TITLE PAGE
- Korean Journal for Food Science of Animal Resources -
Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Title	**Evaluation of probiotic characteristics of newly isolated lactic acid bacteria from dry-aged Hanwoo beef**
Running Title (within 10 words)	Lactic acid bacteria from dry-aged beef
Author	Hayoung Kim, Minhye Shin, Bohyun Yun, Sangnam Oh, Dong-Jun Park, Ham, and Younghoon Kim
Affiliation	Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea Department of Functional Food and Biotechnology, Jeonju University, Jeonju 55069, Korea Korea Food Research Institute, Wanju 55365, Korea
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Hayoung Kim (0000-0002-4142-549X) Minhye Shin (0000-0002-3649-4570) Bohyun Yun (0000-0001-6723-5849) Sangnam Oh (0000-0002-2428-412x) Dong-Jun Park (0000-0001-9452-9391) Younghoon Kim (0000-0001-6769-0657)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A1A02085907) and Microbial Institute for Fermentation Industry (MIFI) through Establishment of Integrated Biobank for Agriculture, Food and Livestock Microbiome Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (MIFI2020KMBB01).
Author's contributions (This field may be published.)	Conceived and designed the experiments: HK, MS, BY, SO, DJP, YK. Performed the experiments: HK, MS, BY, SO, DJP, YK. Analyzed the data: HK, MS, BY, SO, DJP, YK. Wrote the paper: HK, MS, BY, SO, DJP, YK.
Ethics approval (IRB/IACUC) (This field may be published.)	This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Dong-Jun Park and Younghoon Kim
Email address – this is where your proofs will be sent	ykeys2584@snu.ac.kr
Secondary Email address	
Postal address	Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea
Cell phone number	+82-10-4135-2584
Office phone number	+82-2-880-4808
Fax number	+82-2- 873-2271

ABSTRACT

Dry aging is a traditional method that improves meat quality, and diverse microbial communities are changed during the process. Lactic acid bacteria (LAB) are widely present in fermented foods and has many beneficial effects, such as immune enhancement and maintenance of intestinal homeostasis. In this study, we conducted metagenomic analysis to evaluate the changes in the microbial composition of dry-aged beef. We found that lactic acid bacterial strains were abundant in dry-aged beef including *Lactobacillus sakei* and *Enterococcus faecalis*. We investigated their abilities in acid and bile tolerance, adhesion to the host, antibiotic resistance, and antimicrobial activity as potential probiotics, confirming that *L. sakei* and *E. faecalis* strains had remarkable capability as probiotics. The isolates from dry-aged beef showed at least 70% survival under acidic conditions in addition to an increase in the survival level under bile conditions. Antibiotic susceptibility and antibacterial activity assays further verified their effectiveness in inhibiting all pathogenic bacteria tested, and most of them had low resistance to antibiotics. Finally, we used the *Caenorhabditis elegans* model to confirm their life extension and influence on host resistance. In the model system, 12D26 and 20D48 strains had great abilities to extend the nematode lifespan and to improve host resistance, respectively. These results suggest the potential use of newly isolated LAB strains from dry-aged beef as probiotic candidates for production of fermented meat.

INTRODUCTION

Dry aging is a traditional method to store slaughtered livestock under regulated conditions for a certain period (Kim et al., 2017). This process can be affected by the aging period, temperature, relative humidity and air flow (Dashdorj et al., 2016). It has many positive effects on beef quality, such as increased concentrations of beef flavor and improved tenderness and juiciness (Berger et al., 2018). In addition, dry aging also modifies the microbiological, physical, and chemical characteristics of beef (Smaldone et al., 2019). Previous studies have shown that microbiological changes improve quality, safety, palatability and flavor. Changes in the composition of microorganisms, including bacteria, yeast and fungi, result in alterations in the quality of dry-aged beef. In particular, various *Lactobacillus* spp. have been detected on the surface of dry-aged beef, including *L. sakei* and *L. plantarum* (Ryu et al., 2018).

Lactobacilli have been studied for their association with many diseases, attenuating allergic responses and metabolic disorders. Among the *Lactobacillus* strains, *L. sakei* is renowned for its role in the fermentation of meat products (Zagorec and Champomier-Vergès, 2017). In fact, *L. sakei* is found in various fermented foods, such as sake, sourdough and kimchi, and it has several beneficial effects on host immunity and metabolism. Recently, Rather et al.(2018) reported that *L. sakei* has effects on the amelioration of skin diseases such as atopic and psoriasis, and Ji et al.(2019) reported that *L. sakei* can reduce a production of obesity-related biomarkers by increasing the level of short-chain fatty acids. For these reasons, *L. sakei* has been used as a starter culture in fermented foods and optimized for industrial use; however, only a few studies have been performed on dry-aged beef (Barbieri et al., 2020).

The nematode *Caenorhabditis elegans* is a widely used model system for featured biological studies because of its transparency, short generation time, ease cultivation, and availability of numerous mutants. *C. elegans* can also provide insights into the functional aspects of anti-aging and innate immunity (Park et al., 2018). In other studies, *C. elegans* presented extension of lifespan and increased resistance to pathogenic bacteria and oxidative stresses with a supplementation of probiotics (Clark and Hodgkin, 2014; Grompone et al., 2012). In addition, the structure of human intestinal cells is similar to that of the intestinal cells of *C. elegans*, allowing estimation of bacterial adhesion to the host gut system

(Park et al., 2014). Lifespan and killing assays can be applied to screen *L. sakei* subspecies to determine whether they have an effect on antiaging or host defense mechanisms, respectively. For these reasons, we used the *C. elegans* model to evaluate the adhesion ability of bacteria isolated from dry-aged beef.

In the current study, we performed metagenomic analysis and found that dry-aged beef contains the *Lactobacillus* genus as the major portion of the microbiota. During the process of bacterial isolation, one *L. sakei* strain and three *Enterococcus faecalis* strains were isolated. Accordingly, we investigated their capability as probiotics by estimating their acid, bile tolerance and antibiotic sensitivity, antibacterial activity, and adhesion ability. Lastly, we performed *C. elegans* life-span and killing assays.

MATERIALS AND METHODS

Dry-aged Beef and sampling

Longissimus thoracis of first grade Hanwoo cattle were used in this study. Dry aging process was performed at 1–4℃ and a relative humidity of 80–90% for 50 days and microbiological samples (approximately 5.0-cm-thick) were obtained from the surface of carcass. Samples from the dry-aged beef were immediately transported to the laboratory at 4℃, without being vacuum packed.

Isolation and identification of lactic acid bacteria from dry-aging of beef

Lactic acid bacteria including *L. sakei* and *E. faecalis* strains were isolated from fresh samples of dry-aged beef (without any starter cultures). Each samples were homogenized and serially diluted. The diluted solution was plated on De Man-Rogosa-Sharpe (MRS; BD Difco, Sparks, MD, USA) agar and incubated at 37℃ for 48 h (Won et al., 2020). The selected isolates were identified by 16S rRNA sequencing. In every test, all strains were inoculated and sub-cultured in MRS broth at 37℃ for 48 h to reach the appropriate growth phase before the experiment.

Metagenomic analysis

Metagenomic analysis from dry-aged beef samples were performed by the described methods previously. The bacterial DNA were extracted using a Powerfood Microbial DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and subjected to PCR according to 16S metagenomic sequencing library protocols (Illumina, San Diego, CA, USA). FASTQ files obtained from MiSeq data were analyzed using Mothur (v. 1.14). In Mothur, reads were combined using the make contig command and were quality-filtered by the screen.seqs command (Ryu et al., 2020). The Mothur pipeline was used to process sequence data for analysis according to the Mothur SOP manual (https://nothur.org/wiki/miseq_sop/).

Acid and bile tolerance

The preparation of acidic and bile conditions was carried out according to the modified methods previously (Ashraf and Smith, 2016; Park et al., 2014). To make acidic conditions that reflect a stomach, the MRS broth was adjusted to pH 2.5 with 6 N HCl. After autoclaving, pepsin from porcine gastric mucosa (Sigma, St. Louis, MO, USA) was added to a final concentration of 1,000 units per milliliter and was filter-sterilized using 0.45-μm pore size syringe filters. In the acid tolerance test of *L. sakei* and *E. faecalis* strains, 100 μL of isolate was inoculated into 10 ml of acidic solution and incubated at 37°C for 0 h and 3 h. Subsequently, plate counting was performed and compared to 0 h incubated solution as a control.

The bile solution was prepared by suspending oxgall powder (Acumedia, Lansing, MI, USA) in MRS broth to a final concentration of 0.5% (w/v). All isolates were inoculated into 10 ml of bile solution and incubated at 37℃ for 24 h. The survival rate was calculated by counting the final viable population after 3 h of incubation compared with the initial viable counts at 0 h, which were immediately treated.

$$\text{Survival rate (\%)} = (\text{CFU}_{\text{after treatment}}/\text{CFU}_{\text{initial treatment}}) \times 100\%$$

Antibiotic sensitivity

We used the disc diffusion method to examine the antibiotic susceptibility of *L. sakei* isolates following the modified standard Kirby-Bauer method as used by Poonam Sharma et al. (Sharma et al., 2016). One hundred microliters of each isolate grown for 48 h in MRS broth was spread on MRS agar plates, and the discs with antibiotics were placed on the surface, followed by incubation at 37℃ for 24 h. We used ampicillin (10 μg), chloramphenicol (30 μg), kanamycin (30 μg), penicillin (10 μg), tetracycline (30 μg) and vancomycin (30 μg) antibiotic discs. After incubation, inhibition zone diameters were measured, and the degree of inhibition was categorized as resistant (R, zone diameter ≤ 14 mm), intermediate resistant (IR, zone diameter 15-19 mm) or susceptible (S, zone diameter > 20 mm).

Antibacterial activity

To evaluate antibacterial activity, we used 4 pathogenic bacterial strains: *Listeria monocytogenes*

EGD-e, *Staphylococcus aureus* Newman, *Salmonella* Typhimurium SL1344 and *Escherichia coli* ATCC 35150. The pathogenic bacterial strains were inoculated and incubated in LB medium at 37℃ for 24 h except *L. monocytogenes*, which was incubated at 30℃ for 48 h. The pathogenic bacteria were spread on LB agar medium, and then 5 μl of isolated bacterial strains from dry-aged beef was inoculated as a spot (Gomes et al., 2012). The antibacterial activity against pathogenic bacteria was categorized into 6 standards. The diameter of the inhibition zone was classified as < 10 mm, none (−); > 10 mm, weak (+); > 15 mm, middle (+ +); > 20 mm, strong (+ + +); or > 25 mm, very strong (+ + + +) inhibition (Lin et al., 2020).

In vitro adhesion ability

To perform a mucin adhesion assay, we dissolved type III porcine gastric mucin (Sigma-Aldrich, St. Louis, MO) to 1% in distilled water and filter-sterilized the solution using a 0.45-μm pore size syringe filter. After filtration, a 100-μl aliquot of mucin solution was added to a 96-well plate and allowed to attach for 24 h at 4℃. Then, we added bacterial strains to the 96-well plate after removing the mucin solution and incubated at 37℃ for 2 h. Subsequently, we removed the bacterial solution and washed the plate 5 times. Next, we added 200 μl of 0.1% Triton X-100 (DAEJUNG Co. Ltd., South Korea) to detach the mucin and diluted the solution removed from the plate in 0.85% NaCl. Finally, we dropped 5 μL of the solution into MRS agar medium and incubated it at 37°C for 48 h (Valeriano et al., 2014).

In vivo adhesion assay using C. elegans

For the life span and killing assay experiments, we used the *C. elegans fer-15; fem-1* model, which is unable to produce progeny at 25℃ without alteration in the phenotype. Worms were maintained on nematode growth medium (NGM) agar and seeded with *E. coli* strain OP50. Eggs were obtained using sodium hypochlorite-sodium hydroxide solution, and all synchronized L1 worms were grown at room temperature. To identify the degree of bacterial colonization in the *C. elegans* intestinal tract, we measured the number of bacterial cells in worm intestines. After exposing *C. elegans* to

individual bacterial strains on NGM for 24 h, 10 worms were picked randomly and placed on brain heart infusion agar (Sigma-Aldrich, MO, St Louis, USA) plates containing gentamycin (25 μg/mL) for 5 min. Next, worms were transferred to a 1.5-ml Eppendorf tube containing M9 buffer with Triton X-100 and were mechanically disrupted using a pestle (Kontes Glass Inc., Vineland, NJ). These diluted worms were plated on MRS agar medium and incubated at 37℃ for 48 h. *Lactobacillus rhamnosus* GG (LGG) and *E. coli* OP50 were used as positive and negative controls, respectively.

C. elegans life span and killing assay

For the *C. elegans* life span assay, 100-μL volumes of concentrated bacteria were plated on 35-mm-diameter NGM agar plates, and L4 stage *C. elegans fer-15; fem-1* worms were individually transferred with a platinum wire onto OP50 or isolated bacterial strain plates. For each life span assay, we assayed 90 worms per bacterial species in three separate plates, and all were incubated at 25℃. Total number of worms was counted daily and determined whether alive or dead by touching with a loop. To perform a *C. elegans* killing assay, L4 stage worms were placed on conditioning plates with isolated bacterial strains or *E. coli* OP50 for 24 h. Afterward, pathogenic bacteria, including *S. aureus* Newman and *E. coli* O157:H7 EDL933, were prepared and inoculated on NGM agar plates. Prepared nematodes were transferred onto plates with pathogens and incubated at 25℃. Living worms were transferred to fresh pathogen plates every day during the assay periods.

RESULTS

Lactic acid bacteria including Lactobacillus and Enterococcus genus are abundant in dry-aged beef

In our previous study, we found that the dry-aging process altered the microbial composition in beef. Alpha- and beta-diversities that indicate diversity of microbes were altered, especially, the abundance of *Lactobacillus* genus increased until 30 days of dry-aging, and it decreased when it reached at 70 days (Ryu et al., 2020). Based on the findings, we analyzed a 50-day sample to identify certain alterations between 30 and 70 days that are transition points for the microbiota. Samples were aged at 1-4℃ and a relative humidity of 80-90℃ for 50 days. Metagenomic analysis revealed that the *Lactobacillus* genus had the highest abundance in dry-aged beef, followed by *Bifidobacterium*. *Enterococcus* existed at an abundance of approximately 3%. These data were in accordance with findings at the phylum and genus levels (Figure 1). Consequently, we cultured lactic acid bacteria present in dry-aged beef samples on MRS agar plates in order to examine their characteristics. Finally, we isolated one *L. sakei* sample labeled 20D49 and three *E. faecalis* samples labeled 12D26, 20D48, and 30D36.

Isolated probiotic strains from dry-aged beef have acid and bile tolerance

Probiotics must survive in extreme acidic and bile conditions to have a positive impact on host where the gastrointestinal environment is reflected (Mallappa et al., 2019). We compared the isolated bacterial abilities that survive on acid and bile media in a timely manner. LGG has the ability to survive and proliferate under acidic and bile conditions, thus used as a standard along with the test strains (Capurso, 2019). Four bacterial strains isolated from dry-aged beef survived acid MRS media after 3 h of exposure (Figure 2). In particular, the 12D26 isolate showed a remarkable survival rate (88%). Although the 20D48, 20D49 and 30D36 isolates had lower survival rates than LGG in terms of acid tolerance, they had survival rates greater than 70%, suggesting that they have tolerance in acid.

In the bile tolerance test, bacterial strains were exposed to 0.5% bile in MRS broth for 24 h. In our experiment, all isolates survived and they even grew under bile conditions. In particular, the 12D26

isolate had outstanding capacity for enduring bile stress that similar to the small intestine condition. Other three strains showed 74-77% of survival rates which were comparably high than other bacterial strains. Overall, all strains isolated from dry-aged beef presented superior acid and bile tolerance, and 12D26 demonstrated remarkable survival capacity in these experiments.

Isolated probiotic strains from dry-aged beef are mostly susceptible to antibiotics

Antibiotic sensitivity assays verify the susceptibility of bacteria to antibiotics using antibiotic discs. In the current study, we tested bacterial susceptibility to antibiotics, including ampicillin (10 μg), chloramphenicol, kanamycin, penicillin, tetracycline and vancomycin. All isolates indicated different patterns of antibiotic sensitivity and were susceptible to chloramphenicol and penicillin (Table 1). In particular, 20D49 had remarkable results because it did not have antibiotic resistance to most of the antibiotics except for vancomycin and kanamycin. Isolates 12D26 and 30D36 were resistant to kanamycin and tetracycline but sensitive to chloramphenicol, penicillin and vancomycin. Our results suggest that the bacterial isolates are susceptible to a portion of antibiotics, and *L. sakei* 20D49 may have a benefits with respect to the antibiotic susceptibility.

Isolated probiotic strains from dry-aged beef inhibit pathogenic bacterial growth

Probiotic strains must have outstanding antimicrobial properties to eliminate or inhibit pathogenic bacteria. The antagonistic activity of *Lactobacillus* and *Enterococcus* against bacterial pathogens is a characteristic worth consideration (Samot and Badet, 2013). Here, we tested the antibacterial activity of *L. sakei* and *E. faecalis* strains against pathogenic bacteria, including *L. monocytogenes* EGD-e, *S. aureus* Newman, *S.* Typhimurium SL1344 and *E. coli* ATCC 35150. As the result, all isolated bacteria showed outstanding antimicrobial activity against *L. monocytogenes*, *S. aureus*, *S.* Typhimurium and *E. coli*. *L. sakei* and *E. faecalis* strains produced clear zones larger than 17 mm in diameter. These results show that all of the strains isolated from dry-aged beef have antimicrobial properties that can inhibit pathogens.

Isolated probiotic strains from dry-aged beef have adhesion ability to mucin and C. elegans intestine

Probiotics have many beneficial effects on humans under the condition that they survive and proliferate in the environment of the host's gastrointestinal tract (Fernández et al., 2003). When they survive in the human gut, they must have the capability to adhere to the human mucus layer (Tuo et al., 2018). We identified the adhesion ability to mucin compared with a commercial strain as a positive control: LGG, which is well known for its adhesion ability. We found that all of the *L. sakei* strains and *E. faecalis* strains had mucin adhesion profiles similar to LGG (70%, Figure 3A). In conclusion, all of the isolated strains from dry-aged beef showed adhesion ability to mucin.

Next, we attempted to examine the adhesion capacity of the strains by using a *C. elegans* model. Because we defined that all of the isolates had adhesion ability in mucin *in vitro*, we identified their adhesion capacity *in vivo* using *C. elegans*. LGG and *E. coli* OP50 were used as positive and negative controls, respectively. In this study, 12D26, 20D48 and 20D49 indicated outcomes similar to LGG and higher adhesion capacity compared with OP50 by approximately two-folds. Although the 30D36 attached to the nematode intestine less than LGG, the adhesion was considerably improved compared with *E. coli* strain OP50. In conclusion, *L. sakei* and *E. faecalis* strains had remarkable adhesion capacity in the *C. elegans* model compared with the *E. coli* OP50 control strain.

Isolated probiotic strains from dry-aged beef significantly extend the C. elegans lifespan

In previous experiments, we observed that *L. sakei* and *E. faecalis* strains had great potential as probiotics. We next examined whether the isolates had beneficial health effects on the host using a *C. elegans* model system. In the lifespan assay, we used 6 bacterial strains, including one of *L. sakei* and three of *E. faecalis* strains isolated from dry-aged beef. Additionally, LGG and *E. coli* OP50 were used as positive and negative controls, respectively. In this study, we observed that the 12D26 increased the lifespan of *C. elegans*, similar to LGG, and its effect was distinctly distinguished from *E. coli* OP50 (Figure 4). Along with the 12D26, other strains had beneficial effects on life extension compared with *E. coli* OP50, although their effects were slightly lower than the effect of LGG. The 20D49 was also significantly different from *E. coli* and LGG. Therefore, all of the isolated bacteria, especially the *E.*

faecalis 12D26 and the *L. sakei* 20D49, had exceptional effects on *C. elegans* lifespan extension.

Isolated probiotic strains from dry-aged beef enhance resistance against pathogenic bacteria in C. elegans

In addition to the lifespan extension analyses, we further performed killing assays to identify whether the *L. sakei* and *E. faecalis* isolates influenced host resistance against exposure to food-borne pathogenic bacteria (Figure 5). After, *C. elegans* were transferred to the isolated bacterial lawn for 1 day, they were exposed to *S. aureus* Newman or *E. coli* O157:H7 EDL933. These bacteria can kill the nematode through an infection-like process in the intestine (Irazoqui et al., 2010). When worms were exposed to the *E. coli* O157:H7 strain without pretreatment with the isolated strains, they were killed within 14 days after transfer to the pathogen. However, pre-exposure with our isolates attenuated the pathogenic effects, increasing the lifespan of *C. elegans*. In particular, 20D48 had the best ability to influence host resistance against pathogens, while the other three strains had no significant effects on prevention compared with OP50 and LGG. In experiments with *S. aureus*, worms were also killed within 14 days, similar to *E. coli* O157:H7. Pretreatment of the isolates 12D26, 20D49 and 30D36 showed comparably significant improvements in host resistance compared with OP50. In contrast, the 20D48 strain was similar to *E. coli* strain OP50 and did not affect host resistance against *S. aureus*. Based on our results, the isolated strains had different abilities to enhance nematode resistance depending on the type of pathogen. Among the strains, 20D49 and 30D36 had resistance to promote host defense conditioned with *S. aureus*, while they had resistance similar to *E. coli* strain OP50 under pathogenic *E. coli* O157:H7.

DISCUSSION

Dry aging of beef improves flavors due to the absorption of juice and the chemical breakdown of protein and fat (Dashdorj et al., 2016). Through this alteration, the quality and safety of dry-aged beef are improved, and microbiological changes play an important role in these aspects. In our previous study, total bacteria and lactic acid bacteria were significantly increased during the dry-aging period (Ryu et al., 2018). From this perspective, we were able to affirm the changed microorganisms in dry-aged beef, which may influence quality and safety in meat. In our study, isolates from dry-aged beef had superior acid and bile tolerance and adhesion abilities that were similar to LGG. Furthermore, most *L. sakei* and *E. faecalis* strains influenced the lifespan of *C. elegans* compared *with E. coli* strain OP50 in both normal plate conditions and plates inoculated with pathogens.

When we analyzed the microbiota composition of dry-aged beef, the relative abundance of the *Lactobacillus* genus was highest. Accordingly, we isolated the *L. sakei* strain from dry-aged beef and characterized its functionality as a probiotic. *L. sakei* is found in various fermented foods, especially in dry-aged meat, and it has many beneficial effects on the host. Previous studies discovered that *L. sakei* regulates allergic Th2 responses, which enhance Treg gene production and change the relative abundance of gut bacteria (Kwon et al., 2018). Moreover, the treatment of *L. sakei* induced reductions in adipose tissue and various biomarkers associated with obesity resulting from the gut microbiota-modulating ability and stimulation of SCFAs by *L. sakei*.

In this study, our results clearly showed that the *L. sakei* and *E. faecalis* isolates from dry-aged beef possess acid and bile tolerances, anti-bacterial activities, and great adhesion abilities. A previous study demonstrated that the *L. sakei* strain isolated from fresh pork sausage presented a low survival rate under pH 2.5 conditions; however, we confirmed that *L. sakei* isolated from dry-aged beef had a superior survival rate at pH 2.5. In addition to acid tolerance, the bile tolerance of *L. sakei* from fresh pork sausage decreased with time, while *L. sakei* from dry-aged beef increased with time (Gomes et al., 2012). These differences would be due to the dry-aging process, considering that the experimental results of acid and bile tolerance are similar. Furthermore, because of the subspecies differences of isolates from dry-aged beef, the four strains might have different abilities in acid and bile tolerance

compared with previous reports. *L. sakei* is known for producing bacteriocin, which influences the adhesion of pathogenic bacteria (Winkelströter et al., 2011). Previously, *L. sakei* can inhibit the bacterial growth of gram-positive pathogens, including *L. monocytogenes* (Liserre et al., 2002). In addition to *L. sakei*, *E. faecalis* also produce bacteriocins that inhibit gram-positive pathogen growth (Toit et al., 2000). In this respect, we found that all of the isolates from dry-aged beef have the ability to inhibit not only gram-positive pathogens but also gram-negative pathogens. Regarding the adhesion ability, *L. sakei* isolated from healthy humans feces had low or absent adhesion ability in a *C. elegans* model, while the *L. sakei* strain isolated from dry-aged beef had great adhesion ability in a *C. elegans* and a mucin assay (Lee et al., 2015; Park et al., 2014). This implies that the significant *C. elegans* adhesion ability of the isolates might result from differences at the subspecies level.

C. elegans takes bacteria as a source of nutrition, and bacteria play a key role in the regulation of *C. elegans* lifespan (Kim and Mylonakis, 2012). The 12D26 strain had an excellent effect on *C. elegans* lifespan extension in particular. For the killing assay, bacterial isolates showed different abilities with each pathogen. In this study, 20D49 and 30D36 had the ability to attenuate the *S. aureus* infection; however, they had little ability to affect the host resistance against *E. coli* O157:H7. Various studies have investigated the effects of *Lactobacillus* on the lifespan and fitness of *C. elegans* (Kim and Mylonakis, 2012; Park et al., 2014; Park et al., 2018). However, there are a few studies that utilize the *C. elegans* model for *L. sakei* application. In the model system, we identified the effects of *L. sakei* and *E. faecalis* on antiaging and innate immunity using the lifespan and killing assays. These strains extended the lifespan of *C. elegans* and protected them from the bacterial pathogenicity, ultimately prolonging the life of nematodes.

Taken together, *L. sakei* and *E. faecalis* strains exhibited remarkable ability as probiotics, including acid and bile tolerance, good performance in antibiotic sensitivity tests, and antibacterial activity. Additionally, they showed magnificent results based on *C. elegans* lifespan and *C. elegans* killing assays. Based on this study, we identified *L. sakei* and *E. faecalis* strains isolated from dry-aged beef could be attractive probiotic candidates for human wellness as well as production of fermented meat.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A1A02085907) and Microbial Institute for Fermentation Industry (MIFI) through Establishment of Integrated Biobank for Agriculture, Food and Livestock Microbiome Project, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (MIFI2020KMBB01).

REFERENCES

Ashraf R, Smith S. 2016. Commercial lactic acid bacteria and probiotic strains-tolerance to bile, pepsin and antibiotics. International Food Research Journal 23:777.

Barbieri F, Laghi L, Gardini F, Montanari C, Tabanelli G. 2020. Metabolism of lactobacillus sakei chr82 in the presence of different amounts of fermentable sugars. Foods 9:720.

Berger J, Kim YHB, Legako JF, Martini S, Lee J, Ebner P, Zuelly SMS. 2018. Dry-aging improves meat quality attributes of grass-fed beef loins. Meat science 145:285-291.

Capurso L. 2019. Thirty years of lactobacillus rhamnosus gg: A review. Journal of clinical gastroenterology 53:S1-S41.

Clark LC, Hodgkin J. 2014. Commensals, probiotics and pathogens in the Caenorhabditis elegans model. Cellular microbiology 16:27-38.

Das DJ, Shankar A, Johnson JB, Thomas S. 2020. Critical insights into antibiotic resistance transferability in probiotic lactobacillus. Nutrition 69:110567.

Dashdorj D, Tripathi VK, Cho S, Kim Y, Hwang I. 2016. Dry aging of beef; review. Journal of Animal Science and Technology 58:20.

Fernández MF, Boris S, Barbes C. 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. Journal of applied microbiology 94:449-455.

Gomes BC, Rodrigues MR, Winkelstroeter LK, Nomizo A, De Martinis EC. 2012. In vitro evaluation of the probiotic potential of bacteriocin producer lactobacillus sakei 1. Journal of food protection 75:1083-1089.

Grompone G, Martorell P, Llopis S, González N, Genovés S, Mulet AP, Fernández-Calero T, Tiscornia I, Bollati-Fogolín M, Chambaud I. 2012. Anti-inflammatory lactobacillus rhamnosus cncm i-3690 strain protects against oxidative stress and increases lifespan in caenorhabditis elegans. PloS one 7:e52493.

Irazoqui JE, Troemel ER, Feinbaum RL, Luhachack LG, Cezairliyan BO, Ausubel FM. 2010. Distinct pathogenesis and host responses during infection of c. Elegans by p. Aeruginosa and s. Aureus. PLoS Pathog 6:e1000982.

Ji Y, Park S, Chung Y, Kim B, Park H, Huang E, Jeong D, Jung H-Y, Kim B, Hyun C-K. 2019. Amelioration of obesity-related biomarkers by lactobacillus sakei cjls03 in a high-fat diet-induced obese murine model. Scientific reports 9:1-11.

Kim Y, Mylonakis E. 2012. Caenorhabditis elegans immune conditioning with the probiotic bacterium lactobacillus acidophilus strain ncfm enhances gram-positive immune responses. Infection and immunity 80:2500-2508.

Kim YHB, Meyers B, Kim H-W, Liceaga AM, Lemenager RP. 2017. Effects of stepwise dry/wet-aging and freezing on meat quality of beef loins. Meat science 123:57-63.

Kwon M-S, Lim SK, Jang J-Y, Lee J, Park HK, Kim N, Yun M, Shin M-Y, Jo HE, Oh YJ. 2018. Lactobacillus sakei wikim30 ameliorates atopic dermatitis-like skin lesions by inducing regulatory t cells and altering gut microbiota structure in mice. Frontiers in immunology 9:1905.

Lee HK, Choi S-H, Lee CR, Lee SH, Park MR, Kim Y, Lee M-K, Kim G-B. 2015. Screening and characterization of lactic acid bacteria strains with anti-inflammatory activities through in vitro and caenorhabditis elegans model testing. Korean journal for food science of animal resources 35:91.

Lin CF, Lin MY, Lin CN, Chiou MT, Chen JW, Yang KC, Wu MC. 2020. Potential probiotic of lactobacillus strains isolated from the intestinal tracts of pigs and feces of dogs with antibacterial activity against multidrug-resistant pathogenic bacteria. Archives of Microbiology.

Liserre AM, Landgraf M, Destro MT, Franco BD. 2002. Inhibition of listeria monocytogenes by a bacteriocinogenic lactobacillus sake strain in modified atmosphere-packaged brazilian sausage. Meat science 61:449-455.

Mallappa RH, Singh DK, Rokana N, Pradhan D, Batish VK, Grover S. 2019. Screening and selection of probiotic lactobacillus strains of indian gut origin based on assessment of desired probiotic attributes combined with principal component and heatmap analysis. LWT 105:272-281.

Nakagawa H, Shiozaki T, Kobatake E, Hosoya T, Moriya T, Sakai F, Taru H, Miyazaki T. 2016. Effects and mechanisms of prolongevity induced by lactobacillus gasseri sbt2055 in caenorhabditis elegans. Aging cell 15:227-236.

Park M, Yun H, Son S, Oh S, Kim Y. 2014. Development of a direct in vivo screening model to identify potential probiotic bacteria using caenorhabditis elegans. Journal of dairy science 97:6828-6834.

Park MR, Ryu S, Maburutse BE, Oh NS, Kim SH, Oh S, Jeong S-Y, Jeong D-Y, Oh S, Kim Y. 2018. Probiotic lactobacillus fermentum strain jdfm216 stimulates the longevity and immune response of caenorhabditis elegans through a nuclear hormone receptor. Scientific reports 8:1-10.

Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, Gil A. 2019. Mechanisms of action of probiotics. Advances in Nutrition 10:S49-S66.
Rather IA, Bajpai VK, Huh YS, Han Y-K, Bhat EA, Lim J, Paek WK, Park Y-H. 2018. Probiotic lactobacillus sakei probio-65 extract ameliorates the severity of imiquimod induced psoriasis-like skin inflammation in a mouse model. Frontiers in microbiology 9:1021.
Ryu S, Park MR, Maburutse BE, Lee WJ, Park D-J, Cho S, Hwang I, Oh S, Kim Y. 2018. Diversity and characteristics of the meat microbiological community on dry aged beef. J Microbiol Biotechnol 28:105-108.
Ryu S, Shin M, Cho S, Hwang I, Kim Y, Oh S. 2020. Molecular characterization of microbial and fungal communities on dry-aged beef of hanwoo using metagenomic analysis. Foods 9:1571.
Samot J, Badet C. 2013. Antibacterial activity of probiotic candidates for oral health. Anaerobe 19:34-38.
Sharma P, Tomar SK, Sangwan V, Goswami P, Singh R. 2016. Antibiotic resistance of lactobacillus sp. Isolated from commercial probiotic preparations. Journal of Food Safety 36:38-51.
Smaldone G, Marrone R, Vollano L, Peruzy MF, Barone CMA, Ambrosio RL, Anastasio A. 2019. Microbiological, rheological and physical-chemical characteristics of bovine meat subjected to a prolonged ageing period. Italian journal of food safety 8.
Toit MD, Franz C, Dicks L, Holzapfel W. 2000. Preliminary characterization of bacteriocins produced by enterococcus faecium and enterococcus faecalis isolated from pig faeces. Journal of Applied Microbiology 88:482-494.
Tuo Y, Song X, Song Y, Liu W, Tang Y, Gao Y, Jiang S, Qian F, Mu G. 2018. Screening probiotics from lactobacillus strains according to their abilities to inhibit pathogen adhesion and induction of pro-inflammatory cytokine il-8. Journal of dairy science 101:4822-4829.
Valeriano V, Parungao-Balolong M, Kang DK. 2014. In vitro evaluation of the mucin-adhesion ability and probiotic potential of l actobacillus mucosae lm 1. Journal of applied microbiology 117:485-497.
Winkelströter LK, Gomes BC, Thomaz MR, Souza VM, De Martinis EC. 2011. Lactobacillus sakei 1 and its bacteriocin influence adhesion of listeria monocytogenes on stainless steel surface. Food Control 22:1404-1407.
Won S-M, Chen S, Park KW, Yoon J-H. 2020. Isolation of lactic acid bacteria from kimchi and screening of lactobacillus sakei adm14 with anti-adipogenic effect and potential probiotic properties. LWT:109296.
Zagorec M, Champomier-Vergès M-C. 2017. Lactobacillus sakei: A starter for sausage fermentation, a protective culture for meat products. Microorganisms 5:56.
Zhou J, Pillidge C, Gopal P, Gill H. 2005. Antibiotic susceptibility profiles of new probiotic lactobacillus and bifidobacterium strains. International journal of food microbiology 98:211-217.

Table 1. Sensitivity tests for the *L. sakei* and *E. faecalis* strains isolated from dry-aged beef against 6 antibiotics.

	E. faecalis 12D26	*E. faecalis* 20D48	*L. sakei* 20D49	*E. faecalis* 30D36
Ampicillin	I	S	S	I
Chloramphenicol	S	S	S	S
Kanamycin	R	R	I	R
Penicillin	S	S	S	S
Tetracycline	R	R	S	R
Vancomycin	S	S	R	S

*note: R-Resistant; I-Intermediate; S-Susceptible

Antibiotic discs included ampicillin (10 μg), chloramphenicol (30 μg), kanamycin (30 μg), penicillin (10 μg), tetracycline (30 μg) and vancomycin (30 μg).

Antibiotic resistance was evaluated by disc diffusion (inhibition zone diameter); S-Susceptible: >20 (mm), I-Intermediate: 15 -19 (mm), R-Resistant: ≤14 (mm)

FIGURE LEGENDS

Figure 1. (A) Observation of surface and (B) metagenomic composition of dry-aged beef. Dry aging process was performed at 1–4℃ and a relative humidity of 80–90% for 50 days. The relative bacterial abundance was represented by percentage (%).

Figure 2. Viability of *L. sakei* and *E. faecalis* against (A) artificial gastric acid and (B) bile condition. Each strain was inoculated in acidic medium at 37 ℃ for 3 h. This medium has a low pH (pH 2.5) and contains pepsin (1,000 unit/ml). Bile tolerance tests were conducted for 24 h. 12D26, *E. faecalis*; 20D48, *E. faecalis*; 20D49, *L. sakei*; and 30D36, *E. faecalis*. Statistical analysis was performed using ANOVA and differences were considered significant when P was below 0.0001 (****), 0.001 (***), or 0.01 (**). Data are expressed as the mean ± standard deviation (S.D.) of three independent experiments.

Figure 3. Adhesion ability of isolated strains in the mucus layer *in vitro* (A) and *in* the *C. elegans* model *in vivo* (B). 12D26, *E. faecalis*; 20D48, *E. faecalis*; 20D49, *L. sakei*; and 30D36, *E. faecalis*. Statistical analysis was performed using ANOVA, and differences were considered significant when P was below 0.0001 (****), 0.001 (***), or 0.01 (**). Data are expressed as the mean ± standard deviation (S.D.) of three independent experiments.

Figure 4. Extended life span of *C. elegans* fed with *L. sakei* and *E. faecalis* strains (A) 12D26, (B) 20D48, (C) 20D49 and (d) 30D36 compared with *E. coli* OP50 and LGG. Life span assays (n=30 per plate) of *C. elegans* strains *fer-15; fem-1* exposed to *L. sakei* and *E. faecalis* strains. Statistical analysis was performed using Kaplan-Meier method, and differences were considered significant when P was below 0.05. The P value in black is compared with *E. coli* OP50, and the P value in pink is compared with LGG. (a) 12D26, *E. faecalis*, (b) 20D48, *E. faecalis*, (c) 20D49, *L. sakei*, and (d) 30D36, *E. faecalis*.

Figure 5. Killing assay of *C. elegans* fed with *L. sakei* and *E. faecalis* using (a) *E. coli* O157:H7 and (b) *S. aureus* Newman. Preconditioning with isolates from dry-aged beef prolonged the life span of *C. elegans* infected with (a) *E. coli* O157:H7 and (b) *S. aureus*. Statistical analysis was performed using Kaplan-Meier method, and differences were considered significant when P was below 0.05. The P value in black is compared with *E. coli* OP50, and the P value in pink is compared with LGG. 12D26, *E. faecalis*; 20D48, *E. faecalis*; 20D49, *L. sakei*; and 30D36, *E. faecalis*.

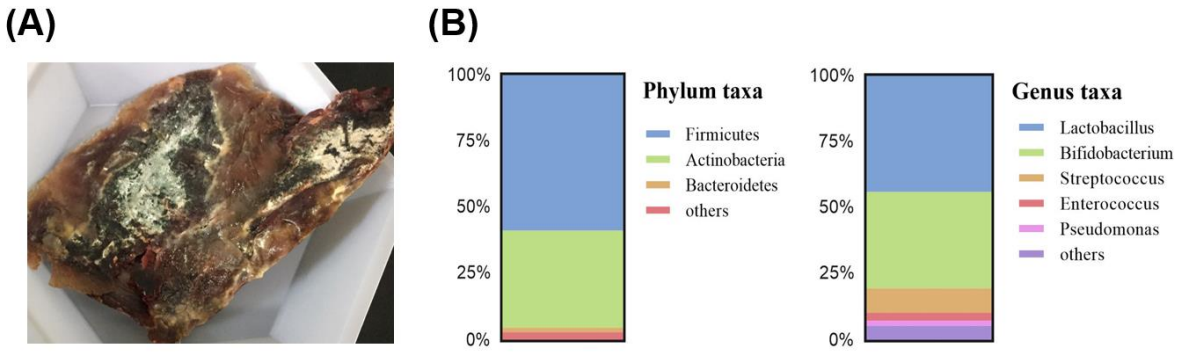
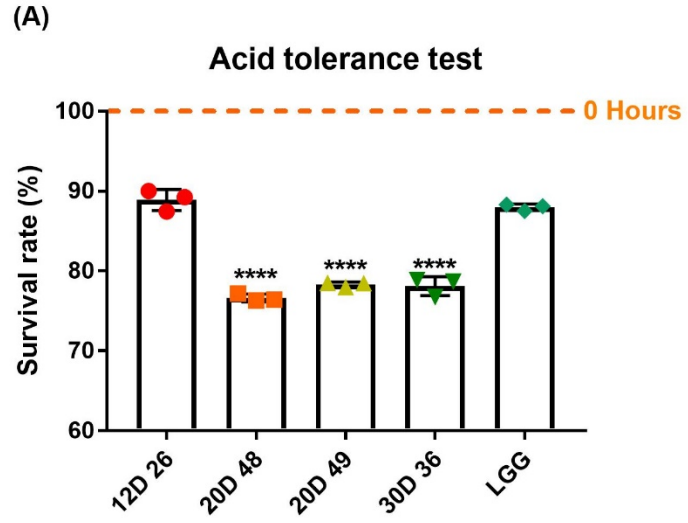


Figure 1.



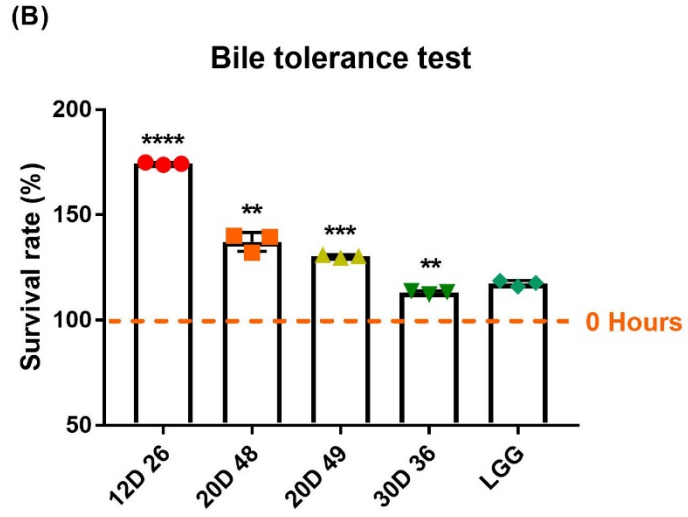
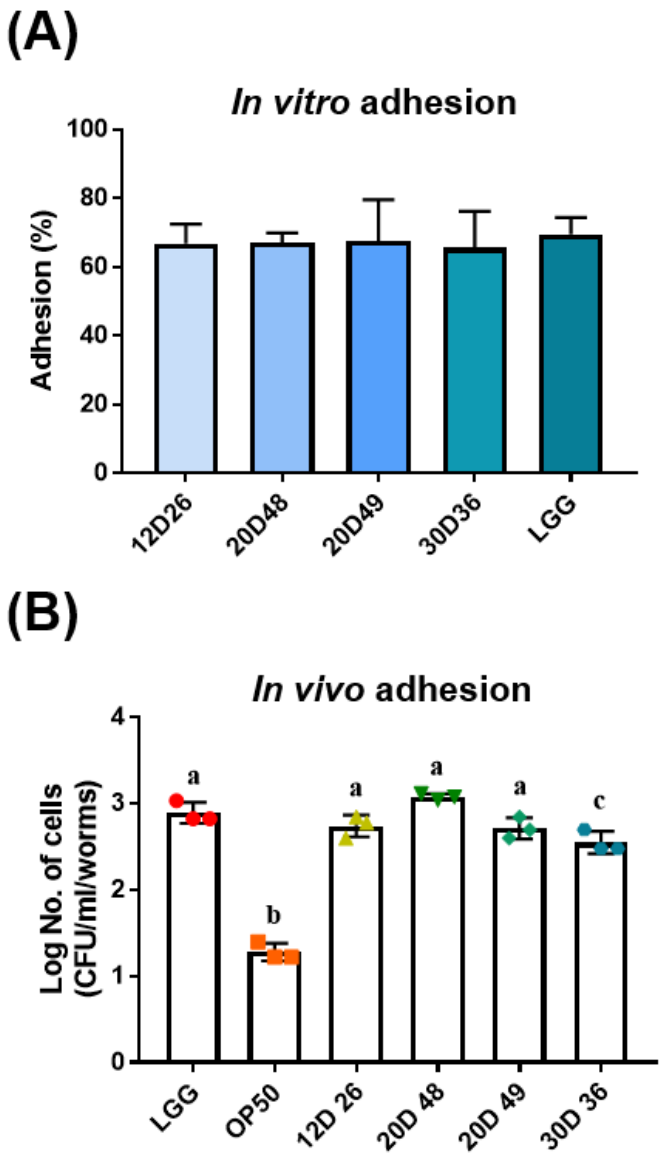


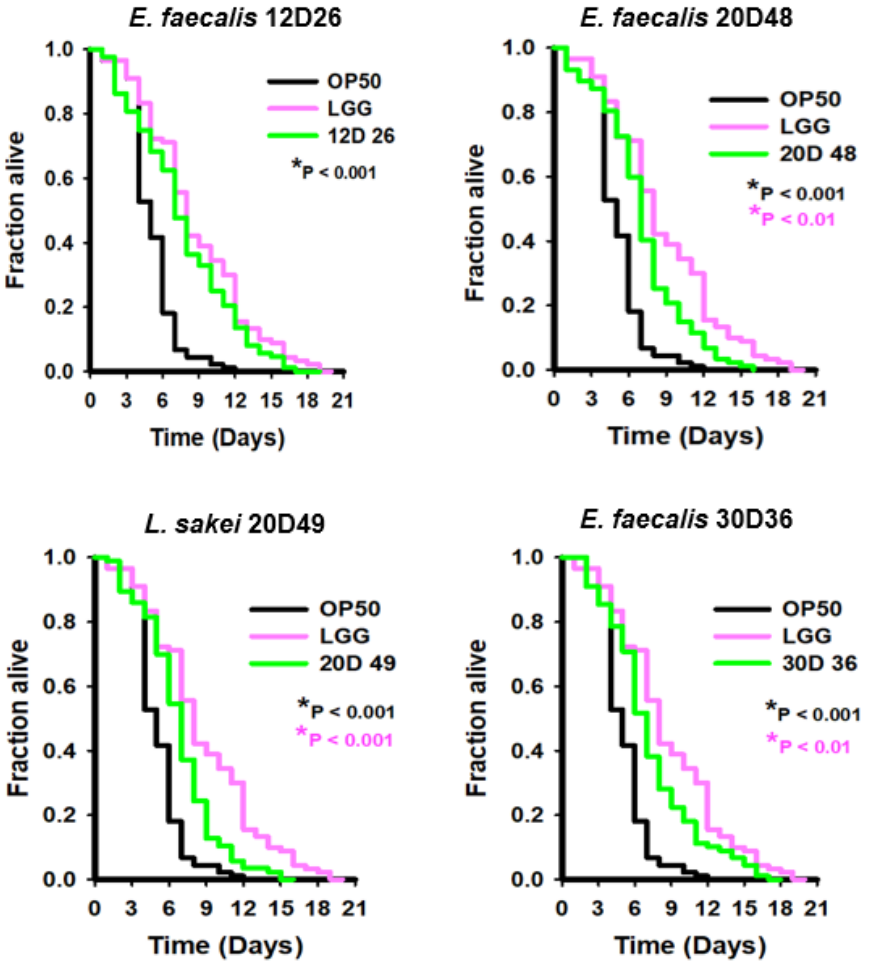
Figure 2.



472

473 **Figure 3.**

474



475

476 **Figure 4.**

477

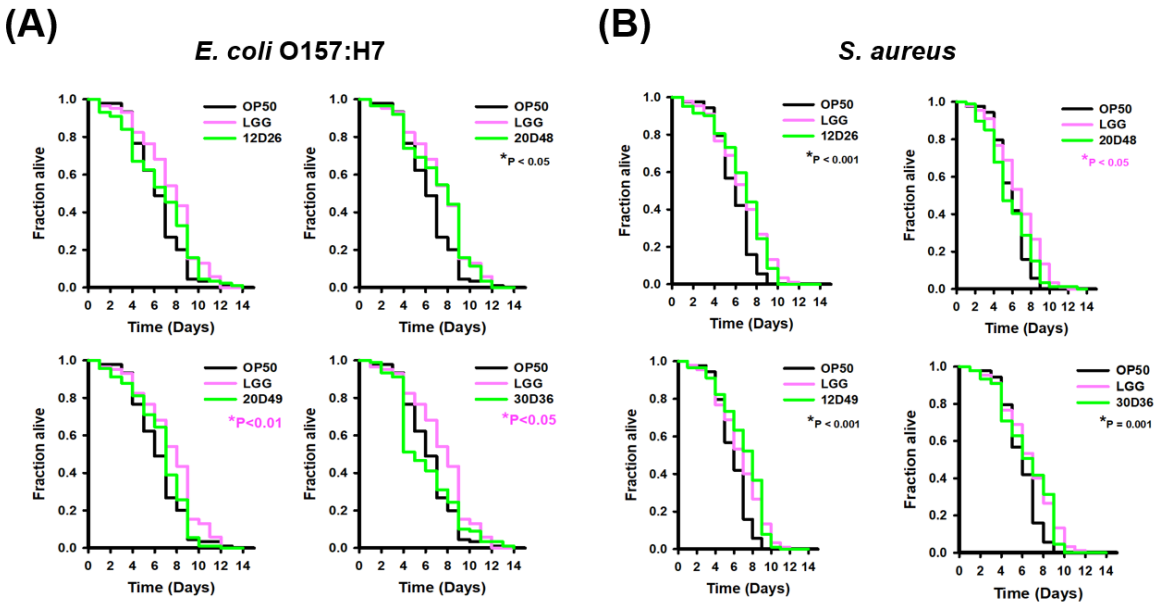


Figure 5.