, 2006; Hallen-Adams and Suhr, 2017). It is a cryo-, xero-, and halotolerant yeast that can survive in highly-saline conditions of up to 4 M NaCl (Aggarwal and Mondal, 2009; Breuer and Harms, 2006). *D. hansenii* is among the yeast species with the highest prevalence on surface-ripened cheeses (Fröhlich-Wyder et al., 2019) and dry-aged beef (Ryu et al., 2018). Importantly, *D. hansenii* produces branched-chain aldehydes and alcohols that accelerate the development of a cheese flavor distinct from the aroma profiles obtained using *Yarrowia lipolytica* and *Saccharomyces cerevisiae* (Sørensen et al., 2011). Considering its long history as food yeast, *D. hansenii* was conferred the Qualified Presumption of Safety status by the European Food Safety Authority, as there were a few human infection cases caused by pathogens found to have been misidentified as *D. hansenii* or human-borne *D. hansenii* (Koutsoumanis et al., 2020; Wagner et al., 2005).

Beyond its role in producing fermented food, such as dairy and fermented meat products, *D. hansenii* is used in several biotechnological processes (Breuer and Harms, 2006; Riley et al., 2016). Food-borne *D. hansenii* produces mycotoxins that are effective against pathogenic *Candida* species (Banjara et al., 2016). The antimicrobial and antifungal activities of *D. hansenii* have been used to prevent the proliferation of undesirable microbes in fermented foods, sausages (Murgia et al., 2019), and olives (Psani and Kotzekidou, 2006). Additionally, D-xylose metabolism in *D. hansenii* has been exploited to produce xylitol from D-xylose and sugarcane bagasse hemicellulose (Prakash et al., 2011). In recent studies, live *D. hansenii* strains have been spotlighted as fish probiotics for their antimicrobial and immunostimulating effects when used in aquaculture (Angulo et al., 2019; Reyes-Becerril et al., 2008). *D. hansenii* strains have also received attention as potential human probiotics because they induced higher levels of IL-10/IL-12 secretion in human

* Corresponding author. Department of Life Science, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul, 156-756, South Korea.
E-mail address: hyunkang@cau.ac.kr (H.A. Kang).

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dendritic cells (hDCs) than *Saccharomyces boulardii* did (Ochangco et al., 2016), which is the only yeast probiotic currently used as a preventive and therapeutic agent for diarrhoea and other gastrointestinal (GI) disorders (Czerucka et al., 2007; Ochangco et al., 2016). A recent study on fungal species in the gut microbiota reported that *D. hansenii* is the most abundant species in the neonatal gut during breastfeeding, whereas *S. cerevisiae* is the most abundant after weaning (Schei et al., 2017).

D. hansenii is a homothallic yeast with a haplontic life cycle dissimilar from *S. cerevisiae* and is grouped as a member of the CUG-Ser clade among the three major Saccharomycotina clades (CUG-Ser, Methylotrophs, and Saccharomycetaceae) in phylogenetic trees generated using genome sequence information (Riley et al., 2016). Complete genome sequencing and assembly of *D. hansenii* CBS767^T has shown that it has a haploid genome with a size of 12.18 Mb consisting of 7 chromosomes with a distinct number of tandem gene repeats compared to the genome of *S. cerevisiae* (Dujon et al., 2004). Notably, *D. hansenii* is a highly heterogeneous species with respect to genetic diversity (Petersen and Jespersen, 2004). Two varieties of *D. hansenii* have been distinguished i.e., *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*, which have different physiological properties such as maximum growth temperatures and glucose-6-phosphate dehydrogenase activity (Nakase and Suzuki, 1985). More recently, intergenic spacer fingerprinting by restriction enzyme digestion re-identified *D. hansenii* varieties as *D. hansenii* var. *hansenii*, *Candida famata* var. *famata*, *D. hansenii* var. *fabryi*, and *Debaryomyces subglobosus* (Nguyen et al., 2009). *Debaryomyces tyrocola* was further subclassified from *D. hansenii* var. *hansenii* by discriminating spliceosomal introns of highly conserved genes (Jacques et al., 2009). The results imply that several cryptic species may potentially constitute *D. hansenii,* forming a *D. hansenii* species complex.

Systematic investigation of the genomic features and food-related properties of prevalent microbial species in fermented foods can provide vital insights into the potential roles of each species in the complex fermentation process as well as the nutritional values of fermented products. In this study, we performed genomic analysis and physiological characterization of two strains (KD2 and C11) belonging to the *D. hansenii* species complex, which were isolated as the most abundant yeast species during fermentation of ganjang, a Korean fermented soy sauce (Chun et al., 2021). Our data revealed distinctive genomic features associated with evolutionary relationships, such as interspecies hybrid formation within the *D. hansenii* species complex, and various physiological traits of the two *D. hansenii* ganjang isolates that may be useful in the food and health industries, such as osmotolerance, flavor production, immunomodulatory activity, and nonvirulence without acute oral toxicity.

2. Materials and methods

2.1. Strains and culture conditions

The bacteria and yeast strains used in this study are listed in Table 1. The *Debaryomyces* spp. strains KD2 and C11 were isolated from ganjang, prepared by separation from meju (dried soybean block) after 60 days of fermentation (Chun et al., 2021). To isolate the yeast strains, the ganjang sample was serially diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.2), spread on yeast extract peptone dextrose (YPD; 1% yeast extract, 2% Bacto peptone, 2% glucose) agar plates containing 7% NaCl, chloramphenicol (50 μg/mL), and tetracycline (10 μg/mL), and aerobically incubated at 30 °C for 2 days. The KD2 and C11 strains were cultured in YPD medium containing 3% (w/v) NaCl at 28 °C with shaking (220 rpm). The *Debaryomyces* spp. strains KD2 and C11 were deposited to the Korean Agricultural Culture Collection (KACC) under deposit numbers 93324P and 49928, respectively. Other yeast strains were cultured in YPD medium at 28 °C (*S. cerevisiae, D. hansenii*, and *Candida albicans*) or 37 °C (*S. boulardii*) with shaking (220 rpm). *Lacticaseibacillus rhamnosus* GG (LGG) and *Leuconostoc mesenteroides* were cultivated in test tubes containing MRS broth (Difco) at 30 °C without shaking. *Vibrio vulnificus* was cultured in Luria-Bertani broth (LB, 0.5% yeast extract, 1% tryptone) supplemented with 3% (w/v) NaCl at 28 °C with shaking (220 rpm).

Table 1
Strains used in this study.

Genus and species	Strain	Characteristics	References
Yeast			
Debaryomyces hansenii	KD2	Isolated from Korean Jang (KACC 93324P)	In this study
	C11	Isolated from Korean Jang (KACC 49928)	In this study
	CBS767^T	Type strain	ATCC
	KCTC27743	Isolated from Korean Nuruk	KCTC
Saccharomyces cerevisiae	BY4741	S288C-derivative laboratory strain (haploid)	Open Biosystems
	BY4742	S288C-derivative laboratory strain (haploid)	Open Biosystems
	BY4743	S288C-derivative laboratory strain (diploid)	Open Biosystems
	CEN.PK2–1C	Laboratory strain with mutated *FDC1* & *PAD1*	Entian and Kötter (2007)
Saccharomyces boulardii (*Saccharomyces cerevisiae* var. *boulardii*)	ATCCMYA-796	Probiotic yeast	ATCC
Candida albicans	ATCC32354	Pathogenic yeast	ATCC
Bacteria			
Lacticaseibacillus rhamnosus GG	ATCC53103	Probiotic bacteria	Park et al. (2019)
Leuconostoc mesenteroides	ATCC8293 (NRRL B-1118)	*Leuconostoc* strain susceptible to bile salt	Kim et al. (2012)
Vibrio vulnificus	M06-24/O	Pathogenic bacteria	Lee et al. (2017)

ATCC: American Type Culture Collection, KCTC: Korean Collection for Type Cultures, KACC: Korean Agricultural Culture Collection.

2.2. Ploidy analysis

Yeast cells were inoculated at OD_{600} = 0.3 in YPD medium, cultivated, and harvested at OD_{600} = 3.0. The cell pellets were washed twice using pure water and fixed in absolute ethanol for at least 12 h. The fixed cells were resuspended in RNase solution (2 mg/mL RNaseA, 15 mM NaCl, and 50 mM Tris-HCl, pH 7.5) supplemented with proteinase K (Sigma-Aldrich, >4 units/mL), washed, and mixed with SYTOX solution (1 μM SYTOX Green nucleic acid stain and 50 mM Tris-HCl, pH 7.5). An Accuri C6 Plus flow cytometer (BD Biosciences) and the BD Accuri C6 software (BD Biosciences) were used for flow cytometry analysis.

2.3. Whole-genome sequencing, assembly, and annotation

Genomic DNA was extracted from spheroplasts obtained after incubation with sorbitol buffer (1 M sorbitol, 0.01 M EDTA, and lyticase (5 KU)) for 1 h at 37 °C. The cell extract was purified using a phenol: chloroform:isoamylalcohol mix (Sigma-Aldrich, 25:24:1) and treated with 4 units/mL of proteinase K from *Tritirachium album* (Sigma-Aldrich) and 8 units/mL of RNase A (Sigma-Aldrich). High-quality genomic DNA was harvested by spooling (Marmur, 1963). For whole-genome sequencing, an SMRT bell library was constructed using the Sequel Binding Kit 1.0 (Pacbio), and the library was sequenced using the Pacbio Sequel SMRT sequencer with 1 cell per library. Reads were trimmed to include high-quality regions using Trim Galore (ver. 0.6.4_dev). Pacbio

long-reads were assembled using the SMARTdenovo (ver. 1.07) and CANU (ver. 2.0) software. SMARTdenovo assemblies of the KD2 strain and CANU assemblies of the C11 strain, respectively, were used for further genomic analysis. The pairwise sequence alignment tool LAST (lastal, ver. 1060) was used to compare the assembled genomes with the *D. hansenii* CBS767^T genome. GC content was calculated using the python script GCcalc.py (https://github.com/WenchaoLin/GCcalc/blob/master/GCcal-c.py), and repeat sequences were predicted using RepeatMasker (ver. 2.10.0+, http://repeatmasker.org). The sequences of the seven chromosomes in the *D. hansenii* KD2 haploid genome were deposited at NCBI with the accession numbers CP046876–CP046882. The sequences of each subgenome in the C11 diploid genome of the *D. hansenii* species complex were deposited at NCBI with the accession numbers JADOBC000000000 (principal haplotype; subgenome A) and JADOBD000000000 (alternate haplotype; subgenome B).

For RNA-Seq analysis, total RNA was isolated from frozen yeast cells that were ground in liquid nitrogen. RNA libraries were constructed using the TruSeq Stranded mRNA LT Sample Prep Kit (ThermoFisher) and sequenced for paired-end reads using the Illumina HiSeq platform. The short reads were trimmed to include high-quality regions using Trim Galore (ver. 0.6.7), and RNA-Seq spliced alignment to assembled scaffolds was processed using the HISAT2 (ver. 2.2.1) aligner. The resulting BAM file was used to generate gene annotation data using BRAKER2 (ver. 2.1.6), along with GeneMark-ET (ver. 4.68_lic) and AUGUSTUS (ver. 3.4.0). Non-coding RNA identification was performed using tRNAscan-SE (ver. 2.0) and Infernal (ver. 1.1.3) with the Rfam database (ver. 14.4).

2.4. Solid-phase microextraction with gas chromatography-mass spectrometry (SPME/GC-MS) analysis

Yeast cells were inoculated at OD_{600} = 0.3 in 50 mL YPD medium. Yeast samples were collected in duplicate after 12, 24, or 48 h incubation at 28 °C with shaking and transferred into glass vials with PTFE/silicone septae (Supelco). A PAL automated GC sampler (Agilent Technologies) transferred each sample to a heater and equilibrated the sample for 5 min at 50 °C. Volatile compounds from yeast samples were absorbed onto a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fiber for 30 min and desorbed in the GC injection port for 2 min at 250 °C. A gas chromatograph-5977E quadrupole mass selective detector (Agilent Technologies, 7820A series) was combined with the HP-INNOWax GC column (Agilent Technologies, 19091N-133; 30 m length × 250 μm i.d. × 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was set to 40 °C at the start, held for 5 min, subsequently increased to 150 °C at 5 °C/min, held for 10 min, increased to 220 °C at 10 °C/min, and held for 5 min. The mass spectra of the volatile compounds were acquired from *m/z* 33–250 at a fragment voltage of 70 eV and identified using a library search (National Institute of Standards and Technology, NIST ver. 11). To test 4-vinylguaiacol production capability, yeast cells were grown in YPD or YPD +15% (w/v) NaCl in the presence of 50 ppm ferulic acid (Suezawa and Suzuki, 2007) for 10 days. Samples were collected after 1, 2, 3, 7, and 10 days of incubation.

2.5. Bile salt and low pH tolerance assays

Yeast cells, cultivated overnight in YPD, were washed twice with PBS and resuspended in freshly-prepared 0.3 or 0.6% (w/v) bovine bile (Sigma-Aldrich) in PBS (Welgene, pH 7.3) or acidic solution (adjusted to pH 2.5 or 3.0 with HCl) (Park et al., 2019). The cell suspension was incubated at 37 °C for 4 h under static conditions, serially diluted with PBS, and spread onto agar plates. As a control, yeast cells were cultivated in PBS. Colony-forming units (CFUs) of each sample were counted after incubation at 30 °C for more than 48 h. The tolerance degree was calculated by log CFU/mL reduction, which was obtained by subtracting the log unstressed-cell CFU/mL from the log stressed-cell CFU/mL values.

2.6. Generation of dendritic cells and cytokine quantification

Human whole blood samples from two different donors were provided by the Korean Redcross Blood Service without identifying information. The use of human-derived products was approved by the Institutional Review Board of Chung-Ang University (Approval number: 1041078-201809-BR-184-01). The experimental process was adapted from previously published methods with a few modifications (Bazan et al., 2018; Ochangco et al., 2016). Peripheral blood mononuclear cells (PBMCs) from the buffy coat were harvested after density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Human monocytes were isolated using the Mojo Sort human CD14-positive selection kit (Biolegend). To generate immature dendritic cells (iDCs), monocytes were cultured for 6 days in RPMI1640 (Welgene) supplemented with 10% fetal bovine serum (Access Biologicals), 100 U/mL penicillin/streptomycin (Welgene), 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, Pepro-Tech), and 20 ng/mL recombinant human interleukin (IL)-4 (Pepro-Tech) at 37 °C and 5% CO_2. To confirm iDC differentiation, the cells were stained with PE/Cy7-conjugated anti-human CD11c (Biolegend, clone 3.9). Data were collected with an Attune NxT Flow Cytometer (ThermoFisher) and analyzed with the Flowjo software (BD Bioscience).

For cytokine quantification, human iDCs were resuspended in RPMI1640 without antibiotics, seeded into 96-well plates at a density of 1×10^5 cells/well, and stabilized at 37 °C, 5% CO_2 for 1 h. The cells were subsequently infected with pre-cultured yeast strains at a multiplicity of infection (MOI) of 10. After a 20 h incubation, cytokine levels in the supernatant were analyzed using the LEGENDplex bead capturing cytokine kit (Biolegend). The cytokine data were obtained from at least three technical replicates from two different donors.

2.7. Animal experiments for safety assessment

Animal studies were approved by the Institutional Animal Care and Use Committee of Chung-Ang University and were conducted at the Chung-Ang University Animal Experiment Center (Approval number: 202000087). During the 14 days of the experiment, mice were monitored and sacrificed by the CO_2 inhalation method if they lost 20% of their original body weight or exhibited signs of morbidity. For fungal pathogenesis analysis, five BALB/c 5-week-old female mice (DBL, Korea) were used per strain and as PBS controls. Yeast strains were cultured for 16 h and washed twice. Prepared cells at an OD_{600} = 0.1 were suspended in 200 μL PBS and intravenously injected into the tail vein. For acute oral toxicity tests, five 9-week-old female ICR mice (DBL, Korea) per strain were administered with the yeast via oral gavage (referred to as the fixed-dose method in the Organisation for Economic Co-operation and Development (OECD) guideline 423 (https://doi.org/10.1787/9789264071001-en). The mice were starved for 12 h, and subsequently, 2000 mg/kg mouse weight of microbial cells were suspended in 800 μL PBS, and 400 μL of the cell suspension was administered twice to each mouse within an hour. After yeast administration, food was withheld for 1–2 h. At the end of the study, a necropsy was performed, and the morphological characteristics of the lung, heart, kidney, spleen, liver, and stomach were assessed.

2.8. Statistical analysis

Statistical differences between multiple groups were evaluated via one-way analysis of variance (ANOVA). An unpaired Student's *t*-test was used for comparing two independent groups. Both tests were performed using GraphPad Prism ver. 7.0.

3. Results

3.1. Growth characterization of yeast isolates KD2 and C11

The yeast strains identified as *Debaryomyces* spp. were isolated as the most highly abundant fungal group at 40 and 60 days during ganjang fermentation (Chun et al., 2021). Among several *Debaryomyces* isolates, the KD2 and C11 strains were selected for further analyses based on their ability to grow on high osmolyte concentrations up to 15% NaCl, 15% KCl, and 2.5 M sorbitol (Supplementary Fig. 1A). The KD2 and C11 yeast isolates grew with yeast-type morphology and showed an egg-like shape and budding without hyphae formation, with a greater cell diameter for

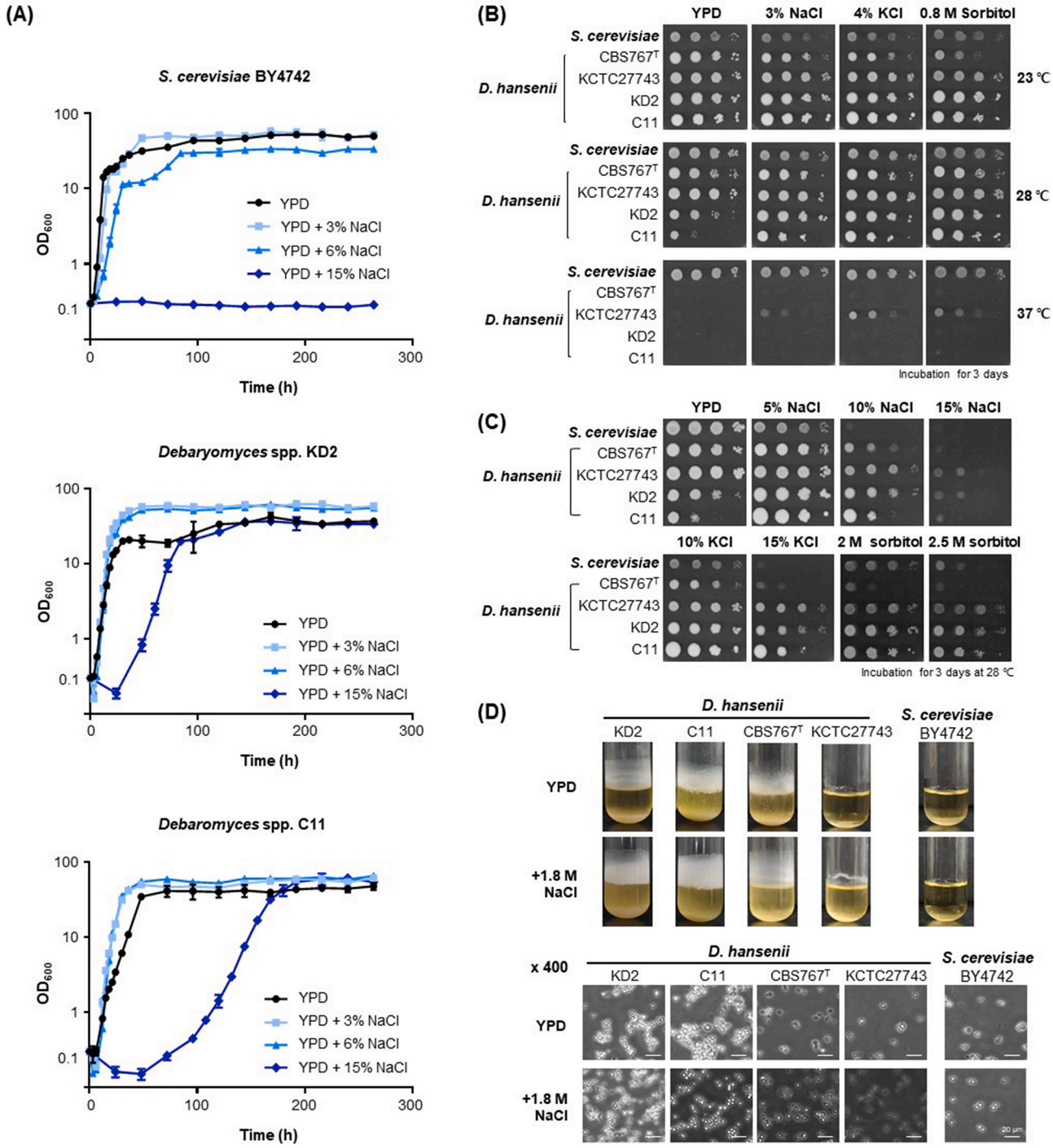

Fig. 1. Growth phenotype of *Debaryomyces* spp. KD2 and C11 isolated from ganjang. (A) Growth curve of *S. cerevisiae* BY4742 and *Debaryomyces* spp. KD2 and C11 on YPD supplemented with 0, 3, 6, and 15% (w/v) NaCl. Yeast cells were precultured overnight and inoculated in 30 mL YPD media. The OD_{600} was measured at each time point using a spectrophotometer. **(B)** Spotting analysis under different salt concentrations and temperatures. Yeast cells, including *S. cerevisiae* BY4742, *D. hansenii* CBS767^T, *D. hansenii* KCTC27743, and *Debaryomyces* spp. KD2 and C11 cells, were subjected to 10-fold serial dilutions from $OD_{600} = 1$, spotted on plates with YPD alone or YPD supplemented with various osmolytes, and incubated for 3 days at 23, 28, and 37 °C. **(C)** Spotting analysis under osmotic stress. Yeast cells were spotted on plates with YPD alone or YPD supplemented with various osmotic stress-inducing components and incubated for 3 days at 28 °C. **(D)** Flor formation and cell aggregation. Yeast was cultured at 28 °C for 5 days without shaking in YPD-containing test tubes in the absence or presence of 1.8 M NaCl. Cell culture photographs were captured using a cell culture microscope (OLYMPUS, CKX53).

C11 (~3–3.5 μm) than for KD2 (~2.5 μm) in normal growth conditions (Supplementary Fig. 1B). The ITS1-5.8S-ITS2 gene sequences of the strains KD2 and C11 (Supplementary Fig. 2A) indicated that the KD2 and C11 strains showed the highest similarities with *D. hansenii* var. *hansenii* CBS767^T (99.84% and 99.35%, respectively) and *D. hansenii* var. *fabryi* CBS789^T (99.83% and 99.67%, respectively). Reflecting their isolation from salty fermented soybean products, both *Debaryomyces* spp. isolates KD2 and C11 exhibited tolerance to hyperosmotic stress up to 15% (w/v) NaCl. Notably, the growth of both *Debaryomyces* spp. strains was improved in YPD supplemented with 3%–6% (w/v) NaCl at 28 °C, relative to the growth of *S. cerevisiae* BY4742 (Fig. 1A). The ganjang isolates KD2 and C11 showed better growth at 28 °C in YPD supplemented with 3% NaCl, 4% KCl, or 0.8 M sorbitol than in YPD without any supplementation. Notably, the growth enhancement effects

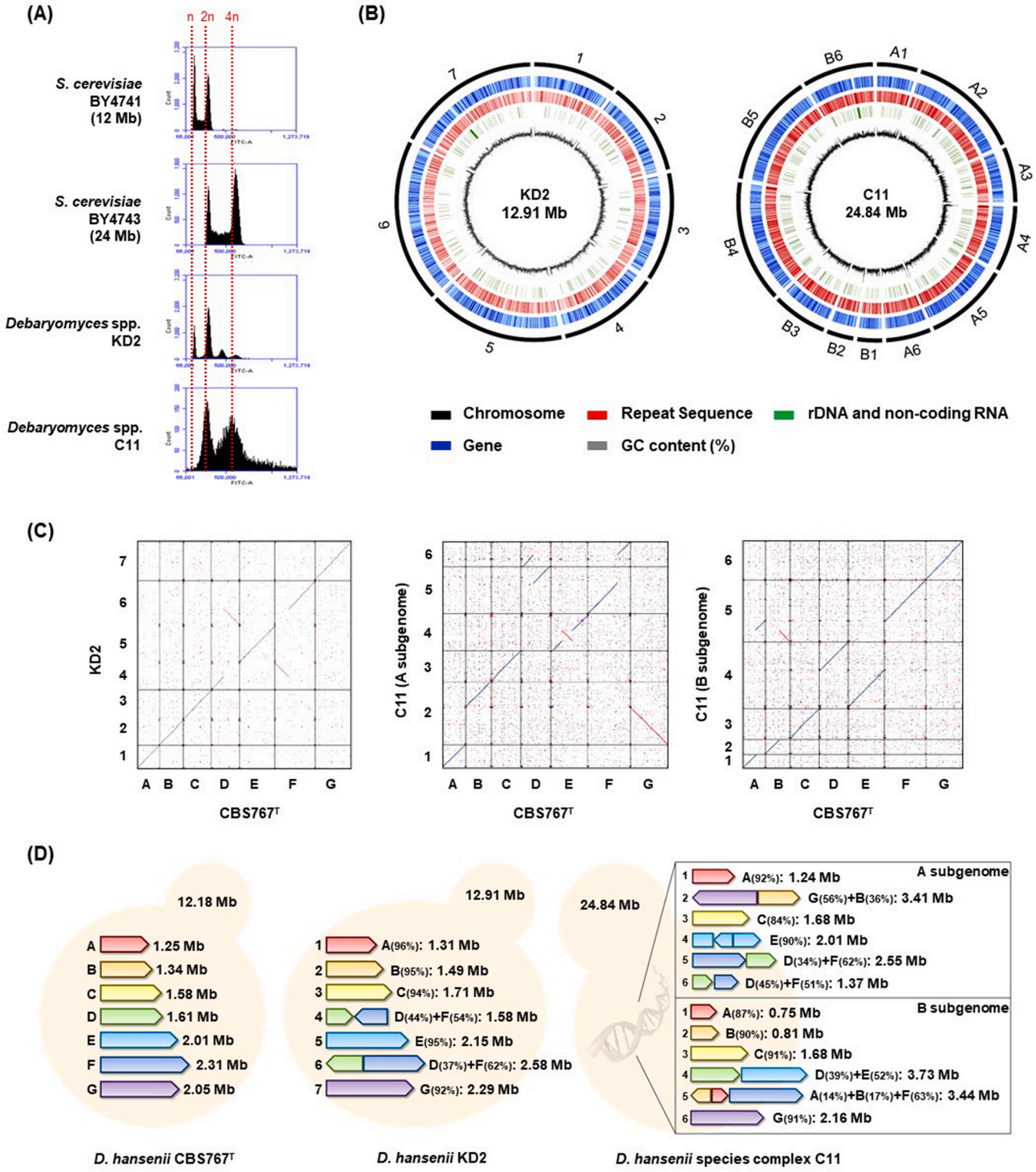

Fig. 2. Analysis of ploidy and synteny between *Debaryomyces* spp. KD2 and C11 genomes. **(A)** Ploidy analysis using flow cytometry. *S. cerevisiae* BY4741 (haploid) and *S. cerevisiae* BY4743 (diploid) were used as controls. **(B)** Circros plot of KD2 and C11 whole genomes. **(C)** Synteny analysis of KD2 and C11 genomes compared to the *D. hansenii* CBS767^T reference genome. The synteny blocks were visualized using dot plots. **(D)** Schematic diagrams of the KD2 and C11 genome assemblies. Brackets indicate the similarity with the *D. hansenii* CBS767^T chromosome.

of osmolytes were absent at 23 °C (Fig. 1B). Although *D. hansenii* KCTC27743 isolated from nuruk, a Korean traditional fermentation starter used to produce starch-based alcoholic beverages, grew at 37 °C with osmolyte supplementation, most of the *D. hansenii* strains tested were unable to grow at 37 °C regardless of the presence of osmolytes. The higher osmolyte concentrations required for optimal growth indicate that the ganjang isolates KD2 and C11, along with *D. hansenii* KCTC27743, are more tolerant to NaCl and sorbitol than *S. cerevisiae* BY4742 and *D. hansenii* CBS767^T (Fig. 1C). Interestingly, the growth of C11 was improved in a wide concentration range of NaCl, KCl, and sorbitol compared to that of KD2, indicating that the two ganjang yeast isolates display distinctive physiological characteristics, with C11 showing a greater osmophilic phenotype.

Under static culture conditions, yeast cells were cultivated in the YPD-containing test tubes with or without 1.8 M NaCl supplementation, and buoyant flor formation was observed with the *D. hansenii* CBS767^T, KD2, and C11 strains, although *D. hansenii* KCTC27743 showed relatively less flor formation (Fig. 1D). Microscopic analysis indicated that the yeast-form cells of *D. hansenii* were aggregated without hyphal formation in static culture with liquid YPD and YPD with 1.8 M NaCl, which is the NaCl concentration commonly present in fermented soybean sauces (Watanabe et al., 2013). In contrast, *S. cerevisiae* BY4742 did not show flor formation as it is a non-flor forming strain due to a nonsense mutation in *FLO8* (Bester et al., 2006). This observation corroborates a previous report that a few *D. hansenii* strains had good adhesion and sliding motility, and these properties are related to greater hydrophobicity of the cell surface rather than the ability to form pseudomycelia (Gori et al., 2011).

3.2. Reassessment of phylogenetic relationships of Debaryomyces spp. KD2 and C11 based on genomic features and intron sequences

Phylogenetic analysis using the ITS1-5.8S-ITS2 gene sequences showed that KD2 was most closely associated with *D. hansenii* CBS767^T, whereas C11 was more closely grouped with *Debaryomyces prosopidis* CBS8450^T and *D. fabryi* CBS789^T (Supplementary Fig. 2B). The results indicate that KD2 belongs to *D. hansenii* var. *hansenii,* whereas C11 may be a member of a recently diverged species of the *D. hansenii* species complex. To examine the genomic features of *D. hansenii* KD2 and *D. hansenii* species complex C11 which show an ambiguous classification based on the ITS1-5.8S-ITS2 gene, we performed *de novo* whole-genome sequencing to obtain PacBio long reads and Illumina short reads. The *D. hansenii* KD2 genome was assembled as seven contigs with a total size of 12.91 Mb, whereas the genome of *D. hansenii* species complex C11 was assembled as twelve contigs with a total size of 24.84 Mb (Supplementary Table 1). Ploidy analysis using flow cytometry showed that the *D. hansenii* C11 genome is twice as large as the KD2 genome (Fig. 2A). The genome assembly results also showed that the *D. hansenii* KD2 has a haploid genome, whereas the *D. hansenii* species complex C11 has a diploid genome (Fig. 2B).

The *D. hansenii* KD2 genome is composed of seven chromosomes with a total length of 12.91 Mb that showed 99% identity to the *D. hansenii* CBS767^T reference genome (Dujon et al., 2004). Synteny analysis revealed the conservation of most gene orders and orientation within the two sets of chromosomes, except for a translocation between chromosomes F and G (Fig. 2C and D, and Supplementary Table 2). Intriguingly, the *D. hansenii* species complex C11 genome consists of two subgenomes A and B, each containing 6 contigs. Chromosome rearrangement was observed in all chromosomes of both C11 subgenomes, except for chromosome 3, which corresponds to chromosome C in *D. hansenii* CBS767^T. A previous study on the population polymorphisms of nuclear mitochondrial DNA insertions reported widespread diploidy in *D. hansenii*, which is associated with a loss of heterozygosity (LOH) (Jacques et al., 2010). Our *de novo* whole-genome data analysis indicated that the *D. hansenii* species complex C11 is a heterozygous diploid which resulted from hybridization between two strains that diverged from the common ancestor of *Debaryomyces* spp., followed by LOH and massive chromosomal rearrangements.

A more recent study using conserved spliceosomal intron sequence comparisons showed that *D. hansenii* is a complex of species that comprises at least four members, i.e., *D. hansenii* (previously *D. hansenii* var. *hansenii*), *D. tyrocola, D. fabryi* (previously *D. hansenii* var. *fabryi*), and *D. subglobosus* (Jacques et al., 2009). To further define the taxonomic classification of KD2 and C11, phylogenetic analysis was performed using the intron sequences of four well-conserved housekeeping genes, *ACT1, TUB2, RPL31,* and *RPL33* (Supplementary Fig. 3). Interestingly, all the tested markers of the C11 strain showed sequence differences (85.4%–93.6%; Supplementary Table 3) between the two alleles from subgenomes A and B, strongly supporting the hypothesis that the C11 genome is heterozygous diploid containing genetic materials from two different ancestors. The phylogenetic tree of partial intron sequences of *ACT1* showed that although KD2 is grouped into the *D. hansenii* cluster containing *D. hansenii* CBS767^T, C11 is more closely clustered with *D. tyrocola*, which may have diverged from the common ancestor with *D. hansenii* (Supplementary Fig. 3A). Analysis using *TUB2*, *RPL31*, and *RPL33* showed good concordance with the phylogenetic analysis based on *ACT1* (Supplementary Fig. 3B, 3C, and 3D). Notably, phylogenetic analysis based on intron sequences indicated that the parental strain with the C11 subgenome B diverged from the common *D. hansenii* ancestor before the divergence of the *D. tyrocola* cluster, in which the C11 subgenome A was grouped. Thus, C11, the heterozygous diploid, may have been generated by hybridization between the unidentified ancestor of subgenome B and the *D. tyrocola* strain, which were grouped in the *D. hansenii* species complex.

3.3. Volatile flavor profiles of D. hansenii KD2 and D. hansenii species complex C11

To examine the potential of *D. hansenii* KD2 and *D. hansenii* species complex C11 to produce flavor components, volatile flavor profiles were analyzed by SPME/GC-MS. KD2 cultivated in rich YPD medium with aeration showed several peaks of flavoring compounds which mostly increased in height with prolonged incubation times (Fig. 3A and Supplementary Table 4). The peaks corresponding to acetoin (sweet, buttery flavor), phenylethyl alcohol (floral and sweet flavor), isoamyl alcohol (alcoholic and fruity flavor), and isovaleraldehyde (fruity and chocolate flavor) were predominant. Cheese-like flavoring components, such as butanoic acids and 3-methylbutanoic acid, were also detected with the highest peak obtained with 24 h cultivation. The production of several other pleasant odor components, including furans (such as musty, sweet, and bready flavor) and butyraldehyde (bready and chocolate flavor), was also detected, although their levels were relatively low. Compared to KD2, C11 exhibited a similar flavor profile, but with few diverse components. Phenylethyl alcohol levels alone were statistically higher in C11 than in KD2. Similarly, when both *D. hansenii* strains were incubated in YPD supplemented with 15% NaCl, KD2 secreted compounds with more diverse flavors, and showed increased levels of butanoic acid, isobutanoic acid, 3-methylbutanoic acid, and valeraldehyde, whereas C11 produced a statistically higher level of phenylethyl alcohol and isoamyl alcohol. Although the diversity of flavor compounds from YPD supplemented with 15% NaCl was decreased compared to that under YPD-rich conditions, 2-ethylhexyl alcohol (fatty and sweet flavor) and 2,5-dimethylpyrazine (cocoa and chocolate flavor) were newly detected.

4-vinylguaiacol (4-VG) is a valuable flavoring agent which has a clove-like, smoky, and spicy flavor, and can be synthesized by microbial metabolism of ferulic acid (FA), an abundant phenolic compound found in several plant cell walls. Highly efficient 4-VG production was detected in both KD2 and C11 strains in the presence of FA (Fig. 3B and Supplementary Fig. 4). The *S. cerevisiae* CEN.PK2–1C and BY4742 strains were used as negative and positive controls for 4-VG production, respectively. Although *S. cerevisiae* BY4742 retains 4-VG conversion

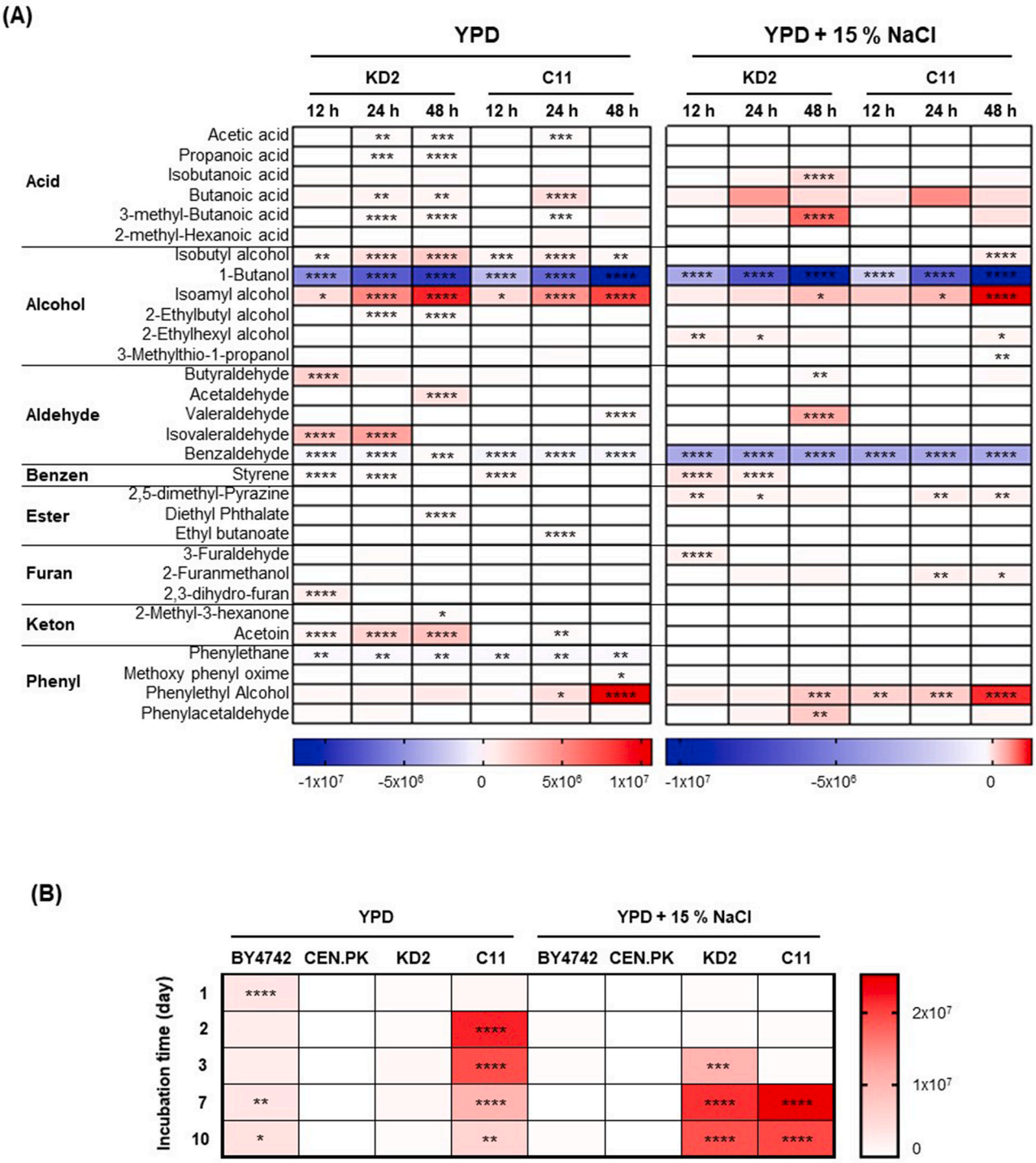

Fig. 3. Production of flavor components by the ganjang yeast isolates KD2 and C11. (A) Volatile flavor profiles determined using SPME-GC/MS. Biologically duplicated yeast cells were cultured in YPD without (left) or with 15% (w/v) NaCl supplementation (right) at 28 °C in shaking incubators. The supernatants were collected after 12, 24, and 48 h. **(B)** Bioconversion of ferulic acid to 4-VG. Biologically duplicated yeast cells were inoculated at OD_{600} of 0.3 in 15 mL YPD containing 50 ppm ferulic acid without and with 15% (w/v) NaCl. Culture supernatants were harvested at the indicated times and analyzed for 4-VG production using SPME-GC/MS. Each heatmap shows the relative peak area extracted from the supernatants of yeast cell cultures compared to media alone (YPD or YPD with 15% NaCl). Significant differences between cell cultures and media without yeast were determined by statistical analyses using one-way ANOVA. GraphPad Prism (ver. 7.0) was used to generate the plots. The asterisk (*) indicates the *P*-value or calculated probability such as *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, and ****, $P \leq 0.0001$.

activity, *S. cerevisiae* CEN.PK2–1C cannot decarboxylate FA owing to a nonsense mutation in *PAD1*, which encodes phenylacrylic acid decarboxylase that is required for the conversion of FA to 4-VG (Ogata et al., 2020; Richard et al., 2015). When cultivated in YPD containing FA, *D. hansenii* species complex C11 showed the highest 4-VG yield among the tested yeast strains. Although *D. hansenii* KD2 showed marginal 4-VG production when cultivated in YPD, it showed highly activated 4-VG production, comparable to that in *D. hansenii* C11, when cultivated under high-salt conditions. The *S. cerevisiae* strains showed no 4-VG production in the presence of 15% NaCl, as they could not grow under such high osmotic stress. The results of the flavor profile analysis clearly showed distinctive flavor production activity in the two *D. hansenii*

ganjang isolates, despite a similar profile for flavor components.

3.4. Evaluation of potential probiotic properties of D. hansenii KD2 and D. hansenii species complex C11

To evaluate the potential of the ganjang isolates *D. hansenii* KD2 and *D. hansenii* species complex C11 to function as probiotics, the tolerance of the strains to low pH and bile salt was analyzed by counting the CFUs and compared with that of two lactic acid bacteria, *Lacticaseibacillus rhamnosus* GG (LGG) and *Leuconostoc mesenteroides*, and two yeast species, *S. cerevisiae* BY4742 and *S. boulardii* (Fig. 4A). *Leu. mesenteroides,* which lacks bile salt hydrolysis activity (Turpin et al., 2011), was used as the negative control in bile salt tolerance analysis. Yeast exhibited greater tolerance to acidic pH than bacteria, except *S. cerevisiae* BY4742, which showed similar acid tolerance to LGG. The *D. hansenii* ganjang strains showed a very low log CFU/mL reduction in the presence of bile salts (0.3% and 0.6%), indicating high tolerance to bile salts.

The immunomodulatory activity of the ganjang isolates was evaluated by analyzing the cytokine profiles of activated human dendritic cells (hDCs) after co-incubation with live or heat-killed yeasts. The production of IL-1β, IL-6, tumor necrosis factor (TNF)-α, IL-8, IL-12p70, and IL-10 was analyzed in the supernatants of hDCs co-incubated for 20 h with viable (Fig. 4B) or heat-killed (Fig. 4C) yeast cells. Although individual variation was observed, the expression levels of the proinflammatory cytokines IL-6, IL-8, and IL-12p70, induced by *D. hansenii* KD2 and *D. hansenii* species complex C11 (either live or heat-killed) were similar to those induced by *S. boulardii*. However, both KD2 and C11 induced significantly higher levels of the anti-inflammatory cytokine IL-10 than *S. boulardii.* These results indicate that the *D. hansenii* ganjang isolates showed beneficial immunomodulatory activity in *in vitro* assays and may have potential probiotic activity to suppress overactivated inflammatory responses.

3.5. Safety assessment of D. hansenii KD2 and D. hansenii species complex C11

Next, we analyzed the virulence and acute oral toxicity of the ganjang yeast isolates. Survival testing of intravenously infected mice validated the non-virulent characteristics of the ganjang isolates *D. hansenii* KD2 and *D. hansenii* species complex C11 (Fig. 5A and Supplementary Fig. 5A). Mice infected with neither strain showed clinical symptoms or death until the end of the experiment, whereas the human pathogenic yeast *Candida albicans* induced death within 6 days after infection. Further, there were no pathogenic phenotypes observed in the organs of the mice after treatment with *D. hansenii* KD2 and *D. hansenii* species complex C11 (Supplementary Fig. 6A). To confirm the acute oral safety of the ganjang isolates, 2000 mg/kg KD2 and C11 cells were administered by oral gavage into healthy mice (Fig. 5B and Supplementary Fig. 5B). During the 14 days of treatment, the yeast-administered mice consumed food and water as actively as PBS-administered mice, except one mouse in the C11-administered group that was dead within 24 h, similar to *Vibrio vulnificus*-administered mice. The dead mice showed abnormal morphologies in the lung, liver, and stomach (Supplementary Fig. 6B).

4. Discussion

The use of fermented products as a functional food is increasing, beyond their use as mere side dishes. In particular, fermented soybean products are becoming popular in the international market due to their nutritive values and many health benefits. The Korean fermented soybean products, ganjang (fermented soybean sauce) and doenjang (fermented soybean paste) are essential seasonings in the Korean diet. In this study, we characterized the genomic and physiological features of two *Debaryomyces* spp. KD2 and C11, which were isolated as dominant yeast species in fermented soy sauce, to obtain insight into their potential roles in the fermentation process and contribution to nutritional properties of ganjang.

Our whole-genome analysis results, combined with ploidy and intron analyses, revealed intriguing aspects of the genetic diversity and dynamic population structure of the *D. hansenii* species complex in the fermented food environment. The *Debaryomyces* ganjang isolates KD2 and C11 were identified as a haploid *D. hansenii* strain and a heterozygous diploid strain generated by inter-clade hybrids within the *D. hansenii* species complex, respectively (Fig. 2 and Supplementary Fig. 3). Although the protein-coding genes of both KD2 and C11 are similar to those of *D. hansenii* CBS767^T, the diploid strain C11 has several sequences homologous to those of *D. fabryi*, which was recently separated as a distinctive group from *D. hansenii* (Nguyen et al., 2009), indicating that C11 might have been generated by hybridization between *D. hansenii* and *D. fabryi*. However, intron sequence analysis showed that the intron sequences of C11 subgenomes A and B were closer to those of *D. tyrocola* and *D. hansenii*, respectively, than of *D. fabryi*. Currently, it is difficult to determine the whole genome similarity between the C11 subgenome B and *D. tyrocola* because genomic information for *D. tyrocola* is not yet available. These findings are consistent with previous reports on the generation of diploid and aneuploid strains by hybridization among *D. hansenii* haploid variants (Jacques et al., 2010), supporting the hypothesis for the existence of populations within the newly delineated *D. hansenii* species and genetic exchanges between these populations (Jacques et al., 2009).

Although *D. hansenii* species can be cultivated in media with up to 25% NaCl (Breuer and Harms, 2006), the ganjang isolates KD2 and C11 exhibited halotolerance up to 15% NaCl and showed improved growth in the presence of salt. Notably, the enhanced growth with salt was less evident with decreased culture temperatures (Fig. 1B). A previous study on the growth of the type strain *D. hansenii* CBS767^T isolated from beer in Denmark reported that growth stimulation by sodium is temperature-dependent in *D. hansenii* (Papouskova and Sychrova, 2007). Thus, the temperature-dependent halophilic phenotype of *D. hansenii* KD2 and *D. hansenii* species complex C11 suggests that they are not strictly halophilic yeast strains, strongly supporting the conception that combined osmotic and high-temperature stresses generally result in improved yeast cell growth (Papouskova and Sychrova, 2007).

The synthesis of pleasant flavor and aroma compounds in fermented soybean products, such as branched alcohols, esters, and aldehydes, stems from amino acid degradation or metabolism by several yeast strains. A few previous studies on yeast community structure and dynamics have reported the presence of *Zygosaccharomyces* and *Debaryomyces* spp. as the dominant species at the initial stage of doenjang fermentation (Kim et al., 2009) and *Candida, Pichia,* and *Rhodotorula* spp. as the major yeasts during the early stage of ganjang fermentation (Song et al., 2015). It was previously reported that as a flavoring agent, *D. hansenii* produces aldehydes such as 2-methylpropanal and 3-methylbutanal at the level of sensory threshold in conditions mimicking various stages of cheese manufacturing (Gori et al., 2012). The overall metabolism of amino acids and the generation of aromatic compounds are related to the origin of isolation of the strain (Flores et al., 2017). Branched aldehydes and ethyl esters are intensively produced by *D. hansenii* isolated from cheese, sausage, and pork, whereas acid compounds are mainly produced by vegetable strains. Our previous metatranscriptomics study on the metabolic features of ganjang fermentation revealed that during gangjang fermentation, carbon sources for cell growth and amino acids for aroma compound formation are generated from the digestion of soybean, mainly by bacteria and filamentous fungi, which are predominant in meju (fermented soybean bricks). The generated peptides and amino acids are subsequently used as substrates for the formation of flavor compounds by yeasts (Chun et al., 2021). We investigated the potential activity of the *D. hansenii* ganjang isolates to form volatile aromatic compounds by cultivating them in rich YPD medium, which can support active cell growth and provide peptide and

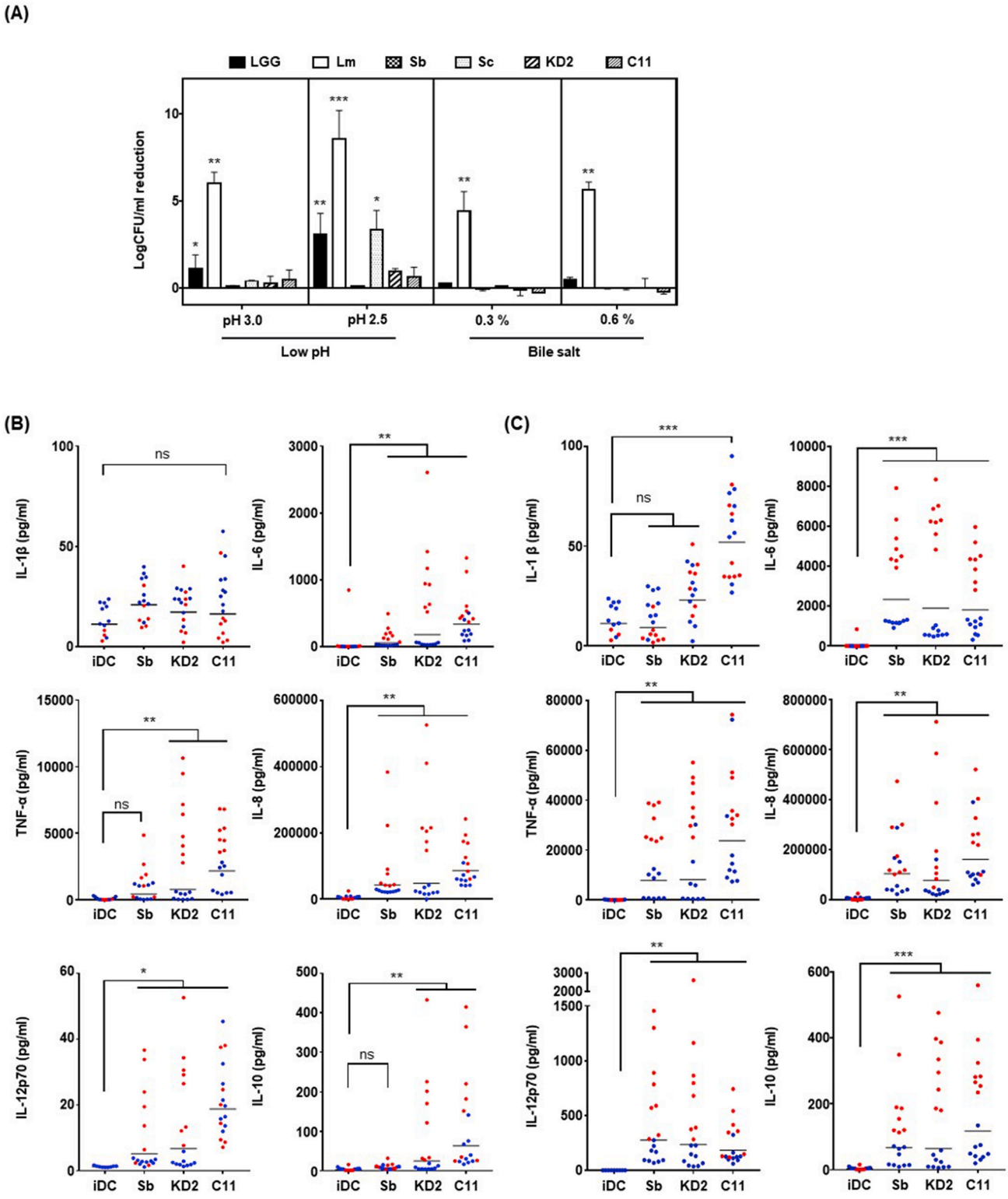

Fig. 4. Assessment of the potential probiotic activity of *D. hansenii* KD2 and *D. hansenii* species complex C11. (A) Tolerance to low pH and bile salt. The data are represented as the mean ± standard deviation from duplicate experiments. Statistical significance was determined between the stressed cell log CFU/mL and the control cell log CFU/mL using unpaired Student's *t*-tests. *: $P < 0.01$, **: $P < 0.001$, ***: $P < 0.0001$. Lm, *Leu. mesenteroides*; Sb, *S. boulardii*; Sc, *S. cerevisiae* BY4742; KD2, *D. hansenii* KD2; C11, *D. hansenii* species complex C11. **(B, C)** Immunomodulatory activity of yeast cells. The secretion of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, IL-8, IL-12p70, and IL-10 was analyzed from human dendritic cell supernatants after co-incubation with viable **(B)** or heat-killed **(C)** yeast cells. The red and blue dots indicate each result from two different human blood donors. The amount of IL-1β, IL-6, TNF-α, IL-8, IL-12p70, and IL-10 was evaluated using the LEGENDplex assay. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

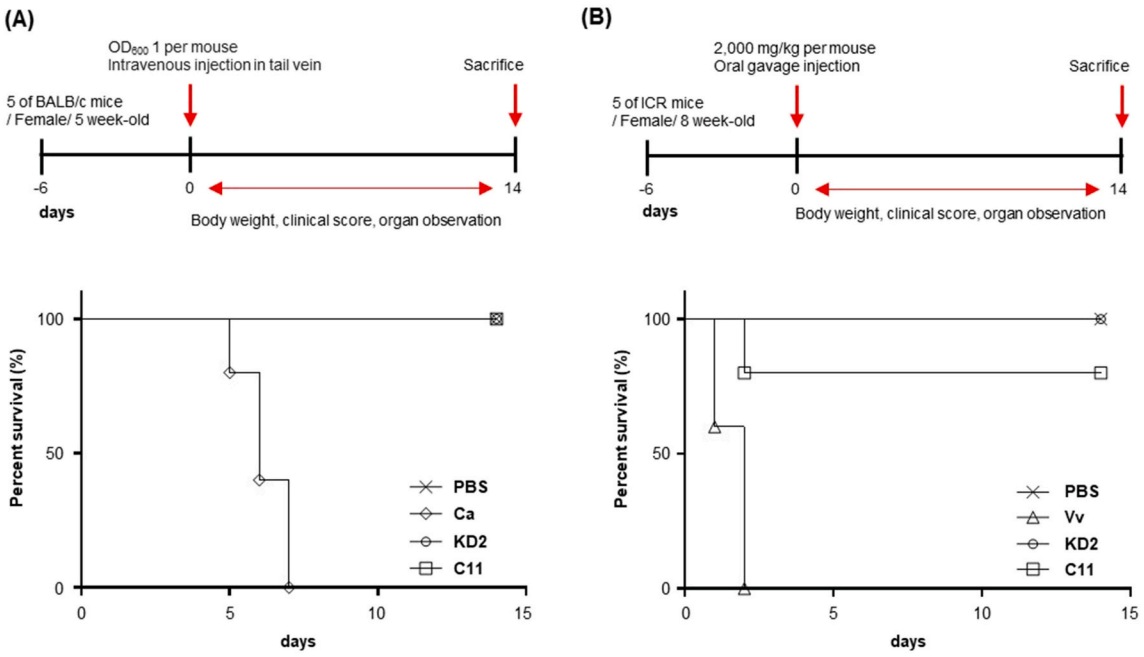

Fig. 5. Analysis of virulence and oral toxicity of *D. hansenii* KD2 and *D. hansenii* species complex C11. (A) *In vivo* virulence tests using mouse models. BALB/c mice were infected with 0.1 OD_{600} yeast cells by intravenous tail vein injection. **(B)** Acute oral toxicity analysis using mouse models. Yeast and bacterial cells [2000 mg (microbe)/kg (mouse weight)] were administered to ICR mice by oral gavage. Percent survival was monitored for 14 days post-infection or administration. The strain names are abbreviated as Ca, *C. albicans*; Vv, *V. vulnificus*; KD2, *D. hansenii* KD2; and C11, *D. hansenii* species complex C11.

amino acid precursors for the formation of volatile aromatic compounds. The *D. hansenii* ganjang isolates produced isoamyl alcohol and phenylethyl alcohol, which are higher alcohols generated from branched amino acids, as the major volatile flavor components in YPD media, whereas butanoic acid, butyraldehyde, and isovaleraldehyde were minor components (Fig. 3). In this study, we employed HS-SPME for the analysis of mixtures of volatile compounds, which may generate biased flavor profiles owing to its limitation in the adsorption selectivity of the fiber. SPME fibers are more sensitive to higher molecular compounds, which displace lower molecular weight compounds because of competition for active sites on the fiber (Murray, 2001). Therefore, more systematic analyses of flavor profiles using culture media that are designed to simulate the amino acid concentration and composition of soybean brick, meju, along with several other techniques for analysis of volatile flavor compounds (Louw, 2021), should be performed to accurately define the aroma generation potential of *D. hansenii* isolates during ganjang fermentation.

Furthermore, the KD2 and C11 strains isolated from ganjang showed strong bioconversion activity from FA to 4-VG, a substantial flavor component in soy sauce (Devanthi and Gkatzionis, 2019). The non-oxidative decarboxylation of FA, which is mediated by the enzymatic reaction of FA decarboxylase, leads to the bioconversion of 4-VG from FA in many yeasts and fungi (Mishra et al., 2014). This result indicates that *D. hansenii* KD2 and *D. hansenii* species complex C11 possess the enzymes responsible for the efficient production of 4-VG from FA. Further metabolism of 4-VG can generate commercially valuable vanillin after spontaneous decarboxylation (Batista, 2014) or 4-ethylguiuacol (4-EG), another flavor component with condimental smell and distinctive soy scent, via NADH dehydrogenation (Suezawa and Suzuki, 2007). However, the subsequent conversion to 4-EG from 4-VG was not detected in our SPME/GC-MS analysis (Supplementary Fig. 4), indicating that *D. hansenii* KD2 and *D. hansenii* species complex C11 cannot produce 4-EG from 4-VG. Intriguingly, KD2 and C11 exhibited a distinctive pattern of 4-VG biosynthetic activity, with KD2 showing high salt-dependent 4-VG biosynthetic activity, whereas C11 displayed constitutive 4-VG conversion activity. It is necessary to elucidate the molecular mechanisms underlying the different FA metabolism between the two *D. hansenii* ganjang isolates.

Although bacterial species in the groups *Bifidobacterium* and *Lactobacillus* originating from the human GI tract are widely used as probiotics, several yeast species have been recently reported to have probiotic activity, including *S. boulardii* (Czerucka et al., 2007; Sen and Mansell, 2020), *Kluyveromyces lactis, K. marxianus, D. hansenii*, and *Y. lipolytica* (Kumura et al., 2004; Maccaferri et al., 2012; Smith et al., 2014). Several *D. hansenii* strains isolated from different environments are also considered probiotic candidates for various animals and human beings (Angulo et al., 2020). The results from this study demonstrate the strong tolerance of the *D. hansenii* ganjang KD2 and C11 isolates to acidic culture conditions. Moreover, the strains showed strong adhesion to Caco-2 cells (Supplementary Fig. 7), as expected from their hydrophobic cell surface. Our result is consistent with previous reports showing that although the adhesion ability of *D. hansenii* varies among strains, *D. hansenii* strains isolated from dairy products have adherent abilities (Kumura et al., 2004). A few *D. hansenii* strains show greater adhesion ability than *S. boulardii*, a commercial probiotic strain (Ochangco et al., 2016). Considering that the anti-inflammatory cytokine IL-10 can suppress the T-helper cell type 1 subset-associated proinflammatory response which is activated by IL-12, the IL-10/IL-12 ratio can predict the effect of probiotics on the immune state (Gad et al., 2011; Watson et al., 2012). Regarding the immunomodulatory activity to suppress overactivated inflammatory responses, both *D. hansenii* ganjang isolates show a higher IL-10/IL-12 ratio that may be superior to that of *S. boulardii*. Interestingly, heat-treated yeast cells induced significantly higher cytokine levels than live yeast cells, likely owing to β-glucan exposure on the cell wall surface (Bazan et al., 2014), resulting in increased secretion of both pro and anti-inflammatory cytokines. However, the immunomodulatory activity of *D. hansenii* ganjang isolates in this study is proposed based on *in vitro* assays, as shown in previous reports on the probiotic potential of several food-borne yeasts. Therefore, further studies should be carried out to demonstrate the probiotic effect on healthy individuals after oral administration of the yeast. Furthermore, the safety of the ganjang yeast isolates was validated using

virulence and acute oral toxicity analyses in mouse models, supporting the potential for enhancing the nutritional properties of fermented foods.

5. Conclusions

Characterization of prevalent microbial species isolated from fermented foods at the genome level and systematic investigation of their food-related properties can provide insight into the roles of each microbial species in the complex fermentation process and nutritional values of fermented products. In this study, our comparative genomic and phylogenetic analyses based on intron sequences showed the distinctive genomic features of two *Debaryomyces* spp. isolates from the Korean fermented soy sauce, ganjang, revealing intriguing aspects of the genetic diversity and population structure within the genus *Debaryomyces* in the fermented food environment. Despite some noticeable differences between the two yeast ganjang isolates, both yeast strains showed beneficial physiological properties, such as the flavor production activity, which contributes to the characteristic flavor of ganjang, and the immunomodulatory activity, associated with the health benefits of fermented soybean products. A better understanding of yeast ganjang isolates as key players in flavor production and potential probiotics in food fermentation would provide information useful for generating Korean traditional ganjang products with higher quality and improved functionality.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104011.

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