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Genomic Analysis and Functional Profiling of Lactic Acid Bacteria: Molecular approaches with High-Activity Promoters for Antioxidant Enhancement

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Abstract

Genomic Analysis and Functional Profiling of Lactic Acid Bacteria: Molecular approaches with High-Activity Promoters for Antioxidant Enhancement

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This study investigates the probiotic potential and improved antioxidant properties of *Lacticaseibacillus paracasei* EG005, with a particular emphasis on its role in reducing oxidative stress through the enhanced expression of antioxidant enzymes. The research is presented in two main chapters, each addressing different but complementary aspects of *L. paracasei* EG005 and its promising applications in health

and biotechnology. By exploring both the theoretical and experimental dimensions, this study aims to provide a comprehensive understanding of EG005's unique capabilities and its potential role as an effective probiotic.

The first chapter of the research paper was an extensive literature review that performed to provide a thorough background on the role of *Lactobacillus* species as probiotics. *Lactobacillus* species are well-known for their beneficial effects on gut health, including modulation of the immune response, enhancement of gut barrier integrity, and reduction of pathogenic microorganisms through the production of antimicrobial substances and lactic acid. This chapter emphasized the role of *Lactobacillus* in maintaining a balanced gut microbiome and improving overall health. Additionally, bioinformatics tools were highlighted as essential for characterizing the genetic and functional attributes of probiotics, which aids in selecting and optimizing strains with enhanced health benefits. Genomic sequencing and gene annotation were discussed as key techniques for identifying functional genes that contribute to probiotic efficacy, including antioxidant enzymes like superoxide dismutase (SOD). Furthermore, the importance of overexpression techniques was outlined, particularly focusing on boosting specific protein activities that contribute to improved probiotic function. Promoters such as Ptuf and Pldh were discussed for their ability to drive high-level expression of target genes, making them valuable tools in enhancing the probiotic properties of *Lactobacillus* strains.

The second chapter was focused on the experimental investigation of the antioxidant capacity of *L. paracasei* EG005, specifically focusing on the activity of

superoxide dismutase (SOD), an enzyme known for its ability to neutralize harmful reactive oxygen species (ROS). The study began by assessing the baseline probiotic characteristics of EG005, including its acid and bile tolerance, which are critical for survival and activity in the gastrointestinal tract. The strain demonstrated significant acid and bile resistance, which is essential for maintaining viability as it passes through the digestive system. Additionally, safety assessments confirmed that EG005 lacked hemolytic activity and antibiotic resistance genes, underscoring its suitability for use as a safe and effective probiotic.

Genetic engineering techniques were then employed to enhance the antioxidant properties of EG005. Potent promoters, such as *Ptuf* and *Pldh*, were used to drive the overexpression of the SOD gene, with the aim of increasing the strain's ability to combat oxidative stress. The Ptuf promoter, in particular, led to a significant two-fold increase in SOD activity compared to the control strain, highlighting its effectiveness in boosting antioxidant enzyme production. This enhanced SOD activity has important implications for the strain's ability to mitigate oxidative stress, which is a key factor in maintaining gut health and reducing the risk of inflammation-related diseases. The Pldh promoter also showed promising results, though its impact was not as pronounced as that of Ptuf. These findings suggest that the choice of promoter plays a critical role in determining the level of gene expression and, consequently, the functional benefits of the probiotic strain. Future studies should focus on in vivo testing to confirm these findings and assess the efficacy of EG005. In vivo trials involving animal models or human subjects would provide critical data on the probiotic's impact on gut microbiota composition, immune modulation, and antioxidant activity within the host.

These research papers will enhance our understanding of the probiotic potential of *L. paracasei* EG005 and its application in enhancing antioxidant defenses. The integration of bioinformatics analysis with genetic engineering approaches has allowed for a deeper understanding of EG005's functional attributes and its suitability for use in therapeutic and functional food applications. This research forms a foundation for future investigations into EG005's use in health-promoting products, particularly for its benefits in combating oxidative stress, reducing inflammation, and enhancing digestive health.

Key Words: Lactic acid bacteria, Superoxide dismutase (SOD), Antioxidant activity, SOD overexpression, Promoter

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Chapter 1. Literature Review

1.1 Probiotics

1.1.1 *Lactobacillus* as probiotics

Lactobacillus is well known as a probiotic that plays an important role in the human gut microbiota. This bacterium contributes not only to improving gut health but also to enhancing the immune system (Quigley, 2013). In particular, *Lactobacillus* produces lactic acid during its metabolic processes, which helps lower the pH of the gut and inhibit the growth of pathogenic microorganisms (Arqués et al., 2015). Through this mechanism, *Lactobacillus* maintains a beneficial gut environment and protects the host's digestive health. Due to these functions, *Lactobacillus* is widely used in various commercial probiotic products (Zhang et al., 2018).

One of the main probiotic effects of *Lactobacillus* is regulating inflammatory responses and enhancing immune function (Tsai et al., 2012). *Lactobacillus* strains interact with immune cells in the gut mucosa to modulate cytokine production, thereby maintaining immune balance in the gut (Wallace et al., 2003). This can have positive effects on immune-related diseases such as inflammatory bowel disease (IBD). Additionally, *Lactobacillus* promotes the production of Immunoglobulin A (IgA), enhancing the defense against pathogens, which contributes not only to gut health but also to overall immune function (Asama et al., 2015).

Another important function of *Lactobacillus* is enhancing resistance to oxidative stress. Oxidative stress is a major cause of various chronic diseases and can lead to cellular damage. *Lactobacillus* produces antioxidant enzymes and substances that help suppress the accumulation of reactive oxygen species (ROS) and protect cells (Feng

and Wang, 2020). Specifically, *Lactobacillus* produces superoxide dismutase (SOD), an enzyme that plays a key role in neutralizing superoxide radicals, one of the most harmful reactive oxygen species (Wang et al., 2017). In addition, some strains of *Lactobacillus* promote the production of intracellular substances such as glutathione, which enhances the host's resistance to oxidative stress (Peran et al., 2006). These properties make *Lactobacillus* an important contributor to maintaining host health by strengthening antioxidant functions.

1.1.2 SOD: A Key Antioxidant Enzyme in Probiotics

Oxidative stress disrupts physiological balance and causes cellular damage, which can lead to various diseases (Rani et al., 2016). ROS, naturally generated during cellular metabolism, can accumulate excessively and damage cellular membranes, proteins, and DNA. To mitigate this, the body relies on an antioxidant system to neutralize ROS (Adwas et al., 2019). Antioxidant enzymes play a central role in this system, working in tandem with non-enzymatic antioxidants to protect cells.

Key antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx), and SOD, function by targeting specific types of ROS. Together, they form a complementary network to counteract oxidative stress in the body (Ali et al., 2020). Among the various antioxidant enzymes, SOD is particularly significant as it directly removes the superoxide anion ($O_2^{-}\cdot$), one of the primary forms of ROS. The superoxide anion can act as a precursor to other, more reactive types of ROS, making its early removal crucial for mitigating oxidative stress. SOD catalyzes the dismutation of

superoxide anions into hydrogen peroxide (H_2O_2) and oxygen (O_2), which are subsequently neutralized by GPx or catalase (Aljuhani, 2017).

SOD was selected as the focus of this study for several key reasons. First, it is a ubiquitous enzyme found in nearly all forms of life, from prokaryotes to higher eukaryotes, emphasizing its fundamental importance to biological systems (Wolfe-Simon et al., 2005). Second, SOD exhibits a remarkably high catalytic efficiency, enabling it to rapidly neutralize superoxide anions and prevent their accumulation. Finally, numerous studies have highlighted the association between impaired SOD activity or mutations in SOD-encoding genes and the development of various diseases, including neurodegenerative conditions such as Alzheimer's and Parkinson's disease, as well as cardiovascular disorders (Guleri and Tiwari, 2020). This strong correlation with disease pathology further underscores the clinical significance of SOD as an antioxidant enzyme.

Superoxide dismutase exists in three major isoforms, which are classified based on their metal cofactors and subcellular localization (Sheng et al., 2014). The first isoform, Cu/Zn-SOD, is predominantly found in the cytoplasm and extracellular fluid and uses copper (Cu) and zinc (Zn) ions as cofactors. The second isoform, Mn-SOD, is localized within mitochondria and relies on manganese (Mn) ions for its catalytic activity. The third isoform, Fe-SOD, is found primarily in prokaryotes and uses iron (Fe) as its cofactor. Each isoform of SOD is strategically localized to provide comprehensive protection against ROS within specific cellular compartments. For instance, Mn-SOD plays a particularly important role in mitochondria, which are the primary sites of ROS

production during cellular respiration (Hu et al., 2005). By neutralizing superoxide anions in mitochondria, Mn-SOD helps maintain mitochondrial function and prevents oxidative damage to mitochondrial DNA, proteins, and membranes (Palma et al., 2020). This localization-based specialization allows SOD to serve as an effective first line of defense against oxidative stress throughout the cell.

1.1.3 Bioinformatics of probiotics

Bioinformatics plays an essential role in analyzing and understanding the characteristics of probiotics. Advances in bioinformatics have greatly improved our ability to investigate the molecular basis of probiotic strains, allowing us to fully exploit their potential in health and industrial applications (Castro-López et al., 2021). With the use of genomic sequencing, gene annotation, and functional gene analysis, researchers can gain insights into the complex biology of probiotic strains (Klaenhammer et al., 2008). Genomic sequencing helps in decoding the complete DNA of probiotic microorganisms, which provides a foundation for understanding their unique genetic traits. This process reveals information about genome size, structure, and organization, as well as the presence of plasmids and mobile genetic elements that may influence probiotic behavior. For instance, the genome of Lactobacillus species often contains genes encoding enzymes involved in carbohydrate metabolism, stress response, and adhesion to intestinal epithelial cells, which are critical for their probiotic functions

Gene annotation further identifies the function of specific genes, allowing researchers to determine which genes contribute to health-promoting activities (Castro-

López et al., 2021). Databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and COG (Clusters of Orthologous Groups) are commonly used in gene annotation to categorize genes into functional pathways and predict their potential roles (Kanehisa and Goto, 2000) (Tatusov et al., 2000). For example, KEGG can map genes to metabolic pathways involved in the synthesis of short-chain fatty acids or bacteriocins, while COG can group genes based on evolutionary relationships, helping to predict their biological functions. This helps in understanding the metabolic capabilities and functional contributions of probiotic strains. Functional gene analysis helps in identifying pathways and regulatory networks that are involved in probiotic functions (Saulnier et al., 2011).

Additionally, bioinformatics tools are used to predict and evaluate the safety and efficacy of probiotic strains. Comparative genomics, for instance, enables the identification of virulence factors, antibiotic resistance genes, or other potentially harmful traits. (dR Altavas et al., 2024). This is particularly critical in the context of probiotics intended for use in food and health products, as the presence of such traits could pose risks to consumers. Using bioinformatics tools, researchers can compare the genomes of probiotic strains with those of pathogenic species, highlighting any genetic similarities that might require further investigation (Cai et al., 2009). As a result, bioinformatics contributes to the selection of safe and reliable probiotic strains for clinical and industrial applications.

Through bioinformatics analysis, researchers can also gain a deeper understanding of the genetic characteristics of *Lactobacillus* strains and clarify their

phylogenetic relationships with other probiotic species (Claesson et al., 2008). By comparing genomic data across different strains, it is possible to identify genetic similarities and differences, which can help in classifying these microorganisms more accurately (Varghese et al., 2015). This phylogenetic analysis not only contributes to the taxonomy of probiotic species but also helps in selecting specific strains with desirable properties for use in health-promoting applications. Ultimately, bioinformatics provides the tools necessary to clarify the genetic basis of probiotic functionality and evolutionary history, leading to more effective utilization of these beneficial microorganisms (Castro-López et al., 2021).

Finally, bioinformatics enables the integration of multi-omics data genomics, transcriptomics, proteomics, and metabolomics to provide a holistic understanding of probiotic functionality (Rebollar et al., 2016). By analyzing these diverse data types, researchers can uncover the molecular mechanisms underlying probiotic host interactions, metabolic contributions, and stress adaptations (Lebeer et al., 2008). This systems biology approach not only clarifies the genetic basis of probiotic activity but also opens new avenues for probiotic engineering and personalized medicine (Lorente-Picón and Laguna, 2021). In conclusion, bioinformatics provides the essential tools and methodologies to unravel the genetic and functional intricacies of probiotics. By combining genomic sequencing, gene annotation, functional analysis, and phylogenetics, researchers can comprehensively characterize probiotic strains, ensuring their safety and efficacy while identifying opportunities for innovation. The continued advancement of bioinformatics technologies will undoubtedly contribute to the effective utilization of probiotics in promoting human health and addressing global

challenges in food and medicine (Castro-López et al., 2021).

1.2 Overexpression

1.2.1 Overexpression Methodology

Gene overexpression is an essential technique in molecular biology, widely employed to investigate gene function, enhance protein production, and explore applications in biotechnology and medicine (Saulnier et al., 2011). By artificially elevating the expression of a specific gene, researchers can study its role under controlled conditions and evaluate its potential contributions to various biological processes (Kunsch and Medford, 1999). To achieve this, engineered expression vectors are designed to introduce the gene of interest into a host organism and drive its transcription and translation at high levels (Papadakis et al., 2004). These vectors are specifically tailored to ensure efficient expression through the inclusion of robust regulatory elements (Mutalik et al., 2013).

Overexpression vectors are constructed to optimize transcription, translation, and stability within the host system (Liu et al., 2013). The most critical component of these vectors is the promoter, which can either be constitutive, driving continuous transcription, or inducible, enabling precise regulation under specific environmental or chemical conditions (Engstrom and Pfleger, 2017). Additionally, multiple cloning sites (MCS) facilitate the insertion of the target gene, while selection markers, such as antibiotic resistance genes, allow for the identification of successfully transformed cells (Yu et al., 2013a). High-copy-number origins of replication ensure that the plasmid is

maintained at elevated levels within the host, further amplifying gene expression (Friehs, 2004). Together, these elements provide the foundation for achieving high levels of gene expression in both prokaryotic and eukaryotic systems.

The construction of an overexpression vector begins with the amplification of the target gene using polymerase chain reaction (PCR) (Bej et al., 1991). Primers are designed to include restriction enzyme recognition sites or other specific sequences to facilitate efficient cloning (Hartley et al., 2000). The vector is then linearized using restriction enzymes corresponding to the primer design, creating compatible ends for the ligation of the amplified gene fragment. The purified gene fragment is ligated into the linearized vector using T4 DNA ligase under optimized conditions to enhance recombination efficiency (Islam et al., 2017). The recombinant plasmid is subsequently introduced into competent cells through transformation methods such as chemical heat-shock or electroporation. Transformed cells are cultured on selective media containing appropriate antibiotics, and positive clones are screened to confirm successful gene insertion using PCR, restriction digestion, or sequencing (Chassy and Flickinger, 1987).

After the recombinant vector is constructed, it is introduced into the desired host organism to achieve overexpression of the target gene. The level of expression depends on the regulatory elements in the vector and the host system used. Inducible expression systems require external inducers, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), to activate transcription, whereas constitutive systems rely on the strength of their promoters for continuous expression (Kirov and Peykov, 2022). Verification of gene overexpression is conducted through a combination of methods, including

quantitative PCR (qPCR) to measure mRNA levels, SDS-PAGE and Western blotting to confirm protein production, and functional assays to evaluate the biological activity of the expressed protein (Guan et al., 2004).

The ability to overexpress specific genes has enabled significant advancements in biotechnology and medicine. Overexpression systems are widely utilized for the production of recombinant proteins, such as enzymes, hormones, and antibodies, which have applications in research, therapeutics, and industrial processes (Schmidt, 2004). Furthermore, overexpression techniques allow researchers to study gene dosage effects on cellular physiology and to investigate metabolic pathways for synthetic biology and metabolic engineering purposes. Recent advancements in vector design and host optimization have further improved the efficiency and versatility of overexpression systems, solidifying their role as a cornerstone of modern molecular biology (Fink et al., 2021).

1.2.2 Overexpression Promoter

Overexpression promoters are crucial elements in cloning technologies, particularly when aiming to increase the production of a specific protein (Liu et al., 2013). These promoters are designed to drive the high-level transcription of target genes, thereby boosting protein synthesis in the host organism. Overexpression is often utilized in various fields, including industrial biotechnology, pharmaceutical production, and research settings where large quantities of a specific protein are needed (Assenberg et al., 2013). The choice of an overexpression promoter is critical, as it directly impacts

the efficiency and levels of target gene expression (Blazeck and Alper, 2013).

Promoters with high activity include several well-characterized examples. For instance, the Ptuf promoter regulates the expression of elongation factor Tu (EF-Tu), which is crucial for protein synthesis (Kim et al., 2009). This promoter is known for its stability and ability to induce high levels of gene expression, making it highly useful for various applications. Another example is the Pldh promoter, which controls lactate dehydrogenase (LDH) involved in lactic acid fermentation. The Pldh promoter can achieve high expression under specific fermentation conditions, providing an effective tool for metabolic engineering (Xin et al., 2017).

These promoters have been shown to exhibit high expression levels in lactic acid bacteria, under certain conditions. Previous studies have demonstrated that the Ptuf promoter can drive consistent and robust gene expression, even during exponential growth phases, which is ideal for maintaining protein production (Kim et al., 2009). The Pldh promoter, on the other hand, has been reported to achieve high expression during lactic acid fermentation, where the metabolic environment favors the activity of LDH (Narita et al., 2006). These findings suggest that selecting appropriate promoters based on the growth conditions and metabolic requirements of LAB can lead to effective overexpression of target genes.

1.2.3 Overexpression of Antioxidant Enzyme

Overexpression of antioxidant enzymes in lactic acid bacteria, such as *Lactobacillus*, has garnered considerable interest due to their potential health benefits

(Khalil et al., 2022). Antioxidant enzymes like SOD and CAT are known for their ability to neutralize ROS, which are implicated in oxidative stress and various chronic diseases (Nandi et al., 2019). Studies have demonstrated that overexpression of these antioxidant enzymes in *Lactobacillus* strains can significantly enhance their antioxidative capacity, providing increased resistance to oxidative stress both in vitro and within host systems (Kong et al., 2020). This enhanced oxidative resistance has practical applications in both food biotechnology and health supplements.

One notable example involves the overexpression of SOD in *Lactobacillus* plantarum (Lin et al., 2016). By increasing the expression of SOD, researchers observed improved survival of the bacteria under oxidative conditions, as well as better stability in gastrointestinal environments. This enhanced resilience allows *Lactobacillus* to deliver antioxidant benefits more effectively when consumed as a probiotic. Upon ingestion, these overexpressed enzymes help reduce oxidative stress in the gut, contributing to a healthier intestinal environment (Vona et al., 2021). This is particularly important for mitigating inflammation and improving gut health, which are crucial factors for overall well-being.

The health benefits of overexpressing antioxidant enzymes extend beyond gut health. Oxidative stress is a major contributing factor in the aging process and in the development of chronic conditions such as cardiovascular diseases, diabetes, and certain cancers (Liguori et al., 2018). Consuming probiotics engineered to overexpress antioxidant enzymes may help reduce oxidative damage throughout the body (Riaz Rajoka et al., 2021). These probiotics could enhance the body's natural antioxidant

defenses, thus reducing systemic inflammation and potentially lowering the risk of chronic diseases (Mahooti et al., 2024). This makes the overexpression of antioxidant enzymes in probiotics a promising approach for promoting human health and preventing age-related diseases.

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Chapter 2. Genomic Insights and Functional Evaluation of *Lacticaseibacillus paracasei* EG005: A Promising Probiotic with Enhanced Antioxidant Activity

2.1 Abstract

Introduction: Probiotics, such as *Lacticaseibacillus paracasei* EG005, are gaining attention for their health benefits, particularly in reducing oxidative stress. The goal of this study was to reinforce the antioxidant capacity of EG005, along with comprehensive genomic analysis, with a focus on assessing superoxide dismutase (SOD) activity, acid resistance and bile tolerance, and safety.

Methods: EG005 was screened for SOD activity and change of SOD activity was tested under various pH conditions. Its survival rates were assessed in acidic (pH 2.5) and bile salt (0.3%) conditions and the antibiotic MIC test and hemolysis test were performed to evaluate safety. Genetic analyses including functional identification and phylogenetic tree construction were performed. The SOD overexpression system was constructed using P_{tuf}, P_{ldh1}, P_{lhd2}, and P_{ldh3} strong promoters.

Results: EG005 demonstrated higher SOD activity compared to *Lacticaseibacillus rhamnosus* GG, with optimal activity at pH 7.0. It showed significant acid and bile tolerance, with survival rates recovering to 100% after 3 hours in acidic conditions. Phylogenetic analysis confirmed that EG005 is closely related to other *L. paracasei* strains with ANI values above 98%. Overexpression of SOD using the P_{tuf} promoter resulted in a two-fold increase in activity compared to the controls. Additionally, EG005 exhibited no hemolytic activity and showed antibiotic susceptibility within safe limits.

Discussion: Our findings highlight EG005's potential as a probiotic with robust antioxidant activity and high tolerance to gastrointestinal conditions. Its unique genetic profile and enhanced SOD activity through strong promoter support its application in probiotic therapies and functional foods. Further research should be investigated to find

the *in vivo* effects of EG005 on gut health and oxidative stress reduction. In addition, attB and attP-based recombination, combined with CRISPR-Cas9 technologies, could offer a more stable alternative for long-term *sodA* gene expression in commercial and medical applications.

2.2 Introduction

Lactobacillus is a genus of gram-positive, facultatively anaerobic, and non-spore-producing bacteria commonly found in fermented dairy foods or gastrointestinal tracts of humans and animals (Hammes and Vogel, 1995). Several *lactobacillus* species are known for their probiotic properties and have been used for food fermentation and preservation for centuries, examples including yogurt, cheese (Plessas et al., 2012), kefir (Zheng et al., 2013), sauerkraut (Yu et al., 2013b), and kimchi (Lee et al., 2011a). Nowadays, they are widely known to be effective in improving digestion (Kim and Gilliland, 1983), promoting intestinal motility, strengthening immunity, and treating human diseases such as inflammatory bowel disease (IBD) (Saez-Lara et al., 2015, Justino et al., 2015, Huang et al., 2022).

Lactobacillus species are capable of producing and secreting a wide range of proteins essential to their survival and interaction with their environment (Lee et al., 2011b). These proteins include enzymes (Danilova and Sharipova, 2020), bacteriocins (Nes et al., 2001), and other bioactive molecules (Griffiths and Tellez, 2013) that contribute to their beneficial effects. In this study, we specifically focused on antioxidant capacity, as it critically involves in resisting oxidative stress and significantly impacts survival (Chooruk et al., 2017).

Naturally formed during cellular metabolism, ROS are chemically reactive molecules originating from oxygen molecules (Bayr, 2005). ROS can damage cell membranes, proteins, and DNA, causing oxidative stress that can induce various diseases such as IBD (Patlevič et al., 2016). This is why we focused on the antioxidant

activity of lactic acid bacteria. More specifically, *Lactobacillus* can produce various antioxidants, that can scavenge free radicals and protect cells from oxidative stress (Suzuki et al., 2013). In antioxidants, *Lactobacillus* produces SOD, which is classified into Mn-SOD, Fe-SOD, Cu-SOD, and Zn-SOD based on the metal cofactor presented in the active site of the enzyme. SOD is derived from species such as *Lactobacillus acidophilus, Lacticaseibacillus casei,* and *Lacticaseibacillus rhamnosus*, and is effective in alleviating oxidative stress in inflammatory bowel disease (IBD) when delivered to the intestines (LeBlanc et al., 2011). Additionally, SOD helps reduce oxidative stress during storage, thereby enhancing the shelf life and maintaining the quality of foods (Arasu et al., 2015). Using *lactobacillus* strains as a natural antioxidant source is gaining attention in the food and nutraceutical industries due to their potential health benefits (Ghiasi et al., 2023, Khubber et al., 2022).

Although many species of lactic acid bacteria with potent probiotic characteristics have been identified through extensive research, they are still insufficient to meet the growing demands of both humans needs and the expanding scale of industry. In this study, we found that strain EG005 exhibited relatively higher SOD activity compared to other strains in our laboratory. To determine the probiotic properties of EG005, the enzyme characterization of SOD in EG005, acid and bile resistance, antibiotic MIC tests, and hemolysis tests were performed. The genome characteristics, phylogenetic analysis of EG005, and the evolutionary patterns of the superoxide dismutase gene (*sodA*) among other *Lacticaseibacillus paracasei* strains were also identified. Additionally, the *sodA* gene from the EG005 genome was cloned with four

selected strong promoters, and the resulting plasmid vectors for *sodA* overexpression were then transformed into EG005. Finally, each overexpressed SOD activities were compared in response to oxidative stress.

This research focuses on investigating the beneficial properties of *L. paracasei* EG005 as a probiotic through safety evaluation experiments and genomic analysis, with a particular emphasis on its superoxide dismutase (SOD) activity along with the overexpression of the antioxidant capability. We aim to determine whether antioxidant capacity not only enhances survival in a harsh gastrointestinal environment but also contributes to reducing oxidative stress.

2.3 Materials and Methods

2.3.1 Sample Collection and Bacterial Species

In this study, EG005, which was isolated from fermented cheese from a local market in Gwanak, Seoul, and previously identified as *L. paracasei,* was used (Jeon et al., 2022). It was stored in de Man, Rogosa, and Sharpe (MRS) medium (Difco, USA) containing 25% glycerol at -80°C. The *E.coli* DH5α (New England Biolabs, USA) strain was used for gene cloning and plasmid propagation. *E.coli* DH5α transformants were grown in Luria-Bertani (LB) medium (Difco, USA) at 37°C in a shaking incubator during overnight (O/N). The medium was supplemented with 100 μg/mL of ampicillin for *E. coli* and 15 μg/mL of erythromycin for *L. paracasei* to select transformed colonies.

2.3.2 Antioxidant Activity Screening

2.3.2.1 Antioxidant Activities of LAB

Various *Lactobacillus* species were grown on the MRS agar plates in an anaerobic container at 37°C for 2 days. A single colony of each *Lactobacillus* species was inoculated in 5 mL of MRS broth medium and cultured overnight at 37°C. After incubation, the intact cells and supernatant were separated by centrifugation at 13,000 rpm for 2 min at 4°C. The cells were washed three times with Phosphate-Buffered Saline (PBS) and resuspended in PBS to adjust the cell amount to 1.0×10^{10} CFU/mL. The cells were disrupted by sonication (Power Sonic 405, Hwashin, Korea) at 40 kHz for 20 min. These cell debris were removed by centrifugation at 13,000 rpm for 10 min at 4°C and the cell-free extracts (CFEs) were obtained for water-soluble tetrazolium 1

(WST-1) assay (Peskin and Winterbourn, 2000).

The SOD activity of *Lactobacillus* species was analyzed by using the EZ-SOD assay kit (Dogen Bio Co. Ltd., Korea). Twenty μL of CFEs were mixed with 200 μL of WST working solution and 20 μL of Xanthine oxidase working solution, then the mixture was incubated at 37°C for 20 min. Absorbance at 450 nm was measured by using a spectrophotometer.

2.3.2.2 Effect of pH on SOD in EG005

To characterize the effects of pH on SOD activity, measurements were taken across various pH conditions, ranging from pH 3 to 10 (Pinmanee et al., 2023). Buffer solutions used for each pH were as follows: sodium acetate buffer for pH 3.0 - 5.0, Tris-HCl buffer for pH 6.0 - 10.0, and PBS for pH 7.0. The experiments were performed in triplicate.

2.3.3 Evaluation of Acid and Bile Tolerance of EG005

2.3.3.1 Acid Tolerance Evaluation

The acid tolerance test of EG005 was performed according to the method of Conway et al (Conway et al., 1987), with minor modifications. 1 mL of overnight broth culture was inoculated into 9 mL of MRS broth adjusted to pH 2.5 using 1 M HCl and incubated at 37°C for 3 hours. Samples were taken at time intervals and were 10-fold serial diluted with sterilized PBS pH 7.4 in 1.7 mL microcentrifuge tubes. 100 μL of

diluted samples were plated on MRS agar plates. Then plates were incubated under anaerobic conditions at 37°C for 48 h. The survival rate was calculated as follows:

$$Survival\ rate\ (\%) = \frac{CFU/mL\ at\ specific\ time\ (C_t)}{CFU/mL\ at\ initial\ ime\ (C_0)} \times 100$$

2.3.3.2 Bile Tolerance Evaluation

The bile tolerance of EG005 was evaluated according to the method of Hassan et al (Hassanzadazar et al., 2012), with minor modifications. The bile solution was prepared by adding powdered oxgall (Difco, USA) to MRS broth to reach a final concentration of 0.3%. 10 mL of cultures, consisting of 1 mL of overnight broth culture and 9 mL of medium, were incubated at 37°C for 6 hours and were taken every hour to calculate the survival rate. Serial dilution and survival rate calculations were carried out in the same way as the acid tolerance experiment previously described.

2.3.4 Safety Assessment

2.3.4.1 Hemolytic Activity

To evaluate the potential hemolytic activity of EG005, the overnight broth culture of EG005 and *Staphylococcus aureus* Cnk100 which was used as positive control were streaked on sheep blood agar plates (MBcell, KisanBio, Korea) and incubated at 37°C for 48 h, under aerobic conditions. Subsequently, the plates were visually inspected for hemolytic activity, characterized by the formation of clear zones

around the bacterial colonies. Hemolytic patterns were classified as α-hemolysis (green zones), ß-hemolysis (clear zones), or γ-hemolysis (no zones) (Mangia et al., 2019).

2.3.4.2 Antibiotic Resistance Assessment

For the antibiotic resistance test, the minimal inhibitory concentration (MIC) of each antibiotic for EG005 was investigated on the European Food Safety Authority (EFSA) recommendations (Additives and Feed, 2012). A total of eight antibiotics (ampicillin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol) with concentrations ranging from 0.25 to 256 ug/mL were used. A single colony of EG005 was inoculated into 5 mL of MRS broth medium and cultured at 37°C for 18 hours. The MIC test was performed using the broth microdilution method (Wiegand et al., 2008). First, 90 uL of broth medium composed of Iso-Sensitest (MBcell, KisanBio, Korea) and MRS at a 9:1 ratio (Klare et al., 2005) was dispensed into a 96-well plate. Then, ten ul of antibiotic solution at each concentration was added to each well so that the final antibiotic concentration of the medium was 0.25 to 256 ug/mL. The strain diluted with PBS at a concentration of 5.0×10^6 CFU/mL was inoculated into each well at 10 uL to achieve a final concentration of 5.0×10^4 CFU/well. The inoculated medium was cultured anaerobically at 37°C for 48 hours. The MIC was established through visual observation, identifying the concentration at which antibiotics suppressed bacterial growth by 80% or more relative to the control.

2.3.5 Genome Characterization

2.3.5.1 Genome Assembly and Annotation

The raw read data of EG005 was obtained from the Sequence Read Archive (SRA, SRA run accession: SRR16961897) (Leinonen et al., 2010) deposit in the National Center for Biotechnology Information (NCBI) database. Adapter trimming was performed using Porechop v0.2.4[1]. Genome *de novo* assembly was implemented using Canu v2.2 with options of "corMhapOptions=--threshold 0.5 --ordered-sketch-size 1000 --ordered-kmer-size 14" and "corErrorRate=0.105" (Koren et al., 2017). Then, the starting point of the genome was rearranged by the fixstart plugin from Circlator v1.5.5 (Hunt et al., 2015). Medaka v1.7.1[2] with the r941_min_hac_g507 model and Homopolish v0.4.1 (Huang et al., 2021) with R9.4.pkl were utilized to convert the draft assembly genome into a higher-quality genome. The quality of the genome assembly was assessed using BUSCO v5.4.2 with the lactobacillales_odb10 dataset (Manni et al., 2021). Gene annotation of EG005 was determined by Prokka v1.14.6 (Seemann, 2014) with the -rfam option.

2.3.5.2 Identification of Gene Function and Safety Analysis

For the Identification of gene functions, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Cluster of Orthologous Groups of Proteins (COG) (Galperin et

[1] https://github.com/rrwick/Porechop.

[2] https://github.com/nanoporetech/medaka

al., 2021) analyses were performed using the eggnog-mapper v2.1.12 (Cantalapiedra et al., 2021) with a 90% identity threshold and an E-value of less than 10^{-3}. The antibiotic resistance genes and virulence factors in the genome of EG005 were verified using ABRicate v1.0.1[3] to determine its safety as a probiotic.

2.3.5.3 Phylogenetic analysis

CVtree was performed using a composition vector (CV) matrix with k = 6 parameters on nucleotide sequences for the whole-genome-based phylogenetic tree (Qi et al., 2004). The evolutionary aspects of EG005 were investigated by analyzing 63 whole genome sequences, including 61 other *L. paracasei* strains retrieved from NCBI (Sayers et al., 2022) and *Bacillus subtilis* DSM10 was used as the outgroup. Additionally, to construct a *sodA* gene tree for EG005, *sodA* genes were extracted from the same 61 *L. paracasei* strains used above. Then, MAFFT v7.525 was used for the multiple sequence alignment (Katoh and Standley, 2013), and the tree was created using IQ-TREE v2.3.3_beta (Minh et al., 2020) with 1,000 bootstrap values (Hoang et al., 2018), employing a quick and efficient stochastic algorithm to infer phylogenetic trees by maximum likelihood (ML). For species delineation, the average nucleotide identity (ANI) was calculated using PYANI v0.2.12 with the ANIm parameter (Pritchard et al., 2019).

[3] https://github.com/tseemann/abricate

2.3.6 SOD overexpression in EG005

2.3.6.1 Construction of SOD overexpression plasmids

The genomic DNA of EG005 was isolated using the G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) for bacteria, following the manufacturer's protocol. Subsequently, DNA fragments necessary for the construction of overexpression plasmids, such as the *sodA* gene and strong promoters (*tuf* promoter (P_{tuf}), *ldh1* promoter (P_{ldh1}), *ldh2* promoter (P_{ldh2}), and *ldh3* promoter (P_{ldh3})), were amplified via PCR. The PCR was conducted using PrimeSTAR® GXL DNA polymerase (Takara, Japan) and several oligonucleotide primer sets, which are listed in Table S2.1. Each PCR product was then subcloned using the pGEM-T Easy Vector Systems (Promega, USA) to obtain pJS-P_{tuf}, pJS-P_{ldh1}, pJS-P_{ldh2}, pJS-P_{ldh3}, pJS-S1, and pJS-S2. To construct pJS2-P_{tuf}, pJS2-P_{ldh1}, pJS2-P_{ldh2}, and pJS2-P_{ldh3}, the enzyme sites on pJS-S1 and pJS-S2 were digested by treatment with HindIII/EcoRV restriction enzymes for pJS-S1, and KpnI/ClaI restriction enzymes for pJS-S2, respectively. Then, each strong promoter DNA fragment was digested from pJS-P_{tuf}, pJS-P_{ldh1}, pJS-P_{ldh2}, and pJS-P_{ldh3} by appropriate enzyme sites which are described in Table S2.2. These DNA fragments harboring each strong promoter were inserted into linearized pJS-S1 or pJS-S2 using T4 DNA ligase (Thermo Fisher Scientific, USA). Consequently, four *sodA* genes conjugated with each strong promoter in pJS2-P_{tuf}, pJS2-P_{ldh1}, pJS2-P_{ldh2}, and pJS2-P_{ldh3} were double-digested again using ApaI/EcoRV or ApaI/ClaI restriction enzymes and inserted into linearized pLEM415-ldhL-mRFP1 (Addgene plasmid #

99842) (Bao et al., 2013) to construct plemJS-P_{tuf}, plemJS-P_{ldh1}, plemJS-P_{ldh2}, and plemJS-P_{ldh3} (Figure S2.1). The recombinant plasmids were confirmed by DNA sequencing using several primers which were listed in Table S2.1 (Cosmogenetech, Korea).

2.3.6.2 Electroporation of EG005

Electroporation of EG005 was conducted as described by Mason et al (Mason et al., 2005) with minor modifications. 2 mL of overnight broth culture of EG005 was inoculated into 100 mL of MRS broth containing 2.5% glycine. The incubation was continued at 37°C, 140 rpm in a shaking incubator until the optical density at 600 nm reached a value between 0.6 and 0.8. The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C and washed three times with ice-cold sterilized deionized water. The harvested cells were resuspended in 50 mM EDTA, incubated on ice for 5 min, and washed once in deionized water again. Then, the cells were washed twice with an electroporation buffer consisting of 0.5 M sucrose and 10% glycerol. After discarding the supernatant, the pellets were resuspended in 800 uL of electroporation buffer solution to endow EG005 with foreign DNA competency. The EG005 competent cell was mixed with 2 μg of plasmid DNA and transferred into a pre-chilled electro cuvette with a 2 mm electrode gap. Then, it was electroporated using an electroporator (Bio-Rad, USA) with a parameter of 1.8 kV pulse voltage. After the electroporation, 1 mL of MRS broth was immediately added and the suspended cells were transferred into a 1.7 mL microcentrifuge tube and subsequently incubated for 3 hours at 37°C. The incubated

cells were spread on an MRS agar plate containing erythromycin and incubated at 37°C for 48 hours under anaerobic conditions.

2.4 Results

2.4.1 Antioxidant Activity of EG005 and pH Influence on SOD

Among the 24 strains which were isolated from several fermented foods in the previous study, EG005 showed the highest antioxidant activity through SOD activity assay (The data is not shown). Compared to *L. rhamnosus* GG (LGG), The inhibition rate for ROS formation of EG005 was 5.4% higher than that of LGG (Figure 2.1A). A Student's t-test was conducted to analyze the difference between EG005 and LGG, revealing a statistically significant *p*-value of 0.0339. The effects of pH on EG005 SOD activity were investigated by incubating the strain under different pH conditions ranging from 3.0 to 10.0 at a constant temperature of 37°C. The result showed that the optimal activity of SOD was observed at a pH level of 7.0 (Figure 2.1B). The relative antioxidant activity of EG005 at pH 7.0 was five times higher than that at pH 3.0, which exhibited the lowest activity. Approximately 70% of the antioxidant activity was maintained at alkaline pH levels of 9.0 and 10.0. As a result, EG005 SOD showed relatively high antioxidant activity across a wide range of pH conditions, from neutral to alkali.

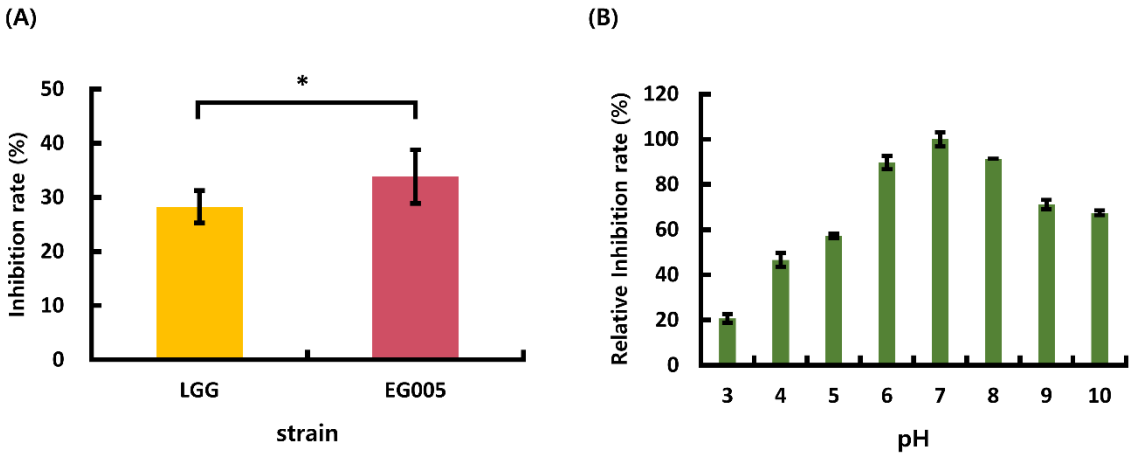


Figure 2.1 Superoxide dismutase (SOD) activity of *L. paracasei* EG005

(A) Superoxide dismutase (SOD) activity of *Lactobacillus rhamnosus* GG (LGG) and strain EG005. The results show that there is a statistically significant difference in the SOD inhibition rate between LGG and EG005 ($p = 0.0339$), indicating a higher antioxidant capacity in strain EG005.

(B) The effect of pH on SOD activity in EG005. EG005 SOD showed the highest activity at pH 7. Error bars represent the standard deviation of three independent experiments.

2.4.2 Survival of EG005 in acidic and bile environment

To evaluate the acid tolerance of EG005 in acidic conditions, such as gastric juice, the survival rates of EG005 and LGG were compared in an acidic MRS medium adjusted to pH 2.5 for 3 hours. EG005 exhibited no decrease in survival rate after the initial 30-minute exposure to pH 2.5 (Figure 2.2A). Afterward, a slight decrease was observed until 2 hours, followed by a subsequent increase in survival rate, nearly returning to the level observed at the outset (0 hours). In contrast, the survival rate of LGG decreased sharply after 30 minutes and declined to 49% after 3 hours. Overall, EG005 exhibited a significantly higher survival rate at acidic conditions than the LGG. To assess the bile tolerance of EG005, the survival rates of EG005 and LGG were compared in MRS broth containing 0.3% bile salt for 6 hours. LGG showed a sharp decrease in survival rate to below 40% after 1 hour, followed by a further decrease to 30% after 6 hours. Conversely, EG005 maintained its survival rate for up to 1 hour, followed by a gradual decline, but still maintaining a survival rate above 80% even after 6 hours (Figure 2.2B). A linear mixed model was applied to statistically analyze the results, incorporating a time-squared term to account for the non-linear pattern of bacterial survival. The analysis revealed a p-value of 0.003 for acid resistance, indicating that EG005's survival rate was significantly less affected compared to LGG. Similarly, for bile resistance, the p-value was 0.013, confirming a significant difference between the two strains.

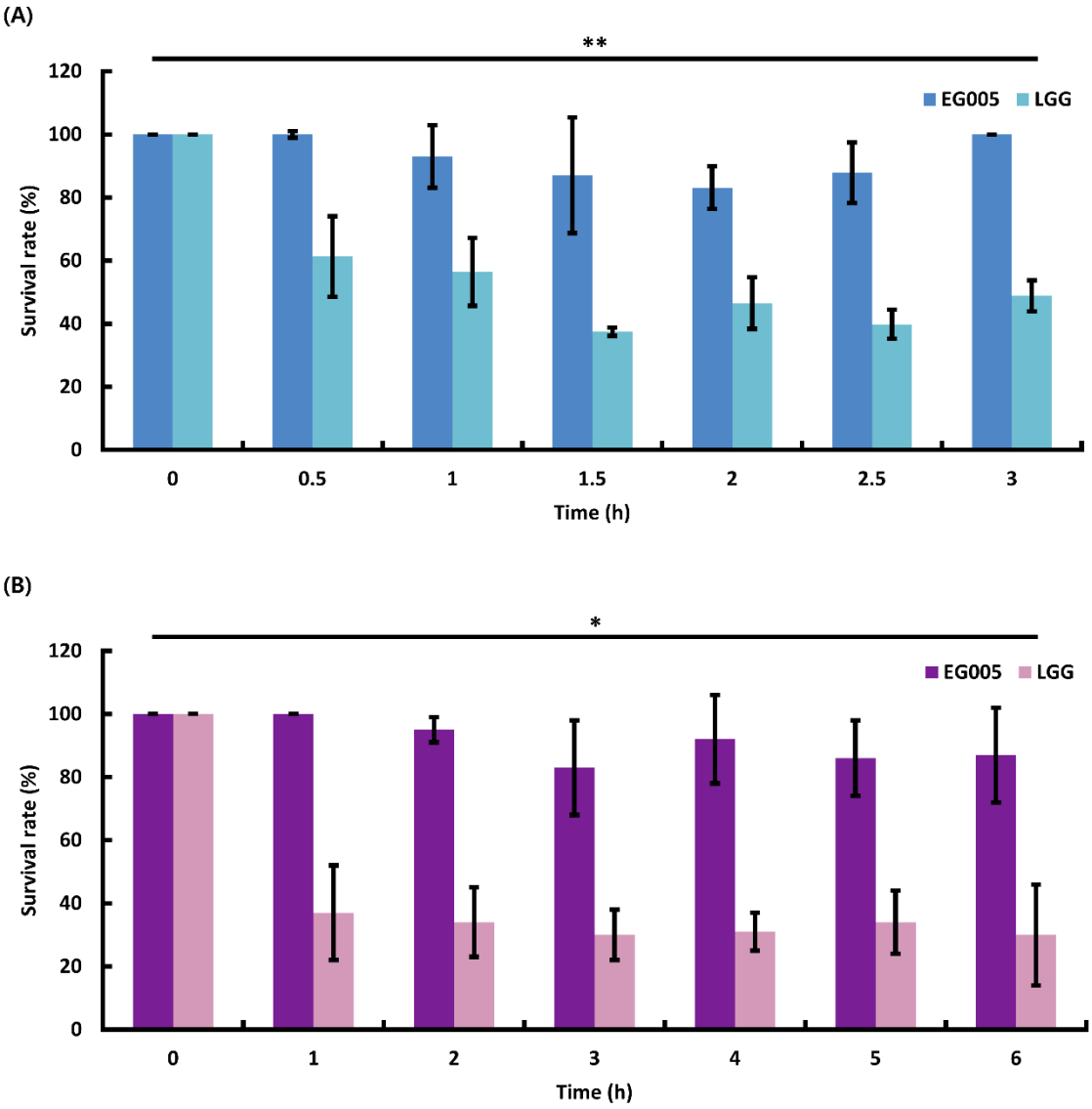


Figure 2.2 Probiotic properties of EG005

(A) The survival rate of EG005 at pH 2.5, was measured at 30-minute intervals for 3 hours. The results show that there is a statistically significant difference between LGG and EG005 ($p = 0.003$, ** $p < 0.01$).

(B) Survival rate of EG005 at 0.3% oxgall, measured at 1-hour intervals for 6 hours. Error bars represent the standard deviation of three independent experiments. The results show that there is a statistically significant difference between LGG and EG005 ($p = 0.013$, * $p < 0.05$).

2.4.3 Hemolytic activity and Antibiotic resistance in EG005

In the hemolytic activity test, EG005 showed neither β-hemolysis nor α-hemolysis, as it did not exhibit any clear zone on the sheep blood agar plate and there was no change around the colonies (Figure 2.3B). This suggested that EG005 did not possess hemolytic properties, which is a desirable trait for probiotics to ensure safety. In contrast, *S. aureus* Cnk100 which was used as a positive control showed clear zones indicating β-hemolysis (Figure 2.3A). To evaluate the antibiotic resistance of EG005, an antibiotic susceptibility test was performed according to the EFSA guidelines for probiotics. Consequently, EG005 was susceptible to most antibiotics, including ampicillin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol, except for kanamycin (Table 2.1A). The susceptibility to these antibiotics met safety standards, as it indicated that EG005 lacked broad antibiotic resistance. At the genomic level, an antibiotic resistance gene analysis was conducted using ABRicate software to determine whether EG005 harbored any antibiotic-resistance genes, but none were identified (Table 2.1B). Additionally, no virulence genes were detected in the ABRicate results.

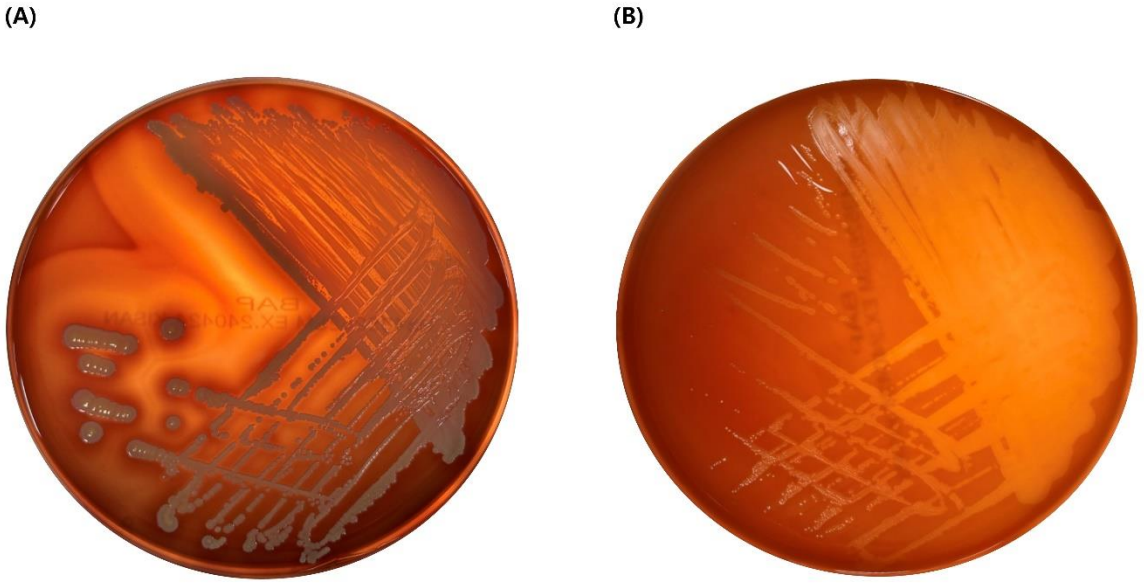


Figure 2.3 Hemolysis test of *L. paracasei* EG005

(A) *Staphylococcus aureus* exhibits β-hemolysis, creating a clear zone due to the complete lysis of red blood cells.

(B) *L. paracasei* EG005 shows γ-hemolysis without a clear zone, indicating no lysis of red blood cells.

Table 2.1 Safety evaluation of *L. paracasei* EG005 as a probiotic

(A)

Antibiotic	MIC (μg/mL)	EFSA cut-off value (μg/mL)	Resistant or sensitive
Ampicillin	4.00	4	S
Gentamicin	8.00	32	S
Kanamycin	256.00	64	R
Streptomycin	32.00	64	S
Erythromycin	≤ 0.25	1	S
Clindamycin	≤ 0.25	1	S
Tetracycline	4.00	4	S
Chloramphenicol	4.00	4	S

(B)

	Database	EG005	*S. aureus* MRSA252
Antibiotic resistance genes	Megares	0	27
	Card	0	16
	Argannot	0	15
	NCBI	0	14
	Resfinder	0	7
Virulence genes	Vfdb	0	63
	Plasmidfinder	0	4
	Ecoli_vf	0	0
	Ecoh	0	0

(A) Minimum inhibitory concentration (MIC) values of EG005 against various antibiotics.

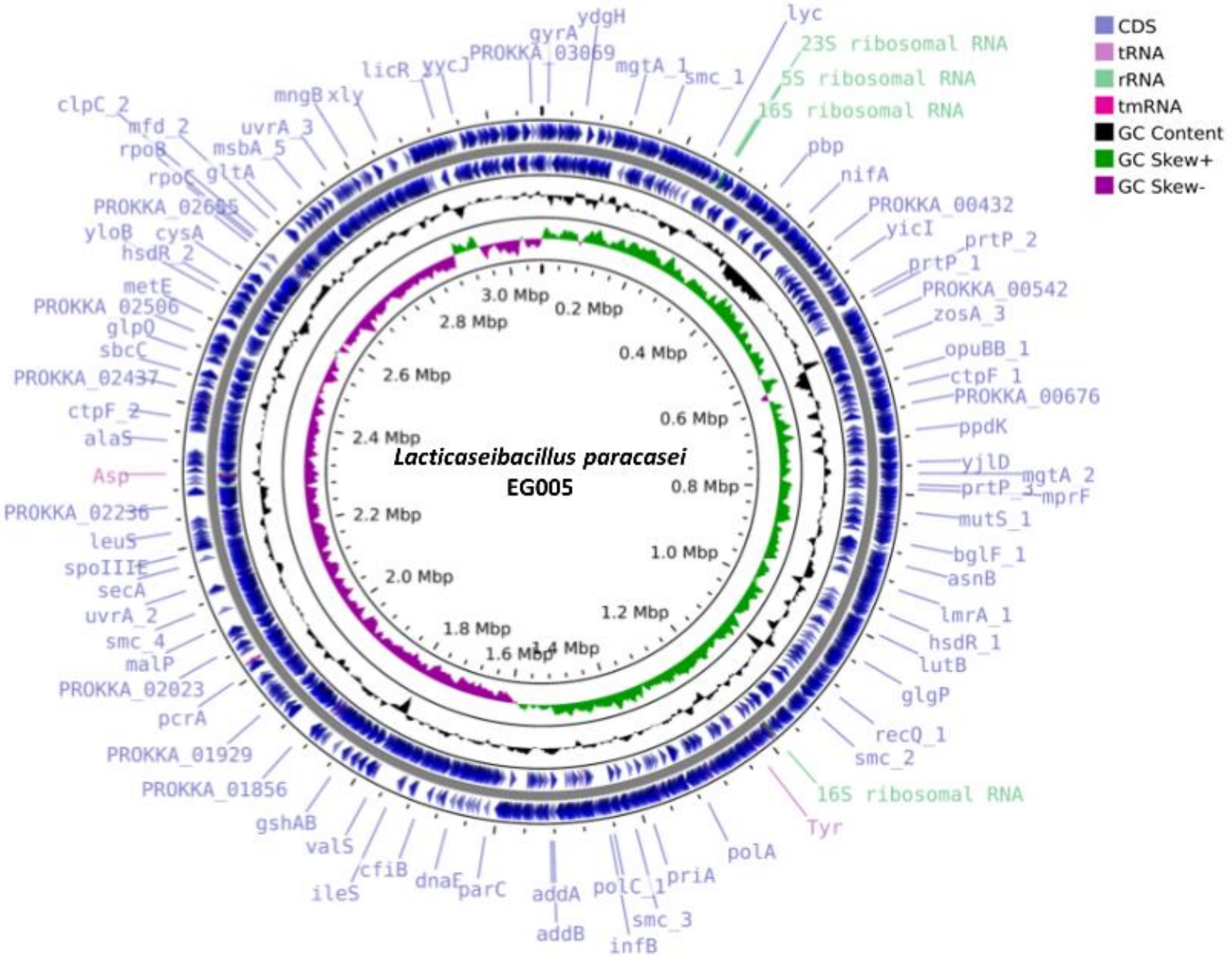
(B) Comparison of detected genes between EG005 and *S. aureus* MRSA252 using ABRicate across various databases. EG005 showed no detected genes, whereas *Staphylococcus aureus* MRSA252 exhibited multiple virulence factors and resistance genes.

2.4.4 Genomic features of EG005

A single contig was generated by performing *de novo assembly*. Following a round of polishing, a full circular genome consisting of 3,073,522 base pairs with a GC content of 46.34% was produced (Figure 2.4A). Prokka annotation predicted a total of 3081 genes, including 3006 coding sequences (CDS), 15 ribosomal RNA (rRNA) genes, 59 transfer RNA (tRNA) genes, and one transfer-messenger RNA (tmRNA) gene. The BUSCO complete value was 99.5%, indicating a highly complete and well-assembled genome with minimal missing gene content. The functional genome analysis of EG005 was analyzed by KEGG and COG databases, classifying all CDS into 29 KEGG functional categories, with "Carbohydrate metabolism" being the most prevalent, highlighting the strain's efficiency in utilizing various carbohydrates. The next most prevalent categories were "Energy metabolism", and "Membrane transport" (Figure 2.4B). Additionally, a total of 2534 genes were classified into 19 categories in the COG function classification, including 535 genes for "Function unknown (S)". Among the COG categories, a significant number of genes (228) were assigned to the "Carbohydrate transport" and "Metabolism (G)", followed by "Transcription (K)" with 228 genes, and "Replication, recombination, and repair (L)" with 212 genes (Figure 2.4C). Through an additional identification of genes involved in the antioxidant activity of EG005, several related genes were discovered, including superoxide dismutase (*sodA*), NADH oxidase (*nox1, nox2*), pyruvate oxidase (*pox5*), glutathione reductase (*gshR*), thioredoxin reductase (*trxB*), nitro reductase (*nfhA*), oxidoreductase (*ydhF)*, and manganese transporter (*psaA1, psaA2*), etc. (data not shown). The presence of these genes underscores the antioxidant activity of EG005, which could have

contributed to its protective effects under oxidative stress conditions.

(A)


CDS
tRNA
rRNA
tmRNA
GC Content
GC Skew+
GC Skew-
Lacticaseibacillus paracasei
EG005
0.2 Mbp
0.4 Mbp
0.6 Mbp
0.8 Mbp
1.0 Mbp
1.2 Mbp
1.4 Mbp
1.6 Mbp
1.8 Mbp
2.0 Mbp
2.2 Mbp
2.4 Mbp
2.6 Mbp
2.8 Mbp
3.0 Mbp
lyc
23S ribosomal RNA
5S ribosomal RNA
16S ribosomal RNA
pbp
nifA
PROKKA_00432
yicI
prtP_2
prtP_1
PROKKA_00542
zosA_3
opuBB_1
ctpF_1
PROKKA_00676
ppdK
yjlD
mgtA_2
prtP_3
mprF
mutS_1
bglF_1
asnB
lmrA_1
hsdR_1
lutB
glgP
recQ_1
smc_2
16S ribosomal RNA
Tyr
polA
priA
polC_1
smc_3
infB
addB
addA
parC
dnaE
cfiB
ileS
valS
gshAB
PROKKA_01856
PROKKA_01929
pcrA
PROKKA_02023
malP
smc_4
uvrA_2
secA
spoIIIE
leuS
PROKKA_02236
Asp
alaS
ctpF_2
PROKKA_02437
sbcC
glpQ
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metE
hsdR_2
cysA
yloB
PROKKA_02605
rpoC
gltA
rpoB
msbA_5
mfd_2
clpC_2
uvrA_3
mngB
xly
licR
yycJ
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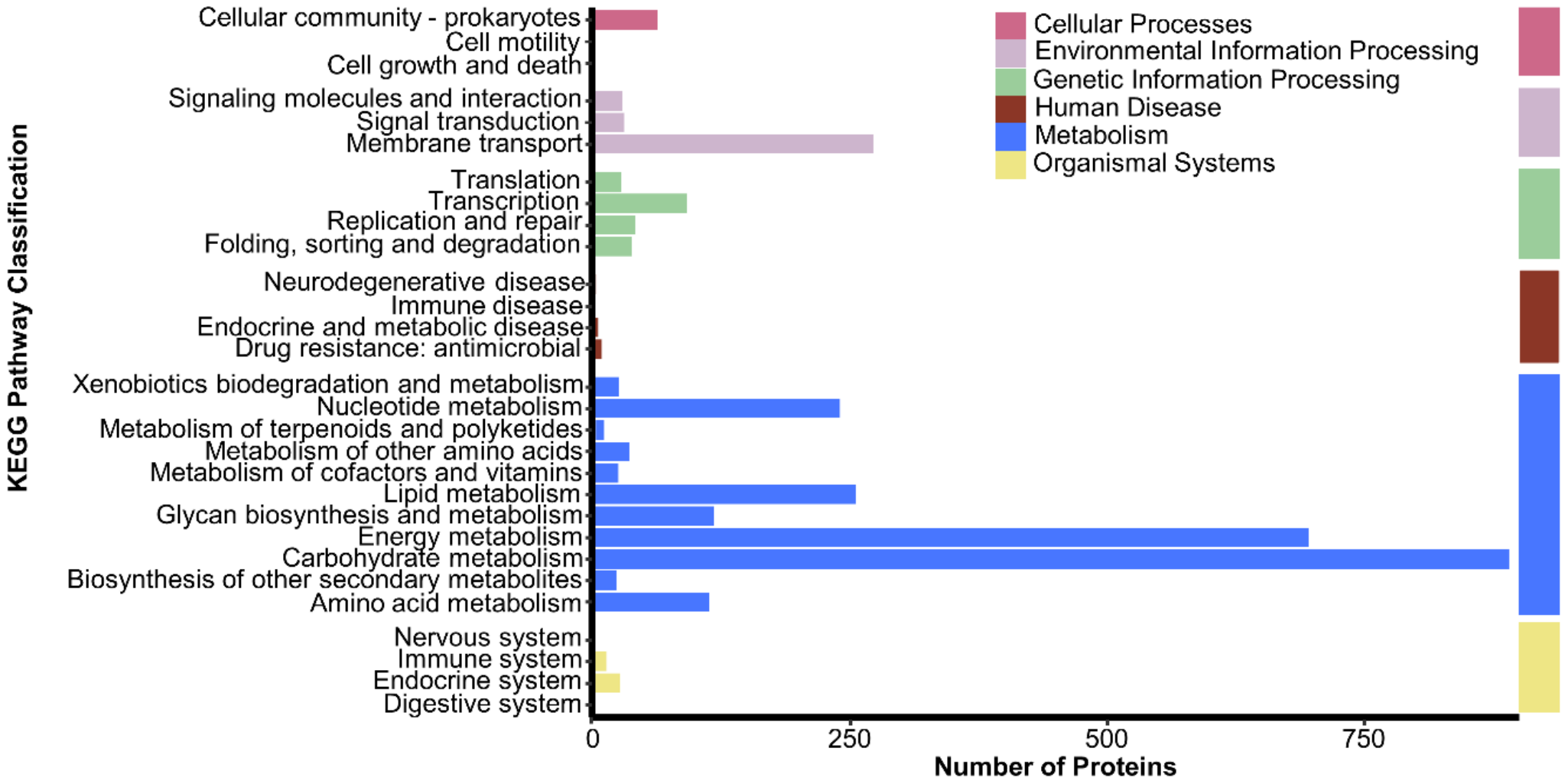
gyrA
ydgH
mgtA_1
smc_1

(B)


Cellular community - prokaryotes
Cell motility
Cell growth and death
Signaling molecules and interaction
Signal transduction
Membrane transport
Translation
Transcription
Replication and repair
Folding, sorting and degradation
Neurodegenerative disease
Immune disease
Endocrine and metabolic disease
Drug resistance: antimicrobial
Xenobiotics biodegradation and metabolism
Nucleotide metabolism
Metabolism of terpenoids and polyketides
Metabolism of other amino acids
Metabolism of cofactors and vitamins
Lipid metabolism
Glycan biosynthesis and metabolism
Energy metabolism
Carbohydrate metabolism
Biosynthesis of other secondary metabolites
Amino acid metabolism
Nervous system
Immune system
Endocrine system
Digestive system
KEGG Pathway Classification
0
250
500
750
Number of Proteins
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Human Disease
Metabolism
Organismal Systems

(C)

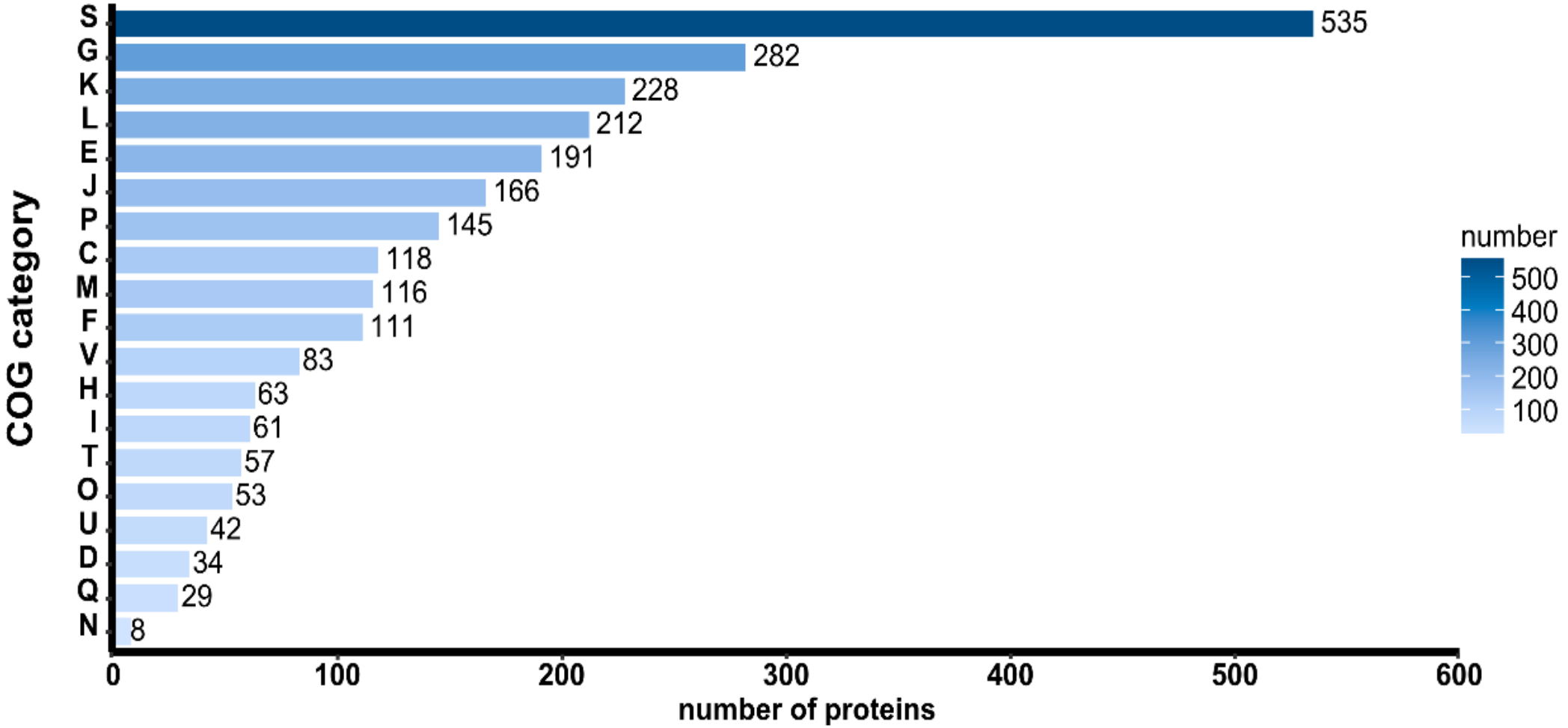

COG category
S
G
K
L
E
J
P
C
M
F
V
H
I
T
O
U
D
Q
N
535
282
228
212
191
166
145
118
116
111
83
63
61
57
53
42
34
29
8
0
100
200
300
400
500
600
number of proteins
number
500
400
300
200
100

Figure 2.4 Genomic characterization of *L. paracasei* EG005

(A) A circular genome map of EG005, generated by CGView. From outside to inside: CDS (light blue), tRNA (light purple), rRNA (emerald green), tmRNA (magenta), GC Content (black), GC Skew+ (green), GC Skew- (purple), GC Content (black).

(B) KEGG pathways distribution of the genes in *L. paracasei* EG005.

(C) COG categories distribution of the genes in *L. paracasei* EG005.

2.4.5 Phylogenetic Relationships of EG005

As described above, multiple genes related to antioxidant activity were found in the genome of EG005. Among them, the *sodA* gene encoding superoxide dismutase (SOD) was selected and a gene tree for *sodA* was constructed to assess the differences between the *sodA* genes present in 61 strains of *L. paracasei*. As a result, the *sodA* gene of EG005 formed a close cluster with those of GM-080, LC2W, CBA3611, 1, VHProbi_F22, BD-II, TCS, and W56 (Figure 2.5). Interestingly, despite the close clustering, none of the *sodA* gene sequences from those strains were 100% identical to the *sodA* gene sequence of EG005. The *sodA* gene sequence of EG005 differed by only one nucleotide from the *sodA* gene sequences of the eight closely related strains. As a result, the adenine (A) base corresponding to the 175th nucleotide on the *sodA* gene of EG005 was replaced by guanine (G) base in the other strains, and consequently, the amino acid sequence in EG005 contained threonine at this position, whereas the other strains had alanine. To investigate the evolutionary relationships among the different strains of *L. paracasei*, phylogenetic and ANI analyses were carried out using the 62 complete genome sequences of *L. paracasei*. According to the phylogenetic tree, *L. paracasei* strains were divided into several clusters, with EG005 positioned very close to CLP-C10, VHprobi OF10, and NJ strains. The short branch lengths, which indicated high genetic similarity (Figure 2.6A). In contrast, EG005 formed a close cluster with GM-080, LC2W, CBA3611, 1, VHProbi_F22, BD-II, TCS, and W56 strains in the ANI analysis, and the ANI values among these eight closest strains were observed to range from 99.66% to 99.69 (Figure 2.6B).

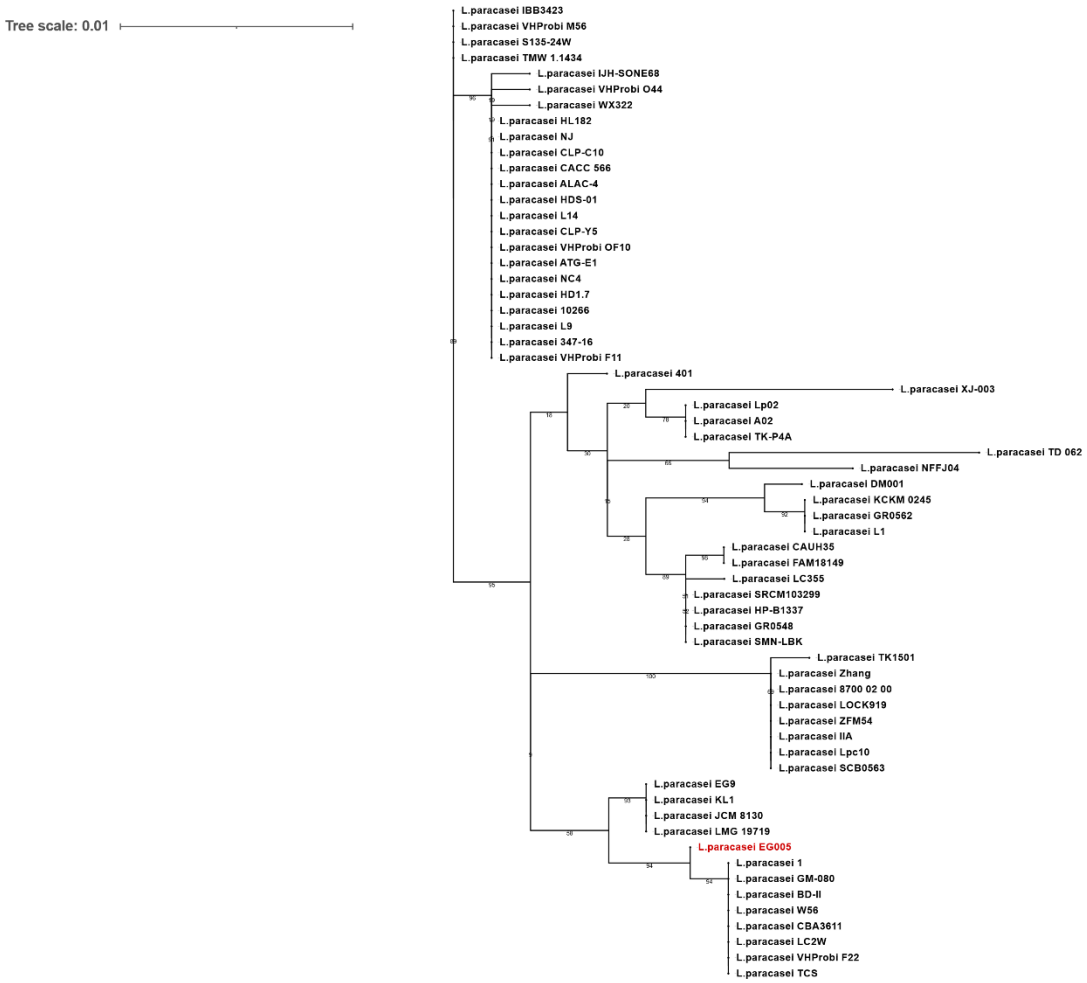


Figure 2.5 The *sodA* gene phylogeny of *L. paracasei* strains

The phylogenetic tree was constructed using the *sodA* gene sequences extracted from 62 strains of *L. paracasei* in the NCBI database. The tree reveals evolutionary relationships among *sodA* genes and EG005 appears in red. The numbers on the branches are bootstrap values with 1,000 replicates and the tree scale is 0.01.

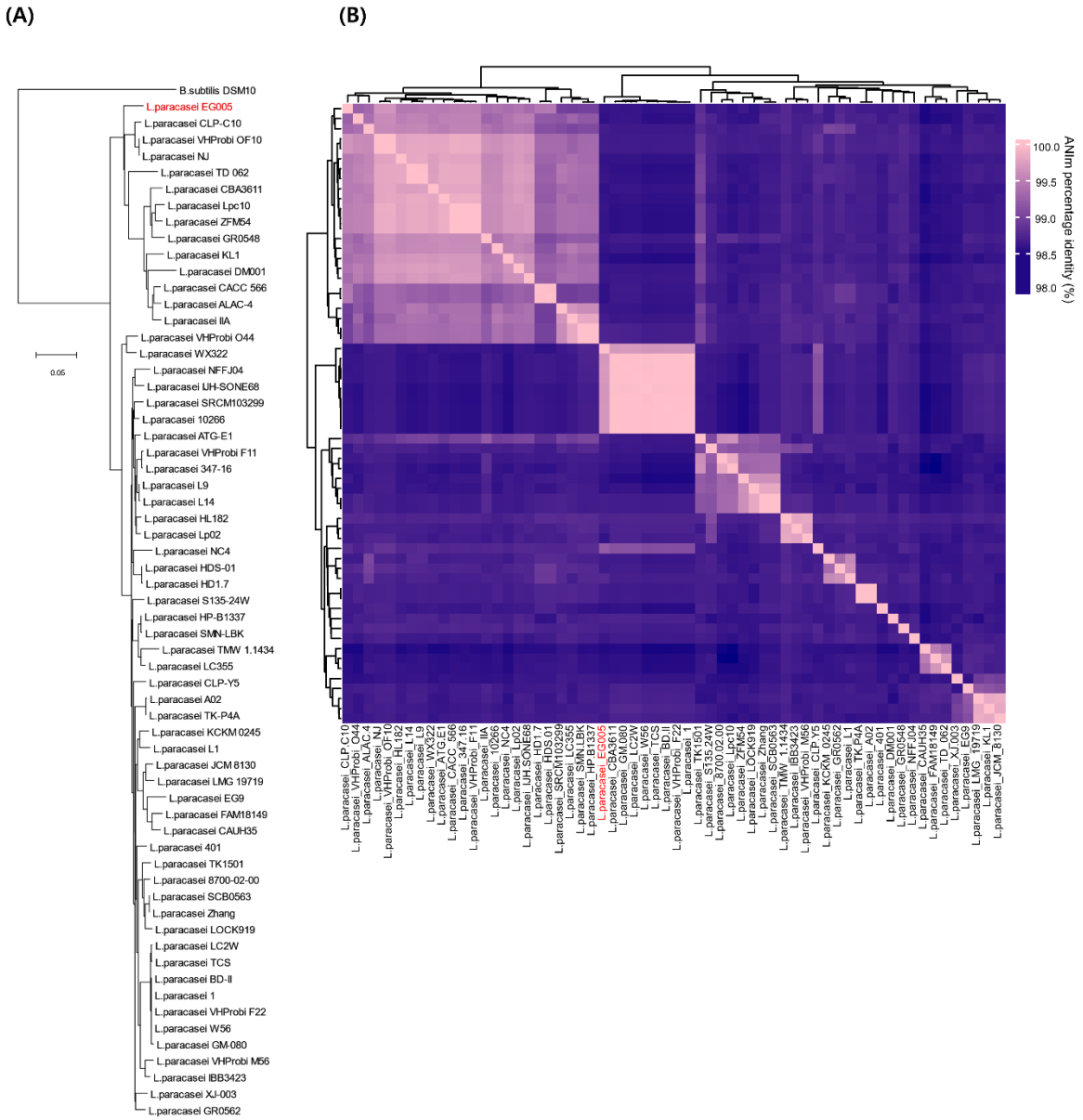


Figure 2.6 The genetic relationships of 62 *L. paracasei* strains

(A) The phylogenetic tree was constructed using CVTree 3.0 based on the whole-genome sequences. *L. paracasei* EG005 appears in red. *B. subtilis* DSM10 was used as an outgroup. The branch length represents overall evolutionary trends among the strains and the tree scale is 0.05. **(B)** ANI heatmap diagram showing the ANI value among 62 *L. paracasei* strains. The highest ANI value was 99.7% between EG005 and GM-080.

2.4.6 Overexpression of SOD in EG005

Four promoters (P_{tuf}, P_{ldh1}, P_{ldh2}, P_{ldh3}) known to be constitutive and highly active were selected to construct the SOD overexpression system in EG005. These promoters were present in the genome of EG005, and the sequence region was selected based on the presence of a TATA box and the Shine-Dalgarno sequence, a ribosomal binding site. All recombinants were constructed correctly as confirmed by restriction enzyme digestion and sequencing. The SOD activity of the EG005 recombinants harboring each strong promoter conjugated with *sodA* was compared to EG005 containing pLEM415, an empty plasmid vector, which was used as a control. As a result, the SOD activity of each EG005 recombinant harboring plemJS-P_{ldh1}, plemJS-P_{ldh2}, or plemJS-P_{ldh3} was only slightly improved compared to the SOD activity of EG005::pLEM415 (30.1%). The SOD activity values of ldh1, ldh2, and ldh3 were 32.0%, 32.1%, and 31.9%, respectively, with no significant differences observed between them and the empty vector. Therefore, the SOD activity among the remaining strains, except for EG005::plemJS-P_{tuf}, was not statistically significant. On the other hand, EG005::plemJS-P_{tuf} showed SOD activity of 61.7%, which was nearly two-fold higher compared to EG005::pLEM415. In addition, it showed a much higher SOD activity than the other three EG005 recombinants harboring plemJS-P_{ldh1}, plemJS-P_{ldh2}, or plemJS-P_{ldh3} (Figure 7). To determine whether these results were statistically significant, a one-way ANOVA test was conducted and revealed a significant effect for strains on SOD activity (p = 0.00018). The p-values between EG005::plemJS-P_{tuf} and the remaining empty vector, plemJS-P_{ldh1}, plemJS-P_{ldh2}, and plemJS-P_{ldh3} were 0.00086, 0.00162, 0.00164, and 0.00156, respectively. These values were all very low and

statistically significant, indicating substantial differences in SOD activity between strains.

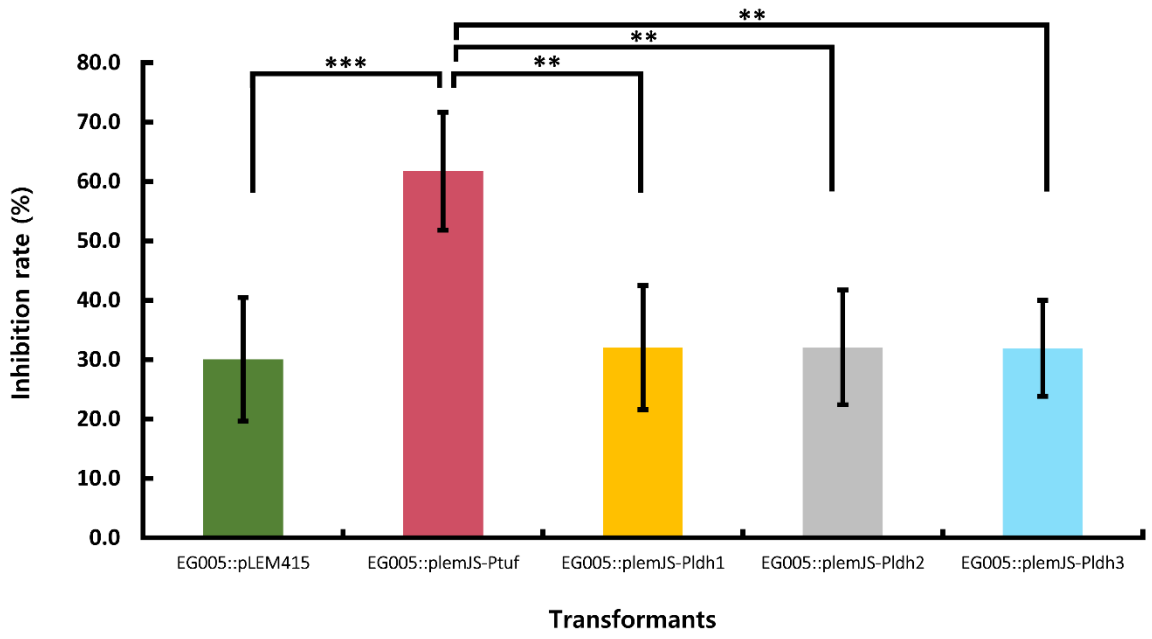


Figure 2.7 The result of SOD activity differences among recombinant strains

The EG005 harboring the pLEM415 vector was used as a control. The recombinant strain involving a *sodA* gene conjugated with P_{tuf} overexpression promoter exhibited the highest SOD activity. Error bars represent the standard deviation of three independent experiments. One-way ANOVA revealed a significant effect of strains on SOD activity. The p-value between all groups was shown on the top bar of the graph, and the p-value between P_{tuf} and the remaining groups is shown below it respectively. (** $p < 0.01$, *** $p < 0.001$).

2.5 Discussion

2.5.1 Antioxidant potential of EG005

The investigation into the SOD activity of EG005 under diverse pH conditions revealed that pH 7.0 is the optimal pH. At neutral pH, superoxide radicals remain stable and can interact most effectively with the SOD active site (Tuteja et al., 2015). Additionally, the amino acid residues composing EG005 SOD exhibit a suitable charge distribution at pH 7.0, contributing to high enzyme activity (Pinmanee et al., 2023). Specifically, Threonine, which contains a hydroxyl group (-OH), can form additional hydrogen bonds (Brockerman et al., 2014). This enhances the stability of the secondary structure of the protein, increasing its resistance to denaturation (Alber et al., 1987). In addition, Threonine's polarity also boosts the stability of proteins across various pH ranges, allowing for better adaptability (Vassall et al., 2013). These findings may reflect natural selection during the evolution of EG005. As EG005 safely traverses through the acidic environment in the stomach and reaches the neutral pH of the small intestine, its enzyme activity will be optimized (Yamamura et al., 2023). Consequently, EG005 SOD has been shown to involve enough potential as an antioxidant, reducing oxidative stress in the gut and improving intestinal health by alleviating inflammation and maintaining microbial balance (Yasui and Baba, 2006, Dam et al., 2019).

2.5.2 Survival and stability of EG005

The properties of probiotics required to survive in various organs within the digestive system depend on their capacity to withstand and adapt to various

environmental conditions, such as changes in pH, salinity, and temperature (Fiocco et al., 2020). Therefore, we evaluated the survival of EG005 under conditions of pH 2.5 and 0.3% bile salt, which closely mimic the environment of the human stomach and intestines (Castro-López et al., 2023). The human stomach typically maintains an acidity ranging from pH 1.5 to 3.5, while bile, produced in the liver and stored in the gallbladder, is released into the small intestine at concentration of 0.3% to 0.5% (Noriega et al., 2004). These conditions are valuable in predicting the survival likelihood of probiotics when consumed by humans. According to our research findings, EG005 demonstrates high acid and bile tolerance. Specifically, the survival rate recovered to approximately 100% after 3 hours in the acid tolerance evaluation. The subsequent increase in survival rate, following an initial slight decrease, may be attributed to the gradual adaptation to acidic conditions through the regulation of membrane permeability or the activation of intracellular enzymes (Hassanzadazar et al., 2012, Wang et al., 2018). In the bile tolerance evaluation, EG005 maintained a survival rate above 80% for up to 6 hours. Its high tolerance to acid and bile suggests that EG005 is well-suited to survive in the intestinal environment, making it a promising candidate for use in future probiotic therapies or functional foods Also, we used LGG as a control, since LGG is widely recognized for its well-established acid and bile tolerance characteristics (Corcoran et al., 2005), it is highly suitable as a control in experiments evaluating probiotics properties (Goldin, 1998, Segers and Lebeer, 2014).

2.5.3 Safety Assessment of EG005

Hemolytic and antibiotic resistance tests are mainly performed to evaluate the safety of probiotics. Hemolysis is a virulence factor found in pathogens that destroys red blood cells, threatening the host's life (Vesterlund et al., 2007). Therefore, it is essential that hemolytic traits are not found in probiotic strains. In this context, EG005 was confirmed to be a safe strain, as it exhibited neither β-hemolytic nor α-hemolytic characteristics. Likewise, evaluating antibiotic resistance is another crucial step in assessing the safety of probiotics. A potential probiotic candidate strain must not exhibit resistance to antibiotics nor possess transposable antibiotic resistance (AR) genes (Gueimonde et al., 2013) because antibiotic resistance genes can be transferred through various mechanisms, including plasmids, transposons, bacteriophages, and horizontal gene transfer (HGT) (Bello-López et al., 2019). EG005 showed resistance only to kanamycin in the MIC test result, but no AR genes were detected from ABRicate analysis. Kanamycin resistance, however, is relatively common in most *lactobacilli* because of its intrinsic resistance to aminoglycosides (including kanamycin), due to features of their cell membranes that limit drug absorption (Campedelli et al., 2019, Lee et al., 2023). Consequently, this inherent resistance is not transferable from EG005 to other bacteria, indicating that EG005 is safe. The absence of hemolytic activity and antibiotic resistance further confirms EG005's suitability as a probiotic strain.

2.5.4 Genomic features and functional annotations of EG005

We generated a new complete genome sequence of *L. paracasei* with a 99.5% BUSCO value. BUSCO is a bioinformatics tool used to assess the completeness of genome assemblies and annotations by searching for conserved single-copy orthologous (Simão et al., 2015). A BUSCO score over 95% typically indicates that the high-quality assembly has been performed (Seppey et al., 2019). The annotation results of EG005 using KEGG and COG databases provide a good overview of the overall distribution of genetic and functional environments (Tatusov et al., 2000, Kanehisa et al., 2006). In our KEGG pathway analysis, CDSs involved in carbohydrate metabolism accounted for the largest proportion with 889 genes. Many of the genes involved in carbohydrate metabolism corresponded to glycolysis, gluconeogenesis, the TCA cycle, and various sugar transport systems (Mailloux et al., 2007). The presence of these genes highlights the ability of EG005 to perform both aerobic and anaerobic respiration (Shan et al., 2012). This flexibility can provide a competitive edge, enabling EG005 to thrive even in rapidly changing environments (Wu et al., 2023). Additionally, the multiple sugar transport system supports EG005's excellent survival ability and environmental adaptability even in ecological niches with diverse carbohydrate availability (Liu et al., 2020). In the COG analysis the “Carbohydrate Transport and Metabolism (G)” category, which accounted for the highest percentage, further supports that EG005 has an excellent ability in carbohydrate metabolism (Ran et al., 2015). Next, the “Transcription (K)” category was notably high as well, which is crucial for managing stress responses by controlling gene expression (Namouchi et al., 2016). These functional annotations of EG005 highlight its evolutionary adaptation for optimizing energy production and

regulatory mechanisms.

2.5.5 Phylogenetic Relationships of EG005

Phylogenetic analysis was performed using CVTree and PYANI programs with whole genome sequences. Both programs were used to understand the evolutionary relationships by quantifying strain similarities (Pritchard et al., 2019). Using nucleotide sequences instead of amino acid sequences provides a comprehensive representation of the entire genome, including non-coding regions. Thus, whole-genome nucleotide data allows for the construction of detailed phylogenetic trees by leveraging the full genomic information (Xu and Hao, 2009). In our comparative phylogenetic tree analysis, however, CVTree and PYANI results showed different clustering among the same strains. This discrepancy arises because CVtree utilizes a composition vector (CV) approach that does not involve sequence alignment (Zuo and Hao, 2015), whereas, PYANI relies on pairwise nucleotide sequence similarities derived from sequence alignments performed by MUMmer and NUCmer (Richter and Rosselló-Móra, 2009). Consequently, these methodological differences may have resulted in the observed discrepancies between the two sets of results.

2.5.6. Promoter Effects on Overexpression of SOD in EG005

The P_{tuf} and P_{ldh} are widely known as promoters that induce high gene expression in Gram-positive bacteria such as *Lactobacillus* (Ma et al., 2018, Anbazhagan et al., 2013). The P_{tuf} regulates the gene for elongation factor Tu (EF-Tu), which plays an important role in transporting aminoacyl-tRNA to ribosomes during protein synthesis (Harvey et al., 2019). The P_{ldh} regulates the expression of lactate dehydrogenase (LDH), which is important in the lactic acid fermentation process (Romero et al., 2007). Based on the above, we constructed overexpression plasmids using a combination of one P_{tuf}, three P_{ldh}, and the *sodA* gene from EG005. These plasmids were then transformed into EG005. According to the result of the SOD activity test using these EG005 transformants, EG005 harboring plemJS-P_{tuf} exhibited the highest performance in inhibit ROS formation. This result suggests that the P_{tuf} is effective in enhancing SOD activity in EG005. However, EG005 transformants harboring plemJS-P_{ldh1}, plemJS-P_{ldh2}, and plemJS-P_{ldh3} showed relatively lower SOD activity than EG005 harboring plemJS-P_{tuf}. This result may be attributed to environmental effects on the expression level of each promoter. In fact, the *tuf* promoter is generally useful when stable and continuous expression is required and is often used to increase protein production (Henke et al., 2021, Kim et al., 2009). On the other hand, the *ldh* promoter originally promotes the expression of genes related to lactic acid fermentation, and its activity increases in fermentation processes such as low oxygen or high sugar concentration conditions (Song et al., 2017).

2.5.7 Potential and Future Directions for EG005

We confirmed that EG005, a strain with relatively high antioxidant activity, demonstrated probiotic properties and survival in simulated gastrointestinal conditions. Additionally, we performed *in silico* predictions to further assess its potential. Furthermore, we created an EG005 strain involving SOD overexpression and found that the P_{tuf} is effective in enhancing the SOD activity for EG005. These findings conclude that EG005, with SOD overexpression, has potential as an antioxidant to reduce oxidative stress in the gut and improve intestinal health by alleviating inflammation. However, further research is required to understand the realistic physiological effects *in vivo* by analyzing the impact of gene overexpression. In addition, as the stability of overexpression plasmid vectors over time cannot be guaranteed, the use of attB and attP-based recombination may offer a more reliable alternative for stable gene integration (Wu et al., 2021). This method would ensure continuous and stable expression of the *sodA* gene by facilitating site-specific recombination into the host genome (Lin et al., 2013). Further research should also focus on integrating this approach with CRISPR-Cas9 technologies to ensure long-term stability and effectiveness in commercial and medical applications (Ran et al., 2013, Doudna and Charpentier, 2014).

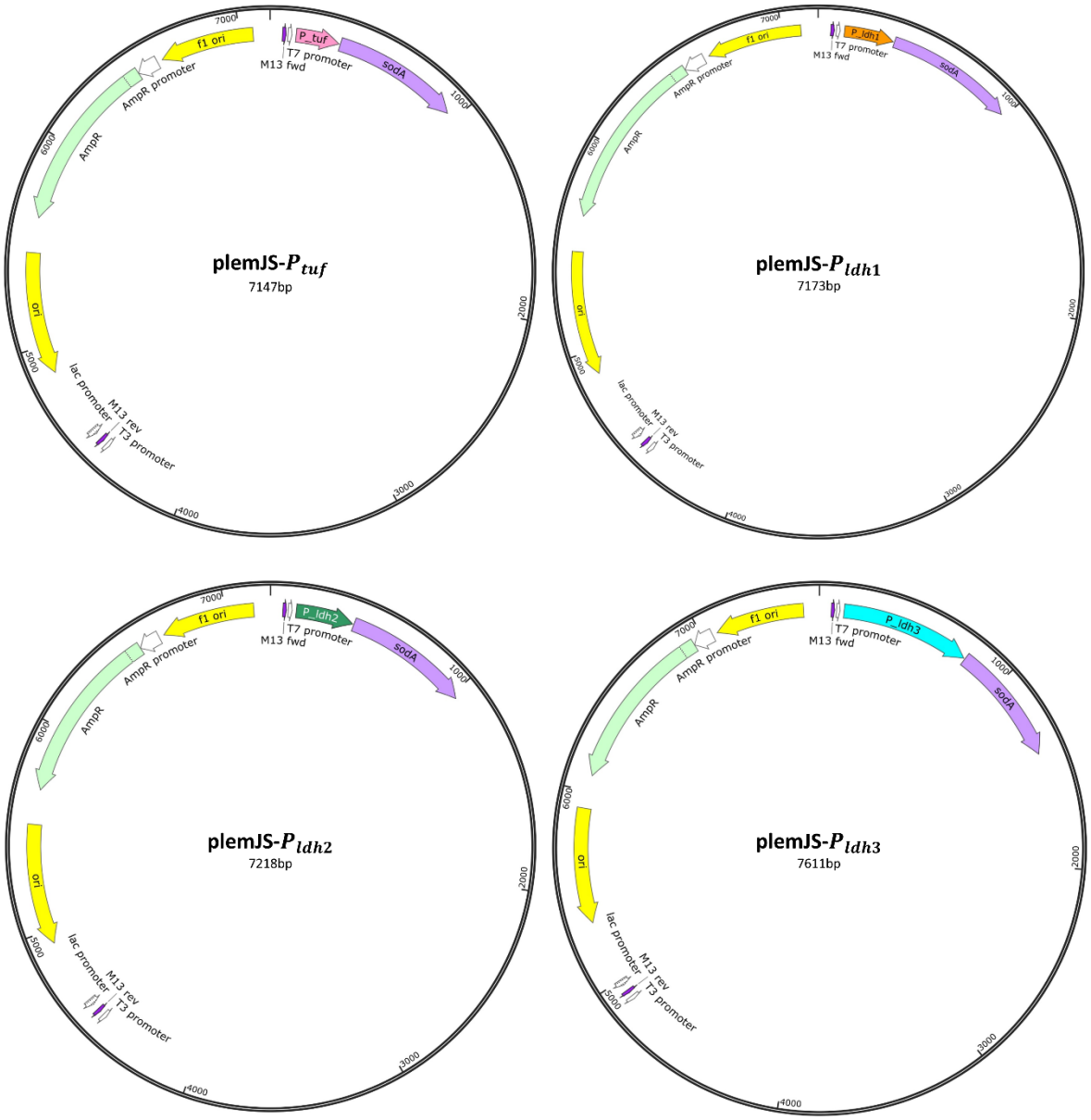
Additional file 1 - Table S2.1 Primers used in this study for construction and amplification

Target	Name	Restriction site	Sequence of primer 5'-3'	Amplicon size
P_{tuf}	F_ptuf	ApaI	**GGGCCC**CGATACGTTTTCACTGTAAATGAAAGA AACTTG	231 bp
	R_ptuf	EcoRV, HindIII	**GATATC**TAT**AAGCTT**GAAAAAAC GAACCTCCTGTATTTTTCGTAAG	
P_{ldh1}	F_pldh1	ApaI	**GGGCCC**TATCTGATAGAGATGTC ATGAAGCAGTCTG	257 bp
	R_pldh1	EcoRV, HindIII	**GATATC**TAT**AAGCTT**GAAAACCC TTCCCCTCCACTC	
P_{ldh2}	F_pldh2	ApaI	**GGGCCC**GAGGAACCACCTTTCT GGAAG	302 bp
	R_pldh2	EcoRV, HindIII	**GATATC**TAT**AAGCTT**CTCAAATT CCTCCTCATGAAGATCTTG	
P_{ldh3}	F_pldh3	ApaI	**GGGCCC**TGGAACTTTTATCCTCC TTGGGG	683 bp
	R_pldh3	ClaI, KpnI	**ATCGAT**TAT**GGTACC**GGTGATAT CATCCTTTCTTATGTGCATGC	
sodA	F_sodA_HindIII	HindIII	TT**AAGCTT**ATGACATTTGTTTTGC CAGATTTACC	634 bp
	R_sodA_EcoRV	EcoRV	TT**GATATC**TCAGGCGTTTGTATCG GG	
	F_sodA_KpnI	KpnI	TT**GGTACC**ATGACATTTGTTTTGC CAGATTTACC	
	R_sodA_ClaI	ClaI	TT**ATCGAT**TCAGGCGTTTGTATCG GG	

Additional file 2 - Table S2.2 Plasmids used in this study

Plasmids	Description	Reference/ Source
pLEM415-ldhL-mRFP1	*Lactobacillus-E.coli* shuttle vector, Erm^R, 7.3kb, f1 and ColE1 origin of replication	Bao et al
pGEM-T Easy	Subcloning vector, Amp^R, 3.0kb, f1 and ColE1 origin of replication	Promega
pJS-P_{tuf}	P_{tuf} promoter, Amp^R, 3.2kb, added HindIII and EcoRV restriction enzyme site, f1 and ColE1 origin of replication	This study
pJS-P_{ldh1}	P_{ldh1} promoter, Amp^R, 3.3kb, added HindIII and EcoRV restriction enzyme site, f1 and ColE1 origin of replication	This study
pJS-P_{ldh2}	P_{ldh2} promoter, Amp^R, 3.3kb, added HindIII and EcoRV restriction enzyme site, f1 and ColE1 origin of replication	This study
pJS-P_{ldh3}	P_{ldh3} promoter, Amp^R, 3.7kb, added KpnI and ClaI restriction enzyme site, f1 and ColE1 origin of replication	This study
pJS-S1	*sodA* gene, Amp^R, 3.6kb, added HindIII and EcoRV restriction enzyme site, f1 and ColE1 origin of replication	This study
pJS-S2	*sodA* gene, Amp^R, 3.6kb, added KpnI and ClaI restriction enzyme site, 7.1kb, f1 and ColE1 origin of replication	This study
pJS2-P_{tuf}	P_{tuf} promoter and *sodA* gene, Amp^R, 3.8kb, f1 and ColE1 origin of replication	This study
pJS2-P_{ldh1}	P_{ldh1} promoter and *sodA* gene, Amp^R, 3.9kb, f1 and ColE1 origin of replication	This study
pJS2-P_{ldh2}	P_{ldh2} promoter and *sodA* gene, Amp^R, 3.9kb, f1 and ColE1 origin of replication	This study

pJS2-P_{ldh3}	P_{ldh3} promoter and *sodA* gene, Amp^R, 4.3kb, f1 and ColE1 origin of replication	This study
plemJS-P_{tuf}	pLEM415 containing P_{tuf} promoter and *sodA* gene, Erm^R, 7.1kb, f1 and ColE1 origin of replication	This study
plemJS-P_{ldh1}	pLEM415 containing P_{ldh1} promoter and *sodA* gene, Erm^R, 7.2kb, f1 and ColE1 origin of replication	This study
plemJS-P_{ldh2}	pLEM415 containing P_{ldh2} promoter and *sodA* gene, Erm^R, 7.2kb, f1 and ColE1 origin of replication	This study
plemJS-P_{ldh3}	pLEM415 containing P_{ldh3} promoter and *sodA* gene, Erm^R, 7.6kb, f1 and ColE1 origin of replication	This study



Additional file 3 - Figure S2.1 Production of plasmid vector containing overexpression promoter and target gene

General Discussion

The overarching goal of this research was to evaluate the probiotic potential and antioxidant activity of *L. paracasei* EG005, with a particular emphasis on enhancing its SOD activity through genetic engineering. This work is presented in two chapters, each with distinct but complementary focuses that together deepen our understanding of EG005 as a robust probiotic strain.

In Chapter 1, we reviewed the fundamental role of *Lactobacillus* species as probiotics, highlighting their contributions to gut health, immune system modulation, and oxidative stress resistance. These benefits are largely attributable to their ability to maintain a balanced gut microbiome, produce antimicrobial substances, and modulate host immune responses. Specifically, the production of antioxidant enzymes such as SOD plays a critical role in mitigating oxidative stress, which is a known contributor to chronic inflammation and various diseases. The importance of bioinformatics tools was highlighted in characterizing and understanding the genetic and functional attributes of these probiotic strains, which are essential for their selection and optimization. Genomic sequencing, gene annotation, and functional pathway analyses provided insights into EG005's genetic composition, supporting the development of strategies to enhance its functionality. Additionally, we discussed overexpression promoters and their potential to enhance protein synthesis in *Lactobacillus*, focusing on Ptuf and Pldh as promising candidates for increasing SOD expression. This chapter established a comprehensive groundwork for subsequent experimental investigations aimed at evaluating the influence of these promoters on the antioxidant activity of EG005.

In Chapter 2, we experimentally investigated the probiotic features of EG005, focusing on SOD activity, acid and bile tolerance, safety evaluations, and overexpression of SOD through various promoters. Our results demonstrated that EG005 possesses significant antioxidant activity, superior acid and bile tolerance, and lacks hemolytic activity or antibiotic resistance genes characteristics essential for safe probiotic application. Notably, overexpression of SOD using the Ptuf promoter led to a significant increase in antioxidant capacity compared to other promoters. This underscores its effectiveness in improving stress resistance, which is a critical factor for probiotics intended for therapeutic or functional food applications.

In conclusion, these findings emphasize the potential of EG005 as an effective probiotic, capable of surviving harsh gastrointestinal conditions and contributing to oxidative stress reduction. The integration of bioinformatics and genetic engineering has provided a deeper understanding of EG005's functional attributes and its suitability for use in therapeutic and functional food applications. The results confirm EG005's potential as an effective probiotic with high survivability in gastrointestinal conditions and robust antioxidant properties. These characteristics position EG005 as a strong candidate for use in functional foods, therapeutic formulations, and health supplements, particularly for mitigating oxidative stress and promoting gut health. Furthermore, the enhanced SOD activity achieved through genetic engineering offers new opportunities for addressing oxidative stress-related conditions, such as inflammatory bowel disease (IBD), cardiovascular disorders, and age-related oxidative damage.

Future research should focus on validating the health benefits of EG005 in

animal models and human clinical trials. This would provide critical insights into its efficacy in reducing oxidative stress, modulating gut microbiota, and improving immune responses in vivo. Also, current study utilized plasmid-based expression systems, which may face challenges in long-term stability and scalability. We should explore genomic integration methods, such as attB/attP-based recombination or CRISPR-Cas9 technologies, to achieve stable and sustained SOD overexpression. These approaches would enhance the commercial viability and consistency of EG005 based products.

References

ADDITIVES, E. P. O.andFEED, P. O. S. U. I. A. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal,* 10, 2740. doi: 10.2903/j.efsa.2012.2740

ADWAS, A. A., ELSAYED, A., AZAB, A. E.andQUWAYDIR, F. A. (2019). Oxidative stress and antioxidant mechanisms in human body. *J. Appl. Biotechnol. Bioeng,* 6, 43-47.

ALBER, T., DAO-PIN, S., WILSON, K., WOZNIAK, J. A., COOK, S. P.andMATTHEWS, B. W. (1987). Contributions of hydrogen bonds of Thr 157 to the thermodynamic stability of phage T4 lysozyme. *Nature,* 330, 41-46. doi: 10.1038/330041a0

ALI, S. S., AHSAN, H., ZIA, M. K., SIDDIQUI, T.andKHAN, F. H. (2020). Understanding oxidants and antioxidants: Classical team with new players. *Journal of food biochemistry,* 44, e13145.

ALJUHANI, N. S. (2017). Role of SOD Peroxidase Activity in Oxidizing Drugs: Potential Modulation of Drug Toxicity.

ANBAZHAGAN, K., SASIKUMAR, P., GOMATHI, S., PRIYA, H.andSELVAM, G. (2013). In vitro degradation of oxalate by recombinant Lactobacillus plantarum expressing heterologous oxalate decarboxylase. *Journal of Applied Microbiology,* 115, 880-887. doi: 10.1111/jam.12269

ARASU, M. V., AL-DHABI, N. A., REJINIEMON, T. S., LEE, K. D., HUXLEY, V., KIM, D. H., et al. (2015). Identification and characterization of *Lactobacillus*

brevis P68 with antifungal, antioxidant and probiotic functional properties. *Indian journal of microbiology,* 55, 19-28. doi: 10.1007/s12088-014-0495-3

ARQUëS, J. L., RODRïGUEZ, E., LANGA, S., LANDETE, J. M.andMEDINA, M. (2015). Antimicrobial activity of lactic acid bacteria in dairy products and gut: effect on pathogens. *BioMed research international,* 2015, 584183.

ASAMA, T., ARIMA, T. H., GOMI, T., KEISHI, T., TANI, H., KIMURA, Y., et al. (2015). *Lactobacillus kunkeei* YB38 from honeybee products enhances IgA production in healthy adults. *Journal of applied microbiology,* 119, 818-826.

ASSENBERG, R., WAN, P. T., GEISSE, S.andMAYR, L. M. (2013). Advances in recombinant protein expression for use in pharmaceutical research. *Current opinion in structural biology,* 23, 393-402.

BAO, S., ZHU, L., ZHUANG, Q., WANG, L., XU, P.-X., ITOH, K., et al. (2013). Distribution dynamics of recombinant *Lactobacillus* in the gastrointestinal tract of neonatal rats. *PloS one,* 8, e60007. doi: 10.1371/journal.pone.0060007

BAYR, H. (2005). Reactive oxygen species. *Critical care medicine,* 33, S498-S501. doi: 10.1097/01.ccm.0000186787.64500.12

BEJ, A. K., MAHBUBANI, M. H.andATLAS, R. M. (1991). Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications. *Critical reviews in biochemistry and molecular biology,* 26,

301-334.

BELLO-LõPEZ, J. M., CABRERO-MARTïNEZ, O. A., IBãóEZ-CERVANTES, G., HERNãNDEZ-CORTEZ, C., PELCASTRE-RODRïGUEZ, L. I., GONZALEZ-AVILA, L. U., et al. (2019). Horizontal gene transfer and its association with antibiotic resistance in the genus Aeromonas spp. *Microorganisms,* 7, 363. doi: 10.3390/microorganisms7090363

BLAZECK, J.andALPER, H. S. (2013). Promoter engineering: recent advances in controlling transcription at the most fundamental level. *Biotechnology journal,* 8, 46-58.

BROCKERMAN, J. A., OKON, M.andMCINTOSH, L. P. (2014). Detection and characterization of serine and threonine hydroxyl protons in Bacillus circulans xylanase by NMR spectroscopy. *Journal of biomolecular NMR,* 58, 17-25. doi: 10.1007/s10858-013-9799-6

CAI, H., THOMPSON, R., BUDINICH, M. F., BROADBENT, J. R.andSTEELE, J. L. (2009). Genome sequence and comparative genome analysis of *Lactobacillus casei:* insights into their niche-associated evolution. *Genome biology and evolution,* 1, 239-257.

CAMPEDELLI, I., MATHUR, H., SALVETTI, E., CLARKE, S., REA, M. C., TORRIANI, S., et al. (2019). Genus-wide assessment of antibiotic resistance in *Lactobacillus spp. Applied and environmental microbiology,* 85, e01738-18. doi: 10.1128/AEM.01738-18

CANTALAPIEDRA, C. P., HERNãNDEZ-PLAZA, A., LETUNIC, I., BORK,

P.andHUERTA-CEPAS, J. (2021). eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Molecular biology and evolution,* 38, 5825-5829. doi: 10.1093/molbev/msab293

CASTRO-LõPEZ, C., GARCIA, H. S., MARTINEZ-AVILA, G. C. G., GONZãLEZ-CõRDOVA, A. F., VALLEJO-CORDOBA, B.andHERNãNDEZ-MENDOZA, A. (2021). Genomics-based approaches to identify and predict the health-promoting and safety activities of promising probiotic strains–A probiogenomics review. *Trends in Food Science & Technology,* 108, 148-163.

CASTRO-LõPEZ, C., ROMERO-LUNA, H. E., GARCïA, H. S., VALLEJO-CORDOBA, B., GONZãLEZ-CõRDOVA, A. F.andHERNãNDEZ-MENDOZA, A. (2023). Key stress response mechanisms of probiotics during their journey through the digestive system: a review. *Probiotics and Antimicrobial Proteins,* 15, 1250-1270. doi: 10.1007/s12602-022-09981-x

CHASSY, B. M.andFLICKINGER, J. L. (1987). Transformation of *Lactobacillus casei* by electroporation. *FEMS Microbiology Letters,* 44, 173-177.

CHOORUK, A., PIWAT, S.andTEANPAISAN, R. (2017). Antioxidant activity of various oral Lactobacillus strains. *Journal of applied microbiology,* 123, 271-279. doi: 10.1111/jam.13482

CLAESSON, M. J., VAN SINDEREN, D.andO'TOOLE, P. W. (2008). *Lactobacillus* phylogenomics–towards a reclassification of the genus. *International*

Journal of Systematic and Evolutionary Microbiology, 58, 2945-2954.

CONWAY, P., GORBACH, S.andGOLDIN, B. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of dairy science,* 70, 1-12. doi: 10.3168/jds.S0022-0302(87)79974-3

CORCORAN, B. M., STANTON, C., FITZGERALD, G.andROSS, R. (2005). Survival of probiotic *lactobacilli* in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and environmental microbiology,* 71, 3060-3067. doi: 10.1128/AEM.71.6.3060-3067.2005

DAM, B., MISRA, A.andBANERJEE, S. (2019). Role of gut microbiota in combating oxidative stress. *Oxidative stress in microbial diseases*, 43-82. doi: 10.1007/978-981-13-8763-0_4

DANILOVA, I.andSHARIPOVA, M. (2020). The practical potential of bacilli and their enzymes for industrial production. *Frontiers in microbiology,* 11, 558639. doi: 10.3389/fmicb.2020.01782

DOUDNA, J. A.andCHARPENTIER, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science,* 346, 1258096. doi: 10.1126/science.1258096

DR ALTAVAS, P. J., AMORANTO, M. B. C., KIM, S. H., KANG, D.-K., BALOLONG, M. P.andDALMACIO, L. M. M. (2024). Safety assessment of five candidate probiotic lactobacilli using comparative genome analysis. *Access Microbiology,* 6, 000715. v4. doi:

ENGSTROM, M. D.andPFLEGER, B. F. (2017). Transcription control engineering

and applications in synthetic biology. *Synthetic and systems biotechnology,* 2, 176-191. doi:

FENG, T.andWANG, J. (2020). Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: a systematic review. *Gut microbes,* 12, 1801944. doi:

FINK, C., BEBLAWY, S., ENKERLIN, A. M., MþHLING, L., ANGENENT, L. T.andMOLITOR, B. (2021). A shuttle-vector system allows heterologous gene expression in the thermophilic methanogen Methanothermobacter thermautotrophicus ΔH. *MBio,* 12, e02766-21. doi:

FIOCCO, D., LONGO, A., ARENA, M. P., RUSSO, P., SPANO, G.andCAPOZZI, V. (2020). How probiotics face food stress: They get by with a little help. *Critical Reviews in Food Science and Nutrition,* 60, 1552-1580. doi: 10.1080/10408398.2019.1580673

FRIEHS, K. (2004). Plasmid copy number and plasmid stability. *New trends and developments in biochemical engineering*, 47-82. doi:

GALPERIN, M. Y., WOLF, Y. I., MAKAROVA, K. S., VERA ALVAREZ, R., LANDSMAN, D.andKOONIN, E. V. (2021). COG database update: focus on microbial diversity, model organisms, and widespread pathogens. *Nucleic acids research,* 49, D274-D281. doi: 10.1093/nar/gkaa1018

GHIASI, F., HASHEMI, S. M. B.andABEDI, E. (2023). Effective enhancement of food oxidative stability induced by Lactobacillus strains: In vitro activity. *Food Control*, 109912. doi:10.1016/j.foodcont.2023.109912

GOLDIN, B. R. (1998). Health benefits of probiotics. *British Journal of Nutrition,* 80, S203-S207. doi: 10.1017/S0007114500006036

GRIFFITHS, M. W.andTELLEZ, A. M. (2013). *Lactobacillus helveticus*: the proteolytic system. *Frontiers in Microbiology,* 4, 40677. doi: 10.3389/fmicb.2013.00030

GUAN, M., PANG, C.-P., YAM, H.-F., CHEUNG, K.-F., LIU, W.-W.andLU, Y. (2004). Inhibition of glioma invasion by overexpression of pigment epithelium-derived factor. *Cancer gene therapy,* 11, 325-332.

GUEIMONDE, M., SãNCHEZ, B., G. DE LOS REYES-GAVILãN, C.andMARGOLLES, A. (2013). Antibiotic resistance in probiotic bacteria. *Frontiers in microbiology,* 4, 202. doi: 10.3389/fmicb.2013.00202

GULERI, S.andTIWARI, A. (2020). Algae and ageing. *Microalgae biotechnology for food, health and high value products*, 267-293.

HAMMES, W. P.andVOGEL, R. F. (1995). The genus *lactobacillus*. *The genera of lactic acid bacteria.* Springer.

HARTLEY, J. L., TEMPLE, G. F.andBRASCH, M. A. (2000). DNA cloning using in vitro site-specific recombination. *Genome research,* 10, 1788-1795.

HARVEY, K. L., JAROCKI, V. M., CHARLES, I. G.andDJORDJEVIC, S. P. (2019). The diverse functional roles of elongation factor Tu (EF-Tu) in microbial pathogenesis. *Frontiers in microbiology,* 10, 2351. doi: 10.3389/fmicb.2019.02351

HASSANZADAZAR, H., EHSANI, A., MARDANI, K.andHESARI, J. Investigation of

antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Veterinary Research Forum, (2012). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, 181.

HENKE, N. A., KRAHN, I.andWENDISCH, V. F. (2021). Improved plasmid-based inducible and constitutive gene expression in Corynebacterium glutamicum. *Microorganisms,* 9, 204. doi: 10.3390/microorganisms9010204

HOANG, D. T., CHERNOMOR, O., VON HAESELER, A., MINH, B. Q.andVINH, L. S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Molecular biology and evolution,* 35, 518-522. doi: 10.1093/molbev/msx281

HU, Y., ROSEN, D. G., ZHOU, Y., FENG, L., YANG, G., LIU, J., et al. (2005). Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. *Journal of Biological Chemistry,* 280, 39485-39492.

HUANG, R., WU, F., ZHOU, Q., WEI, W., YUE, J., XIAO, B., et al. (2022). Lactobacillus and intestinal diseases: Mechanisms of action and clinical applications. *Microbiological Research,* 260, 127019. doi: 10.1016/j.micres.2022.127019

HUANG, Y.-T., LIU, P.-Y.andSHIH, P.-W. (2021). Homopolish: a method for the removal of systematic errors in nanopore sequencing by homologous polishing. *Genome biology,* 22, 1-17. doi: 10.1186/s13059-021-02282-6

HUNT, M., SILVA, N. D., OTTO, T. D., PARKHILL, J., KEANE, J. A.andHARRIS, S. R. (2015). Circlator: automated circularization of genome assemblies using long sequencing reads. *Genome biology,* 16, 1-10. doi: 10.1186/s13059-015-0849-0

ISLAM, M. N., LEE, K. W., YIM, H.-S., LEE, S. H., JUNG, H. C., LEE, J.-H., et al. (2017). Optimizing T4 DNA Polymerase Conditions Enhances the Efficiency of One-Step Sequence-and Cligation-Independent Cloning. *Biotechniques,* 63, 125-130. doi:

JEON, S., KIM, H., KIM, J., SEOL, D., JO, J., CHOI, Y., et al. (2022). Positive effect of Lactobacillus acidophilus EG004 on cognitive ability of healthy mice by fecal microbiome analysis using full-length 16S-23S rRNA metagenome sequencing. *Microbiology Spectrum,* 10, e01815-21. doi: 10.1128/spectrum.01815-21

JUSTINO, P. F., MELO, L. F., NOGUEIRA, A. F., MORAIS, C. M., MENDES, W. O., FRANCO, A. X., et al. (2015). Regulatory role of Lactobacillus acidophilus on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. *Cancer chemotherapy and pharmacology,* 75, 559-567. doi: 10.1007/s00280-014-2663-x

KANEHISA, M.andGOTO, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic acids research,* 28, 27-30.

KANEHISA, M., GOTO, S., HATTORI, M., AOKI-KINOSHITA, K. F., ITOH, M., KAWASHIMA, S., et al. (2006). From genomics to chemical genomics: new

developments in KEGG. *Nucleic acids research,* 34, D354-D357. doi: 10.1093/nar/gkj102

KATOH, K.andSTANDLEY, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution,* 30, 772-780. doi: 10.1093/molbev/mst010

KHALIL, M. A., SONBOL, F. I., AL-MADBOLY, L. A., ABOSHADY, T. A., ALQURASHI, A. S.andALI, S. S. (2022). Exploring the therapeutic potentials of exopolysaccharides derived from lactic acid bacteria and bifidobacteria: antioxidant, antitumor, and periodontal regeneration. *Frontiers in Microbiology,* 13, 803688.

KHUBBER, S., MARTI-QUIJAL, F. J., TOMASEVIC, I., REMIZE, F.andBARBA, F. J. (2022). Lactic acid fermentation as a useful strategy to recover antimicrobial and antioxidant compounds from food and by-products. *Current Opinion in Food Science,* 43, 189-198. doi: 10.1016/j.cofs.2021.11.013

KIM, E. B., PIAO, D. C., SON, J. S.andCHOI, Y. J. (2009). Cloning and characterization of a novel tuf promoter from *Lactococcus lactis* subsp. lactis IL1403. *Current microbiology,* 59, 425-431. doi: 10.1007/s00284-009-9455-2

KIM, H. S.andGILLILAND, S. E. (1983). *Lactobacillus acidophilu*s as a dietary adjunct for milk to aid lactose digestion in humans. *Journal of dairy*

science, 66, 959-966. doi: 10.3168/jds.S0022-0302(83)81887-6

KIROV, B.andPEYKOV, S. (2022). Design and characterization of synthetic promoters. *New frontiers and applications of synthetic biology.* Elsevier.

KLAENHAMMER, T. R., ALTERMANN, E., PFEILER, E., BUCK, B. L., GOH, Y.-J., O'FLAHERTY, S., et al. (2008). Functional genomics of probiotic Lactobacilli. *Journal of clinical gastroenterology,* 42, S160-S162. doi:

KLARE, I., KONSTABEL, C., MU¨LLER-BERTLING, S., REISSBRODT, R., HUYS, G., VANCANNEYT, M., et al. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of *lactobacilli, pediococci, lactococci*, and *bifidobacteria*. *Applied and Environmental Microbiology,* 71, 8982-8986. doi: 10.1128/AEM.71.12.8982-8986.2005

KONG, L., XIONG, Z., SONG, X., XIA, Y., ZHANG, H., YANG, Y., et al. (2020). Enhanced antioxidant activity in *Streptococcus thermophilus* by high-level expression of superoxide dismutase. *Frontiers in Microbiology,* 11, 579804.

KOREN, S., WALENZ, B. P., BERLIN, K., MILLER, J. R., BERGMAN, N. H.andPHILLIPPY, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome research,* 27, 722-736. doi: 10.1101/gr.215087.116

KUNSCH, C.andMEDFORD, R. M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circulation research,* 85, 753-766.

LEBEER, S., VANDERLEYDEN, J.andDE KEERSMAECKER, S. C. (2008). Genes and

molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews,* 72, 728-764.

LEBLANC, J. G., DEL CARMEN, S., MIYOSHI, A., AZEVEDO, V., SESMA, F., LANGELLA, P., et al. (2011). Use of superoxide dismutase and catalase producing lactic acid bacteria in TNBS induced Crohn's disease in mice. *Journal of Biotechnology,* 151, 287-293. doi: 10.1016/j.jbiotec.2010.11.008

LEE, H., YOON, H., JI, Y., KIM, H., PARK, H., LEE, J., et al. (2011). Functional properties of *Lactobacillus* strains isolated from kimchi. *International Journal of Food Microbiology,* 145, 155-161. doi: 10.1016/j.ijfoodmicro.2010.12.003

LEE, H. B., BANG, W. Y., SHIN, G. R., JEON, H. J., JUNG, Y. H.andYANG, J. (2023). Isolation, Characterization, and Safety Evaluation of the Novel Probiotic Strain *Lacticaseibacillus paracasei* IDCC 3401 via Genomic and Phenotypic Approaches. *Microorganisms,* 12, 85. doi: 10.3390/microorganisms12010085

LEE, K., RHO, B.-S., PI, K., KIM, H.-J.andCHOI, Y.-J. (2011). Proteomic analysis of protein expression in *Lactobacillus plantarum* in response to alkaline stress. *Journal of biotechnology,* 153, 1-7. doi: 10.1016/j.jbiotec.2011.02.008

LEINONEN, R., SUGAWARA, H., SHUMWAY, M.andCOLLABORATION, I. N. S. D. (2010). The sequence read archive. *Nucleic acids research,* 39, D19-D21.

doi: 10.1093/nar/gkq1019

LIGUORI, I., RUSSO, G., CURCIO, F., BULLI, G., ARAN, L., DELLA-MORTE, D., et al. (2018). Oxidative stress, aging, and diseases. *Clinical interventions in aging*, 757-772.

LIN, C.-F., LO, T.-C., KUO, Y.-C.andLIN, T.-H. (2013). Stable integration and expression of heterologous genes in several lactobacilli using an integration vector constructed from the integrase and attP sequences of phage ΦAT3 isolated from *Lactobacillus casei* ATCC 393. *Applied microbiology and biotechnology,* 97, 3499-3507.

LIN, J., ZOU, Y., CAO, K., MA, C.andCHEN, Z. (2016). The impact of heterologous catalase expression and superoxide dismutase overexpression on enhancing the oxidative resistance in *Lactobacillus casei*. *Journal of Industrial Microbiology and Biotechnology,* 43, 703-711.

LIU, L., YANG, H., SHIN, H.-D., CHEN, R. R., LI, J., DU, G., et al. (2013). How to achieve high-level expression of microbial enzymes: strategies and perspectives. *Bioengineered,* 4, 212-223.

LIU, Y., SHANG, L., ZHAN, Y., LIN, M., LIU, Z.andYAN, Y. (2020). Genome-wide analysis of sugar transporters identifies the gtsA gene for glucose transportation in Pseudomonas stutzeri A1501. *Microorganisms,* 8, 592. doi: 10.3390/microorganisms8040592

LORENTE-PICõN, M.andLAGUNA, A. (2021). New avenues for Parkinson's disease therapeutics: disease-modifying strategies based on the gut

microbiota. *Biomolecules,* 11, 433.

MA, Y., CUI, Y., DU, L., LIU, X., XIE, X.andCHEN, N. (2018). Identification and application of a growth-regulated promoter for improving L-valine production in *Corynebacterium glutamicum*. *Microbial Cell Factories,* 17, 1-10. doi: 10.1186/s12934-018-1031-7

MAHOOTI, M., ABDOLALIPOUR, E., SANAMI, S.andZARE, D. (2024). Inflammatory Modulation Effects of Probiotics: A Safe and Promising Modulator for Cancer Prevention. *Current microbiology,* 81, 372.

MAILLOUX, R. J., BERIAULT, R., LEMIRE, J., SINGH, R., CHENIER, D. R., HAMEL, R. D., et al. (2007). The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. *PloS one,* 2, e690. doi: 10.1371/journal.pone.0000690

MANGIA, N. P., SALIBA, L.andDEIANA, P. (2019). Functional and safety characterization of autochthonous *Lactobacillus paracasei* FS103 isolated from sheep cheese and its survival in sheep and cow fermented milks during cold storage. *Annals of Microbiology,* 69, 161-170. doi: 10.1007/s13213-018-1416-1

MANNI, M., BERKELEY, M. R., SEPPEY, M., SIMåO, F. A.andZDOBNOV, E. M. (2021). BUSCO update: novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of eukaryotic, prokaryotic, and viral genomes. *Molecular biology and evolution,* 38, 4647-4654. doi: 10.1093/molbev/msab199

MASON, C. K., COLLINS, M. A.andTHOMPSON, K. (2005). Modified electroporation protocol for Lactobacilli isolated from the chicken crop facilitates transformation and the use of a genetic tool. *Journal of microbiological methods,* 60, 353-363. doi: 10.1016/j.mimet.2004.10.013

MINH, B. Q., SCHMIDT, H. A., CHERNOMOR, O., SCHREMPF, D., WOODHAMS, M. D., VON HAESELER, A., et al. (2020). IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular biology and evolution,* 37, 1530-1534. doi: 10.1093/molbev/msaa131

MUTALIK, V. K., GUIMARAES, J. C., CAMBRAY, G., LAM, C., CHRISTOFFERSEN, M. J., MAI, Q.-A., et al. (2013). Precise and reliable gene expression via standard transcription and translation initiation elements. *Nature methods,* 10, 354-360.

NAMOUCHI, A., GõMEZ-MUóOZ, M., FRYE, S. A., MOEN, L. V., ROGNES, T., TúNJUM, T., et al. (2016). The *Mycobacterium* tuberculosis transcriptional landscape under genotoxic stress. *BMC genomics,* 17, 1-13. doi: 10.1186/s12864-016-3132-1

NANDI, A., YAN, L.-J., JANA, C. K.andDAS, N. (2019). Role of catalase in oxidative stress-and age-associated degenerative diseases. *Oxidative medicine and cellular longevity,* 2019, 9613090.

NARITA, J., ISHIDA, S., OKANO, K., KIMURA, S., FUKUDA, H.andKONDO, A. (2006). Improvement of protein production in lactic acid bacteria using

5′-untranslated leader sequence of slpA from *Lactobacillus acidophilus*: Improvement in protein production using UTLS. *Applied microbiology and biotechnology,* 73, 366-373.

NES, I. F., HOLO, H., FIMLAND, G., HAUGE, H. H.andNISSEN-MEYER, J. (2001). Unmodified peptide-bacteriocins (class II) produced by lactic acid bacteria. *Peptide antibiotics: discovery, modes of action and application, section B. Distribution of antimicrobial peptides. Marcel Dekker, Inc., New York, NY*, 81-115.

NORIEGA, L., GUEIMONDE, M., SãNCHEZ, B., MARGOLLES, A.andDE LOS REYES-GAVILãN, C. G. (2004). Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium*. *International journal of food microbiology,* 94, 79-86. doi: 10.1016/j.ijfoodmicro.2004.01.003

PALMA, F. R., HE, C., DANES, J. M., PAVIANI, V., COELHO, D. R., GANTNER, B. N., et al. (2020). Mitochondrial superoxide dismutase: what the established, the intriguing, and the novel reveal about a key cellular redox switch. *Antioxidants & redox signaling,* 32, 701-714.

PAPADAKIS, E., NICKLIN, S., BAKER, A.andWHITE, S. (2004). Promoters and control elements: designing expression cassettes for gene therapy. *Current gene therapy,* 4, 89-113.

PATLEVIČ, P., VAŠKOVã, J., ŠVORC JR, P., VAŠKO, L.andŠVORC, P. (2016). Reactive oxygen species and antioxidant defense in human gastrointestinal

diseases. *Integrative medicine research,* 5, 250-258. doi: 10.1016/j.imr.2016.07.004

PERAN, L., CAMUESCO, D., COMALADA, M., NIETO, A., CONCHA, A., ADRIO, J. L., et al. (2006). *Lactobacillus fermentum*, a probiotic capable to release glutathione, prevents colonic inflammation in the TNBS model of rat colitis. *International journal of colorectal disease,* 21, 737-746.

PESKIN, A. V.andWINTERBOURN, C. C. (2000). A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). *Clinica chimica acta,* 293, 157-166. doi: 10.1016/S0009-8981(99)00246-6

PINMANEE, P., SOMPINIT, K., JANTIMAPORN, A., KHONGKOW, M., HALTRICH, D., NIMCHUA, T., et al. (2023). Purification and Immobilization of Superoxide Dismutase Obtained from *Saccharomyces cerevisiae* TBRC657 on Bacterial Cellulose and Its Protective Effect against Oxidative Damage in Fibroblasts. *Biomolecules,* 13, 1156. doi: 10.3390/biom13071156

PLESSAS, S., BOSNEA, L., ALEXOPOULOS, A.andBEZIRTZOGLOU, E. (2012). Potential effects of probiotics in cheese and yogurt production: A review. *Engineering in Life Sciences,* 12, 433-440. doi: 10.1002/elsc.201100122

PRITCHARD, L., COCK, P.andESEN, Ö. (2019). pyani v0. 2.8: average nucleotide identity (ANI) and related measures for whole genome comparisons. doi: 10.5281/zenodo.2584238

QI, J., LUO, H.andHAO, B. (2004). CVTree: a phylogenetic tree reconstruction tool based on whole genomes. *Nucleic acids research,* 32, W45-W47. doi:

10.1093/nar/gkh362

QUIGLEY, E. M. (2013). Gut bacteria in health and disease. *Gastroenterology & hepatology,* 9, 560.

RAN, F., HSU, P. D., WRIGHT, J., AGARWALA, V., SCOTT, D. A.andZHANG, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols,* 8, 2281-2308. doi: 10.1038/nprot.2013.143

RAN, S., LIU, B., JIANG, W., SUN, Z.andLIANG, J. (2015). Transcriptome analysis of *Enterococcus faecalis* in response to alkaline stress. *Frontiers in microbiology,* 6, 795. doi: 10.3389/fmicb.2015.00795

RANI, V., DEEP, G., SINGH, R. K., PALLE, K.andYADAV, U. C. (2016). Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. *Life sciences,* 148, 183-193.

REBOLLAR, E. A., ANTWIS, R. E., BECKER, M. H., BELDEN, L. K., BLETZ, M. C., BRUCKER, R. M., et al. (2016). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Frontiers in microbiology,* 7, 68.

RIAZ RAJOKA, M. S., THIRUMDAS, R., MEHWISH, H. M., UMAIR, M., KHURSHID, M., HAYAT, H. F., et al. (2021). Role of food antioxidants in modulating gut microbial communities: Novel understandings in intestinal oxidative stress damage and their impact on host health. *Antioxidants,* 10, 1563.

RICHTER, M.andROSSELLõ-MõRA, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the*

National Academy of Sciences, 106, 19126-19131. doi: 10.1073/pnas.0906412106

ROMERO, S., MERINO, E., BOLI´VAR, F., GOSSET, G.andMARTINEZ, A. (2007). Metabolic engineering of *Bacillus subtilis* for ethanol production: lactate dehydrogenase plays a key role in fermentative metabolism. *Applied and environmental microbiology,* 73, 5190-5198. doi: 10.1128/AEM.00625-07

SAEZ-LARA, M. J., GOMEZ-LLORENTE, C., PLAZA-DIAZ, J.andGIL, A. (2015). The role of probiotic lactic acid bacteria and bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. *BioMed research international,* 2015, 505878. doi: 10.1155/2015/505878

SAULNIER, D. M., SANTOS, F., ROOS, S., MISTRETTA, T.-A., SPINLER, J. K., MOLENAAR, D., et al. (2011). Exploring metabolic pathway reconstruction and genome-wide expression profiling in Lactobacillus reuteri to define functional probiotic features. *PloS one,* 6, e18783.

SAYERS, E. W., BOLTON, E. E., BRISTER, J. R., CANESE, K., CHAN, J., COMEAU, D. C., et al. (2022). Database resources of the national center for biotechnology information. *Nucleic acids research,* 50, D20-D26. doi: 10.1093/nar/ gkaa892

SCHMIDT, F. (2004). Recombinant expression systems in the pharmaceutical industry. *Applied microbiology and biotechnology,* 65, 363-372.

SEEMANN, T. (2014). Prokka: rapid prokaryotic genome annotation.

Bioinformatics, 30, 2068-2069. doi: 10.1093/bioinformatics/btu153

SEGERS, M. E.andLEBEER, S. (2014). Towards a better understanding of Lactobacillus rhamnosus GG-host interactions. *Microbial cell factories,* 13, S7. doi: 10.1186/1475-2859-13-S1-S7

SEPPEY, M., MANNI, M.andZDOBNOV, E. M. (2019). BUSCO: assessing genome assembly and annotation completeness. *Gene prediction: methods and protocols*, 227-245. doi: 10.1007/978-1-4939-9173-0_14

SHAN, Y., LAI, Y.andYAN, A. (2012). Metabolic reprogramming under microaerobic and anaerobic conditions in bacteria. *Reprogramming microbial metabolic pathways*, 159-179. doi: 10.1007/978-94-007-5055-5_8

SHENG, Y., ABREU, I. A., CABELLI, D. E., MARONEY, M. J., MILLER, A.-F., TEIXEIRA, M., et al. (2014). Superoxide dismutases and superoxide reductases. *Chemical reviews,* 114, 3854-3918.

SIMåO, F. A., WATERHOUSE, R. M., IOANNIDIS, P., KRIVENTSEVA, E. V.andZDOBNOV, E. M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics,* 31, 3210-3212. doi: 10.1093/bioinformatics/btv351

SONG, A. A.-L., IN, L. L., LIM, S. H. E.andRAHIM, R. A. (2017). A review on *Lactococcus lactis*: from food to factory. *Microbial cell factories,* 16, 1-15. doi: 10.1186/s12934-017-0669-x

SUZUKI, Y., KOSAKA, M., SHINDO, K., KAWASUMI, T., KIMOTO-NIRA,

H.andSUZUKI, C. (2013). Identification of antioxidants produced by *Lactobacillus plantarum. Bioscience, biotechnology, and biochemistry,* 77, 1299-1302. doi: 10.1271/bbb.121006

TATUSOV, R. L., GALPERIN, M. Y., NATALE, D. A.andKOONIN, E. V. (2000). The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic acids research,* 28, 33-36.

TSAI, Y.-T., CHENG, P.-C.andPAN, T.-M. (2012). The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. *Applied microbiology and biotechnology,* 96, 853-862.

TUTEJA, N., MISHRA, P., YADAV, S., TAJRISHI, M., BARAL, S.andSABAT, S. C. (2015). Heterologous expression and biochemical characterization of a highly active and stable chloroplastic CuZn-superoxide dismutase from Pisum sativum. *BMC biotechnology,* 15, 1-14. doi: 10.1186/s12896-015-0117-0

VARGHESE, N. J., MUKHERJEE, S., IVANOVA, N., KONSTANTINIDIS, K. T., MAVROMMATIS, K., KYRPIDES, N. C., et al. (2015). Microbial species delineation using whole genome sequences. *Nucleic acids research,* 43, 6761-6771.

VASSALL, K. A., BESSONOV, K., DE AVILA, M., POLVERINI, E.andHARAUZ, G. (2013). The effects of threonine phosphorylation on the stability and dynamics of the central molecular switch region of 18.5-kDa myelin basic protein. *PloS one,* 8, e68175. doi: 10.1371/journal.pone.0068175

VESTERLUND, S., VANKERCKHOVEN, V., SAXELIN, M., GOOSSENS, H., SALMINEN, S.andOUWEHAND, A. C. (2007). Safety assessment of *Lactobacillus* strains: presence of putative risk factors in faecal, blood and probiotic isolates. *International journal of food microbiology,* 116, 325-331. doi: 10.1016/j.ijfoodmicro.2007.02.002

VONA, R., PALLOTTA, L., CAPPELLETTI, M., SEVERI, C.andMATARRESE, P. (2021). The impact of oxidative stress in human pathology: Focus on gastrointestinal disorders. *Antioxidants,* 10, 201.

WALLACE, T. D., BRADLEY, S., BUCKLEY, N. D.andGREEN-JOHNSON, J. M. (2003). Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. *Journal of Food Protection,* 66, 466-472.

WANG, C., CUI, Y.andQU, X. (2018). Mechanisms and improvement of acid resistance in lactic acid bacteria. *Archives of microbiology,* 200, 195-201.

WANG, Y., WU, Y., WANG, Y., XU, H., MEI, X., YU, D., et al. (2017). Antioxidant properties of probiotic bacteria. *Nutrients,* 9, 521.

WIEGAND, I., HILPERT, K.andHANCOCK, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature protocols,* 3, 163-175. doi: 10.1038/nprot.2007.521

WOLFE-SIMON, F., GRZEBYK, D., SCHOFIELD, O.andFALKOWSKI, P. G. (2005). The role and evolution of superoxide dismutases in algae 1. *Journal of Phycology,* 41, 453-465.

WU, J., XIN, Y., KONG, J.andGUO, T. (2021). Genetic tools for the development of recombinant lactic acid bacteria. *Microbial Cell Factories,* 20, 118.

WU, Z., LIANG, X., LI, M., MA, M., ZHENG, Q., LI, D., et al. (2023). Advances in the optimization of central carbon metabolism in metabolic engineering. *Microbial Cell Factories,* 22, 76. doi: 10.1186/s12934-023-02090-6

XIN, Y., GUO, T., MU, Y.andKONG, J. (2017). Development of a counterselectable seamless mutagenesis system in lactic acid bacteria. *Microbial Cell Factories,* 16, 1-13.

XU, Z.andHAO, B. (2009). CVTree update: a newly designed phylogenetic study platform using composition vectors and whole genomes. *Nucleic acids research,* 37, W174-W178. doi: 10.1093/nar/gkp278

YAMAMURA, R., INOUE, K. Y., NISHINO, K.andYAMASAKI, S. (2023). Intestinal and fecal pH in human health. *Frontiers in Microbiomes,* 2, 1192316. doi: 10.3389/frmbi.2023.1192316

YASUI, K.andBABA, A. (2006). Therapeutic potential of superoxide dismutase (SOD) for resolution of inflammation. *Inflammation Research,* 55, 359-363. doi: 10.1007/s00011-006-5195-y

YU, Y., WANG, Y., TONG, Q., LIU, X., SU, F., QUAN, F., et al. (2013a). A site-specific recombinase-based method to produce antibiotic selectable marker free transgenic cattle. *PloS one,* 8, e62457.

YU, Z., ZHANG, X., LI, S., LI, C., LI, D.andYANG, Z. (2013b). Evaluation of probiotic properties of *Lactobacillus plantarum* strains isolated from

Chinese sauerkraut. ***World Journal of Microbiology and biotechnology,*** **29, 489-498. doi: 10.1007/s11274-012-1202-3**

ZHANG, Z., LV, J., PAN, L.andZHANG, Y. (2018). Roles and applications of probiotic ***Lactobacillus*** **strains.** ***Applied microbiology and biotechnology,*** **102, 8135-8143.**

ZHENG, Y., LU, Y., WANG, J., YANG, L., PAN, C.andHUANG, Y. (2013). Probiotic properties of Lactobacillus strains isolated from Tibetan kefir grains. ***PloS one,*** **8, e69868. doi:10.1371/journal.pone.0069868**

ZUO, G.andHAO, B. (2015). CVTree3 web server for whole-genome-based and alignment-free prokaryotic phylogeny and taxonomy. ***Genomics, Proteomics and Bioinformatics,*** **13, 321-331. doi: 10.1016/j.gpb.2015.08.004**

국문 초록

유산균의 유전체 및 기능 분석과 고효율 프로모터를 활용한 항산화 능력 향상의 분자생물학적 접근

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본 연구는 *Lacticaseibacillus paracasei* EG005의 프로바이오틱스 잠재력과 향상된 항산화 특성을 조사하였으며, 항산화 효소의 발현을 증진시켜 산화 스트레스를 줄이는 역할에 중점을 두었다. 연구는 EG005의 건강 증진 및 생명공학적 응용 가능성을 다룬 내용으로 구성되어 있다. 이론 및 실험적 연구 수행을 모두 탐구하여 EG005만의 독특한 기능과 효과적인 프로바이오틱스 역할에 대한 포괄적인 이해를 제공하고자 한다.

첫 번째 챕터에서는 *Lactobacillus* 속이 프로바이오틱스로서 가지는

역할에 대한 배경을 제공하기 위해 문헌 조사를 수행하였다. *Lactobacillus* 속은 면역 반응 조절, 장벽 강화, 항균 물질과 유산을 생산하여 병원성 미생물의 억제 등 장 건강에 유익한 효과로 잘 알려져 있다. 이 장에서는 *Lactobacillus*가 장내 미생물 균형 유지와 전반적인 건강 증진에 중요한 역할을 한다는 점을 강조하였다. 또한, 프로바이오틱스의 유전적 및 기능적 특성을 분석하기 위한 생물정보학 도구의 중요성을 강조하여 향상된 건강 효과를 가진 균주의 선택과 최적화에 도움을 줄 수 있음을 설명하였다. 유전체 시퀀싱과 유전자 기능 규명은 항산화 효소인 초산화물 불균등화효소(SOD)를 포함한 기능적 유전자를 확인하기 위한 핵심 기술로 논의되었다. 더불어, 특정 단백질 활성 증진에 기여하는 과발현 기술의 중요성도 언급되었으며, 특히 Ptuf와 Pldh와 같은 프로모터가 높은 수준의 목표 유전자 발현을 유도하는 능력을 가지는 유용한 도구로서 *Lactobacillus* 균주의 프로바이오틱스 특성을 향상시키는 데 가치가 있음을 설명하였다.

두 번째 챕터에서는 *L. paracasei* EG005의 항산화 능력에 대한 실험적 연구를 다루었으며, 특히 유해한 활성 산소종(ROS)을 중화하는 데 중요한 역할을 하는 SOD의 활성에 중점을 두었다. 연구는 EG005의 기본적인 프로바이오틱스 특성 평가부터 시작하였으며, 여기에는 위장관에서의 생존과 활성을 위해 필수적인 산 및 담즙 내성이 포함되었다. 균주는 소화 시스템을 통과하면서 생존력을 유지하는 데 중요한 산과 담즙 내성을 보여주었다. 또한, 안전성 평가를 통해 EG005가 용혈 활성과 항생제 내성 유전자를 가지지 않음을 확인하여 안전하고 효과적인 프로바이오틱스로서의 적합성을 강조하였다.

EG005의 항산화 특성을 강화하기 위해 유전자 공학 기술이 적용되었다. Ptuf와 Pldh와 같은 강력한 프로모터가 SOD 유전자의 과발현을 유도하기 위해 사용되었으며, 이를 통해 산화 스트레스에 대항하는 균주의 능력을 높이고자 하였다. 특히 Ptuf 프로모터는 대조군에 비해 SOD 활성을

두 배로 증가시켜 항산화 효소 생산을 증진하는 데 효과적임을 강조하였다. 이러한 SOD 활성 증진은 산화 스트레스를 줄이고 장 건강을 유지하며 염증 관련 질환의 위험을 낮추는 데 중요한 의미가 있다. Pldh 프로모터 또한 유망한 결과를 보였으나, 그 효과는 Ptuf에 비해 다소 덜 두드러졌다. 이러한 결과는 프로모터 선택이 유전자 발현 수준과 결과적으로 프로바이오틱 균주의 기능적 이점을 결정하는 데 중요한 역할을 한다는 점을 시사한다. 향후 연구는 이러한 결과를 확인하고 EG005의 효과를 평가하기 위해 *in vivo* 실험에 중점을 두어야 한다. 동물 모델이나 인간을 대상으로 한 *in vivo* 실험은 유산균이 장내 미생물 구성, 면역 조절, 그리고 체내 항산화 활성에 미치는 영향을 확인하는 데 중요한 데이터를 제공할 것이다.

이 연구 논문들은 *L. paracasei* EG005의 프로바이오틱 잠재력과 항산화 방어력 증진에 대한 이해를 높이는 데 기여할 것이다. 생물정보학 분석과 유전공학 접근법의 접목은 EG005의 기능적 특성과 치료 및 기능성 식품으로서의 적합성에 대한 깊은 이해를 가능하게 하였다. 본 연구는 EG005의 산화 스트레스 완화, 염증성 장 질환 감소, 소화기 건강 증진과 같은 효과를 위한 건강 촉진 제품에서의 활용을 위한 향후 연구의 기초를 형성한다.

주요어: 유산균, 초산화물 불균등화효소, 항산화 활성, SOD 과발현, 프로모터

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