

Development of a yeast-based platform for bacteriophage applications

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Development of a yeast-based platform for bacteriophage applications

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and

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Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

O aumento global da resistência aos antibióticos evidencia a necessidade urgente de alternativas eficazes, particularmente contra patógenos gastrointestinais como *Listeria monocytogenes*, *Staphylococcus aureus* e *Clostridioides difficile*. Entre as soluções emergentes, as endolisinas de bacteriófagos destacam-se pela elevada especificidade e pelo reduzido impacto sobre a microbiota comensal. As leveduras, em especial a probiótica *Saccharomyces boulardii*, constituem chassis atrativos para a produção e entrega de endolisinas, combinando segurança, sobrevivência no trato gastrointestinal, benefícios probióticos e potencial para engenharia genética. Nesta tese, ferramentas de CRISPR-Cas9 foram aplicadas para modificar geneticamente estirpes de *S. cerevisiae* e *S. boulardii* visando a integração cromossómica e expressão da endolisina anti-*Listeria* Ply511.

Em *S. cerevisiae*, a secreção de Ply511 revelou atividade antimicrobiana eficaz contra *L. monocytogenes*, inclusive em matrizes alimentares, ao passo que a sua exibição na superfície não resultou em redução do patógeno. A extensão desta abordagem a *S. boulardii* demonstrou a capacidade da levedura probiótica de secretar Ply511 com atividade detetável, ainda que inferior a observada em *S. cerevisiae*. As estirpes modificadas mantiveram efeitos antimicrobianos após digestão gastrointestinal simulada e reduziram *L. monocytogenes* numa comunidade definida SIHUMI, porém falharam em controlar *L. monocytogenes* numa simulação colónica *in vitro* com fezes humanas. A plataforma foi ainda explorada para outros patogénicos Gram-positivos, através da engenharia de *S. boulardii* para expressar LysSA11, direcionada a *S. aureus*, e CD27L(1-179), direcionada a *C. difficile*. A ausência de atividade destas endolisinas evidenciou a complexidade da secreção e da estabilidade, revelando variações dependentes da própria endolisina e do desenho dos peptídos sinal.

Em conjunto, este trabalho demonstra a viabilidade da engenharia de leveduras, incluindo a probiótica *S. boulardii*, para a produção e entrega de endolisinas derivadas de fagos no trato gastrointestinal. Apesar de persistirem desafios relacionados com a eficiência de secreção e a otimização das estirpes, os resultados apresentados estabelecem as bases para o desenvolvimento de sistemas probióticos produtores de antimicrobianos de precisão, contribuindo para alternativas inovadoras aos antibióticos baseadas em fagos.

Palavras-chave: CRISPR-Cas9; endolisina; *Listeria monocytogenes*; probióticos*; Saccharomyces boulardii*.

Abstract

The global rise of antimicrobial resistance highlights the urgent need for alternatives to antibiotics, particularly against gastrointestinal pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridioides difficile*. Bacteriophage endolysins have emerged as promising candidates due to their high specificity and minimal impact on the commensal microbiota. Yeast, and particularly the probiotic *Saccharomyces boulardii*, offers an attractive chassis for endolysin production and delivery, combining safety, survival in the gastrointestinal tract, probiotic benefits, and amenability to genetic engineering. In this thesis, CRISPR-Cas9 tools were applied to engineer *S. cerevisiae* and *S. boulardii* strains for the chromosomal integration and expression of the anti-*Listeria* endolysin Ply511.

In *S. cerevisiae*, secretion of Ply511 proved effective against *L. monocytogenes*, maintaining activity in food matrices, whereas surface display did not result in pathogen reduction. Extending this approach to *S. boulardii* demonstrated that the probiotic yeast can secrete Ply511 with detectable activity, although at lower levels than *S. cerevisiae*. Engineered strains retained antimicrobial activity after simulated gastrointestinal digestion and reduced *L. monocytogenes* in a defined SIHUMI community; however, failed to control *L. monocytogenes* in an *in vitro* colonic simulation with human feces. The creation of a platform was further explored against other clinically relevant Gram-positive pathogens by engineering *S. boulardii* to express LysSA11, targeting *S. aureus*, and CD27L(1-179), targeting *C. difficile*. The lack of detectable activity of these endolysins highlighted the complexity of secretion and stability, with outcomes depending on both the endolysin and the signal peptide design.

Altogether, this work demonstrates the feasibility of engineering yeast, including probiotic *S. boulardii*, for the production and delivery of phage-derived endolysins to the gastrointestinal tract. While secretion efficiency and strain optimization remain key challenges, these findings establish a foundation for probiotic-based systems delivering precision antimicrobials and contribute to the development of phage-based alternatives to antibiotics.

Keywords: CRISPR-Cas9; endolysin; *Listeria monocytogenes*; probiotics*; Saccharomyces boulardii*.

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List of abbreviations and acronyms

ANP	Atrial Natriuretic Peptide	**IRES**	Internal Ribosome Entry Site
AP	Alkaline Phosphatase	**LAB**	Lactic Acid Bacteria
ARS	Autonomously Replicating Sequence	**lacZ**	β-galactosidase
ATP	Adenosine Triphosphate	**LiAc**	Lithium Acetate
BP	Base Pairs	**mRNA**	Messenger Ribonucleic Acid
Cas9	CRISPR associated protein 9	**mSA**	Monomeric Streptavidin A
CBD	Cell Binding Domain	**NPA**	*Clostridium difficile* Toxin A Neutralising Peptide
CDI	Clostridium difficile Infection	**OD600**	Optical Density
CEN	Centromeric	**OVA-CPE**	Chicken Ovalbumin and *Clostridium perfringens* Enterotoxin
CFU	Colony Forming Unity	**PAM**	Protospacer Adjacent Motif
CHAP	Cysteine Histidine-dependent Amidohydrolase/Peptidase	**PCR**	Polymerase Chain Reaction
Chr	Chromosome	**PEG**	Polyethylene Glycol
CRISPR	Clustered Regularly Interspaced Palindromic Repeats	**RACE**	Rapid Amplification of cDNA Ends
DNA	Deoxyribonucleic Acid	**RNA Pol II**	RNA Polymerase II
EMCV	Encephalomyocarditis Virus	**SCFA**	Short Chain Fatty Acids
ER	Endoplasmic Reticulum	**SpCas9**	*Streptococcus pyogenes* Cas9
ereA	Erythromycin Destructase	**T7 RNAP**	T7 RNA Polymerase

FDA	US Food and Drug Administration	**V5-tag**	Epitope Tag from Simian Virus 5
GFP	Green Fluorescent Protein	**VBNC**	Viable but Non-culturable Cells
GI	Gastrointestinal	**WT**	Wild-type
GIT	Gastrointestinal Tract	**YCp**	Centromeric Plasmids
GRAS	Generally Regarded as Safe	**yEGFP**	Yeast-enhanced Green Fluorescent Protein
HA	Hemagglutinin	**YEp**	Yeast Episomal Plasmid
HCV	Hepatitis C Virus	**YIp**	Yeast-integrating Plasmids
HRV3C	Human Rhinovirus 3C protease	**YPD**	Yeast Extract, Peptone, Dextrose
IL-10	Interleukin 10	**YRp**	Yeast-replicating Plasmids
IMAC	Immobilized Metal Affinity Chromatography		

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Scientific output

According to the 2nd paragraph of article 8 of the Portuguese Decree-Law no. 388/70, the scientific outputs of this thesis are listed below. The results presented in this thesis have been partially published elsewhere.

Peer-reviewed journal articles:

- **Moreno DS,** Qimron U, Azeredo J, Domingues L. Towards T7 RNA polymerase (T7RNAP)-based expression system in yeast: challenges and opportunities. Bioengineered. 2022 Jul-Dec;13(7-12):14947-14959. doi: 10.1080/21655979.2023.2180579. PMID: 37105766; PMCID: PMC10234217.

- Carvalho JP, **Moreno DS**, Domingues L. Genetic engineering of *Saccharomyces boulardii*: Tools, strategies and advances for enhanced probiotic and therapeutic applications. Biotechnol Adv. 2025 Jul 30;84:108663. doi: 10.1016/j.biotechadv.2025.108663. PMID: 40750061.

- **Moreno DS**, Cunha J, de Melo LDR, Tanaka K, Bamba T, Hasunuma T, Azeredo J, Domingues L. CRISPR-Cas9 engineered Saccharomyces cerevisiae for endolysin delivery to combat Listeria monocytogenes. Appl Microbiol Biotechnol. 2025 Apr 2;109(1):81. doi: 10.1007/s00253-025-13464-8. PMID: 40175837; PMCID: PMC11965161.

- **Moreno DS**, Carvalho, JP, Murray E., Rios, NS, Lamas, A, Cobas, AC, Hill, C, Azeredo J, Domingues L. Evaluation of the delivery of an anti-*Listeri*a endolysin via CRISPR-Cas9 engineered probiotic *Saccharomyces boulardii* , **Under review.**

Posters in conferences:

- **Congress of Microbiology & Biotechnology 2023** (Poster), Covilhã, Portugal – *Yeast surface display of Ply511 against* L. monocytogenes.

- **XXIX Spanish Society of Microbiology Congress** (Poster), Burgos, Spain – *Endolysin display activity of recombinant yeast*

Oral communications in conferences:

- **III Fagoma Meeting** (Oral), Valencia, Spain – *CRISPR-Cas9 engineering of yeast to deliver Ply511 against* L. monocytogenes.

- **XXII Jornadas de Leveduras** (Flash), Curia, Portugal – *CRISPR-Cas9 edited yeast to reduce* L. monocytogenes.

- **IMFAHE International Conference 2024** (Oral, Online) – *Yeast surface display & secretion of Ply511 against* L. monocytogenes.

- **FoodMicro 2024** (Flash), Burgos, Spain – *Yeast surface display of Ply511 against* L. monocytogenes

Thesis outline

The research activities resulting in this doctoral thesis were mainly developed at CEB – Centre of Biological Engineering, University of Minho (Braga, Portugal) under the supervision of Professor Lucília Domingues and Professor Joana Azeredo. Part of this work was developed at the EGBCR - Engineering Biology Research Centre in Kobe University (Kobe, Japan) under the supervision of Prof. Tomohisa Hasunuma, at APC Microbiome Ireland in University College Cork (Cork, Ireland) under the supervision of Prof. Colin Hill and at the University of Santiago de Compostela under the supervision of Prof. Alejandra Cardelle. Part of this work , particularly the tasks related to genomic analysis of *S. boulardii* and evaluation of insertion sites were carried out as part of MSc Thesis of J.P. Carvalho. This doctoral thesis is divided into five chapters, three of them describing experimental research published or in preparation for submission to international peer-reviewed journals.

Chapter I (Introduction: state of the art) encompasses a comprehensive review of the development of a yeast-based platform for phage applications. This chapter aims to introduce the antibiotic resistance crisis, with a focus on the need for alternatives to antibiotics in the context of gastrointestinal bacterial pathogens. Phages and their endolysins are presented as promising tools for the control of these infections. In this context, we explore the potential of yeasts as a platform for producing phages and phage-derived antimicrobials. Initially, yeast is considered as a production host for phages, but its limitations led us to investigate a second application: using probiotic yeast as a vehicle for delivering endolysins directly to the gastrointestinal tract.

Chapter II (CRISPR Cas9 engineered *Saccharomyces cerevisiae* for endolysin delivery to combat *Listeria monocytogenes*) focuses on the engineering of *S. cerevisiae* strains, using CRISPR-Cas9, to express Ply511, an anti-*Listeria* endolysin. We prove that the surface display of the protein does not lead to a reduction in *Listeria* numbers and we demonstrate that secreting the endolysin to the extracellular media, did lead to a reduction in Listeria, using the whole cells, the supernatant and the cell extracts. Part of the tasks related to surface display were performed in the EGBCR - Engineering Biology Research Centre in Kobe University (Kobe, Japan) under the supervision of Prof. Tomohisa Hasunuma.

Chapter III (Evaluation of the delivery of an anti-*Listeria* endolysin via CRISPR-Cas9 engineered probiotic *Saccharomyces boulardii)* is dedicated to our efforts towards creating a platform for endolysin delivery, engineering *S. boulardii* strains, using the CRISPR-Cas9 tools used to engineer *S. cerevisiae*. We demonstrate the effects of the delivery of the endolysin Ply511 into the human gut and its microbiota. We show the activity of the yeast extract in a defined bacterial consortium (SIHUMI) and evaluated the delivery of Ply511 under gastrointestinal conditions, and its effect in two different scenarios, as whole-cell and as yeast-extract, studying its impact against Listeria against different simulations resembling the human gut environment. Part of the tasks described in this chapter were performed by JP Carvalho under my co-supervision and by myself but at APC Microbiome Ireland in University College Cork (Cork, Ireland) under the supervision of Prof. Colin Hill and part at the University of Santiago de Compostela under the supervision of Prof. Alejandra Cardelle.

Chapter IV (Exploring the use of yeast as a platform for the delivery of endolysins) is dedicated to our efforts towards creating a platform for endolysin delivery, engineering *S. boulardii* strains, using the CRISPR-Cas9 tools previously optimized, to engineer *S. boulardii* strains to express anti- *S. aureus*

LysSA11 and anti-*C. difficile* CD27. The tasks described in this chapter related to *C. difficile* testing were performed at APC Microbiome Ireland in University College Cork (Cork, Ireland) under the supervision of Prof. Colin Hill and those related to simulated intestinal fermentations were performed at the University of Santiago de Compostela under the supervision of Prof. Alejandra Cardelle.

Chapter V offers the major conclusions from this doctoral project and highlights the next steps of this work, emphasising its contribution to the development of phage-based alternatives to antibiotics using genetically engineered probiotics.

Chapter I.

Introduction: state of the art

This chapter aims to introduce the antibiotic resistance crisis, with a focus on the need for alternatives to antibiotics in the context of gastrointestinal bacterial pathogens. Phages and their endolysins are presented as promising tools for the control of these infections. In this context, we explore the potential of yeasts as a platform for producing phages and phage-derived antimicrobials. Initially, yeast is considered as a production host for phages, but its limitations led us to investigate a second application: using probiotic yeast as a vehicle for delivering endolysins directly to the gastrointestinal tract.

This chapter is based on the following review articles:

- **Moreno DS**, Qimron U, Azeredo J, Domingues L. Towards T7 RNA polymerase (T7RNAP)-based expression system in yeast: challenges and opportunities. Bioengineered. 2022 Jul-Dec;13(7-12):14947-14959. doi: 10.1080/21655979.2023.2180579. PMID: 37105766; PMCID: PMC10234217.

- Carvalho JP, **Moreno DS**, Domingues L. Genetic engineering of *Saccharomyces boulardii*: Tools, strategies and advances for enhanced probiotic and therapeutic applications. Biotechnol Adv. 2025 Jul 30;84:108663. doi: 10.1016/j.biotechadv.2025.108663. PMID: 40750061.

1.1. The Pressing need for alternatives to antibiotics. Scope and consequences.

Since the introduction of antibiotics in 1910 they have been used as the standard of care of many different infections because of their efficacy and broad spectrum of action [1]. Therefore, antibiotics have been utilized for the treatment of human bacterial infections, but also diseases in crops and animals [2]. Naturally arising from these treatments are bacteria that randomly survive the antibiotic treatment, are able to reproduce and pass on their resistance traits to the next generation. After several cycles of this selective pressure, this can lead to a resistant population: evolution that would normally occur, but which has been fast forwarded due to the wide use, together with the sometimes misuse and abuse. An example of this misuse is the use of sub-clinical doses of antibiotics for growth enhancement in animals. A practice that has been banned completely in 2006 in Europe (European Commission, 2006) and slowly followed by other countries in the world, such as USA (medically important antibiotics, banned in 2017) or China in 2020 [4]. Another example is the misuse in humans, for example taking or prescribing antibiotics for viral infections [5]; today in Europe, pharmacies are not allowed to sell antibiotics without a prescription.

Some measures have therefore been implemented to release some of the selective pressure which favours antibiotic resistance, but these measures have not been long in place and are difficult to ensure globally, which means we need further actions to stop the antibiotic resistance crisis or other ways of treatment against bacterial infections.

The consequences of the antibiotic resistance crisis are often described in human deaths, which in 2019 accounted for almost 5M, and are predicted to rise year by year [6]. Its impact, however, extends well beyond human health, threatening crop production, animal agriculture, food security, and the stability of entire ecosystems, as recognized within the One Health framework.

Another factor aggravating this crisis is the prevailing economic model in Western countries, and indeed in most parts of the world, under which anti-bacterials face what is often described as a “dry pipeline”. Since the early 2000s, the development of new antibiotics has steadily declined, and since 2010, fewer than 15 antibiotics have been approved globally [7]. Moreover, the most recent approvals are typically reserved as “last resort” antibiotics, used only when other treatments fail. Consequently, sales volumes remain low, making

them unprofitable for developers, despite the high and risky investments required, a situation that is unlikely to be sustainable. This economic reality partly explains why Bayer has withdrawn from producing anti-infectives [8].

Climate change is one of the most pressing challenges facing humanity and is likely also exacerbating the antibiotic resistance crisis, a link that has been increasingly recognized. Among other factors, this connection can be explained by the fact that rising temperatures promote the proliferation of microorganisms, including bacteria [9],[10].

Antibiotic resistance is shaped not only on a global scale but also by individual behaviours. Collective awareness is essential to improve education on this issue. Interestingly, higher dietary fibre intake and greater dietary diversity are associated with a lower abundance of antibiotic resistance genes in healthy adults. In contrast, higher consumption of animal protein and saturated fats, common in many modern diets, correlates with an increased abundance of these genes [11]. These findings highlight the role that individual behaviours play in driving this crisis. To address the antibiotic resistance crisis, only a few realistic strategies remain: (i) significant governmental investment to stimulate pharmaceutical interest in antibiotic development, (ii) structural reforms to healthcare systems, (iii) broader societal transformation in how we approach infection control and education, and (iv) the development of alternative antimicrobial agents. The work developed within the scope of this thesis is focused on efforts to tackle antibiotic resistance by the development of Yeast-Based platforms to efficiently produce alternative antimicrobial agents, such as bacteriophage's endolysins, a promising antimicrobial agent derived from the natural enemies of bacteria, bacteriophages.

1.2. Gastrointestinal bacterial pathogens.

The gastrointestinal tract (GIT) is a key interface between the human body and the environment. The human gut hosts a diverse community of microorganisms, including bacteria, viruses, fungi and protozoa. A healthy gut microbiome supports digestion and nutrient adsorption, synthesizes vitamins, regulates the immune system and protects against invading pathogens. Disruptions to this microbial balance have been linked to a wide range of diseases, including obesity, diabetes, cardiovascular conditions, gastrointestinal disorders and mental health issues [12]. As such, gut health is widely recognized as central to overall human health. However, the gut also serves as the primary entry point of different bacterial pathogens, which can invade the GIT

through the ingestion of contaminated food or water. Despite protective barriers like gastric acid, antimicrobial peptides, and competition with the resident microbes, some bacteria can survive and cause infection. This can occur particularly in vulnerable individuals, with weakened immune systems, when the GIT does not function properly, or when the gut microbiota is disrupted (dysbiosis) - for example, by antibiotic use – and when the pathogen levels are higher than what our defenses can handle.

Several bacterial pathogens—such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridioides difficile*—can reach or colonize the gut through contaminated food ingestion or environmental exposure. These pathogens have been associated with gastrointestinal infections that range from mild symptoms to severe outcomes, such as systemic listeriosis, sepsis and colitis, respectively. This thesis focuses on these three gut-associated pathogens since they all are candidates for putative endolysin interventions.

1.2.1. *Listeria monocytogenes*.

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, a disease producing a range of symptoms from febrile gastroenteritis to meningitis, often leading to fatality [13],[14]. The overall case-fatality rate in high-risk groups can reach 20–30%, making *L. monocytogenes* one of the deadliest foodborne pathogens.

Upon consumption of contaminated food, the bacterium is able to survive the different gastrointestinal (GI) stressors such us mechanical digestion, saliva, the low stomachal pH, enzymes and bile acids [15],[16]. Once in the intestine it competes with the commensal flora. To initiate its infection cycle, it must survive these interactions and translocate across the GI barrier, reaching the bloodstream [17]. Its ability to cross epithelial barriers (not only intestinal, but also placental and blood-brain) is central to its virulence, which is facilitated by virulence factors like internalins, listeriolysin O, and actin-based motility proteins.

Though it is generally considered a transient colonizer rather than a permanent resident[15], *L. monocytogenes* has been found in human faeces, something which indicates that, apart from its transient colonization of the intestine for the initiation of infection, it can also reside for long periods of time in the gut [18]. In fact, its presence has been reported in approximately 10% of healthy, asymptomatic individuals, and its detection has also been documented in oral, sputum, skin, and even gall bladder samples [18]. The abundance of *L. monocytogenes* in human faecal samples has been correlated with a low α-diversity in humans, indicating the importance of a varied microbiome for protection [18]. In fact, antibiotic treatment has been shown to

decrease α-diversity in the gut microbiota [19], which combined with the worldwide appearance of antibiotic-resistant clones of *L. monocytogenes* [20],[21] highlights the need for development of alternatives to antibiotics for the treatment of *L. monocytogenes* infections.

Even relatively low doses of *L. monocytogenes* can cause infection. In an ice cream-associated outbreak, the estimated number of viable cells per contaminated serving ranged from as few as 55 CFU to over 11,000 CFU, with infections occurring in individuals consuming mean doses of just 310–620 CFU [22]. This highlights the pathogen's ability to initiate infection from modest contamination levels, especially in susceptible hosts.

L. monocytogenes has been associated with contamination of dairy products, particularly soft cheeses and unpasteurized milk, which have been implicated in numerous foodborne outbreaks ([23]. The fact that *L. monocytogenes* is able to grow at refrigeration temperatures and tolerate low pH [24] gives it a competitive advantage during the early stages of cheese ripening. During this early period the pH is lower and yeast dominate, consuming lactic acid and leading to a rise in pH. The production of protective bacteriocins and other antimicrobial compounds by cheese-ripening bacteria often depends on this increase in pH, from around 5.0 to 6.0 [24]. Thus, *L. monocytogenes*—being more pH-tolerant than many beneficial bacteria—can grow and establish itself early in the production process. In this regard, a fast-acting molecule or an engineered yeast capable of inhibiting the pathogen at these early stages, thereby preventing its multiplication, could be highly valuable for the prevention of listeriosis.

1.2.2. *Clostridioides difficile*

Clostridioides difficile (formerly known as *Clostridium difficile*) is a Gram-positive, spore-forming anaerobe and a leading cause of nosocomial gastrointestinal infections. It causes *C. difficile* infection (CDI), its symptoms range from mild diarrhea to severe colitis. CDI is often recurrent and is associated with the prior use of antibiotics, which cause the disruption of the gut microbiota allowing *C. difficile* colonization and toxin production [25],[26].

In the United States alone, over 500,000 cases of CDI are estimated to occur each year [27],[28]. Although the initial infection can be treated with targeted antibiotics, this approach commonly leads to recurrence—20–30% of patients experience at least one relapse, and risk increases with each subsequent episode [26] . The main barrier against *C. difficile* posed by the human GIT is known as colonization resistance and is mediated

by a healthy gut microbiome. This helps explain why antibiotic treatment and its posterior induced dysbiosis weakens this protective barrier, allowing the infection and/or recurrence. The main contamination route of *C. difficile* is through spores, which are acquired via fecal-oral transmission or from contaminated surfaces in healthcare settings. Once they germinate and the bacterium establishes, it releases toxins (TcdA and TcdB) which can damage the intestinal epithelium, leading to inflammation, diarrhea, and in severe cases, pseudomembranous colitis.

Alternatives to antibiotics, such as fecal microbiota transplantation and probiotics such as *Saccharomyces boulardii* have shown efficacy for the prevention and treatment of CDI [26]. In fact, engineered probiotic strategies that help restore microbiota balance and deliver targeted anti-*C. difficile* molecules, may be promising alternatives to traditional antibiotics in the control of CDI.

1.2.3. *Staphylococcus aureus*

Although *Staphylococcus aureus* is most often associated with skin, soft tissue, prosthetic-joint infections and other biofilm-related infections, it has been found to colonize the GIT, particularly in hospitalized or immunocompromised individuals. While the anterior nares in the nose are considered the primary reservoir for *S. aureus*, the gut is recognized as a persistent site of colonization, and a potential source for transference to other body niches [29]–[31]. In fact, faecal carriage rates of *S. aureus* have been reported in approximately 20% of healthy adults, with typical levels reaching 10^6 CFU per 100 g of faeces, surpassing nasal carriage levels [31],[32]. The highest bacterial burdens are found in the distal gastrointestinal tract, such as the cecum and colon [33].

S. aureus gut colonization is concerning because carriers, even when asymptomatic, face a higher risk of developing infections, especially under conditions such as immunosuppression. In this regard, there is evidence suggesting that probiotic interventions can suppress *S. aureus* gut colonization through mechanisms related to the inhibition of quorum-sensing, i.e. not related to direct killing. For instance, oral administration of *Bacillus subtilis* spores in humans led to a significant reduction in *S. aureus* levels in both the gut and nose, without disrupting beneficial microbes [33]. This suppression was attributed to the probiotic's secretion of fengycin lipopeptides, which interfere with *S. aureus* quorum-sensing pathways and prevent colonization.

Although *S. aureus* has not been traditionally viewed as an enteric pathogen, *S. aureus* colonization of the GI tract can be a target of interest for probiotic interventions, that might help restore a healthy microbiota balance and prevent the establishment of the pathogen.

1.3. Phage-based antibacterial therapies

Bacteriophages, or phages**,** are viruses of bacteria. Lytic bacteriophages infect and kill their bacterial host as part of their reproductive cycle. Once inside the host bacterium, phages hijack their cellular machinery to replicate and cause bacterial lysis, releasing progeny phages that can continue the infection cycle. Over the past decade, the interest in phages as alternatives or adjuvants to antibiotics has increased in light of the antimicrobial resistance crisis.

Several characteristics of phages make them attractive as antibacterial agents: specificity [34], activity against the bacterial biofilms [35], propagation at the site of infection and activity against antimicrobial-resistant strains [36]–[39]. Contrary to antibiotics, phages are highly specific and infect only within the species range, something that enables to selectively target pathogenic bacteria while preserving the host microbiota.

Phages are being actively investigated in clinical applications and have shown positive results in compassionate use cases and early clinical studies against different bacterial pathogens [40] known as phage therapy. Due to the relatively newness of phage therapy and the expensive and long path for approval, only a few clinical trials have been or are being completed. Meanwhile, some countries (such as USA, Belgium, France, Australia, Portugal) have opened up to the personalized approach and allowed the compassionate use of phages [41]. In other countries like Georgia and Poland phages have been routinely used for decades [42],[43].

1.4. Endolysins: mode of action and therapeutic potential

After bacteriophage infection and virion production inside of the bacterial cell, at the end of the phage lytic cycle, the newly formed virions degrade the peptidoglycan of the bacterial host from within, lysing the cells and being released to the exterior (Figure 1.1). The phage enzymes responsible of cleaving the peptidoglycan (peptidoglycan hydrolases) are termed lysins. They have gained attention in the recent years for their potential as antimicrobials when applied exogenously as recombinant proteins [44]–[46]. Their main advantages over

antibiotics are not only the retention of the high specificity of phage infectivity but also the low propensity of bacterial resistance [45],[47].

The use of whole phages does present regulatory and biological challenges, including limited host range, potential for horizontal gene transfer, and difficulty in standardization—challenges that endolysins could potentially circumvent, since they can be treated as small antimicrobial molecules and are subject to engineering towards different targets.

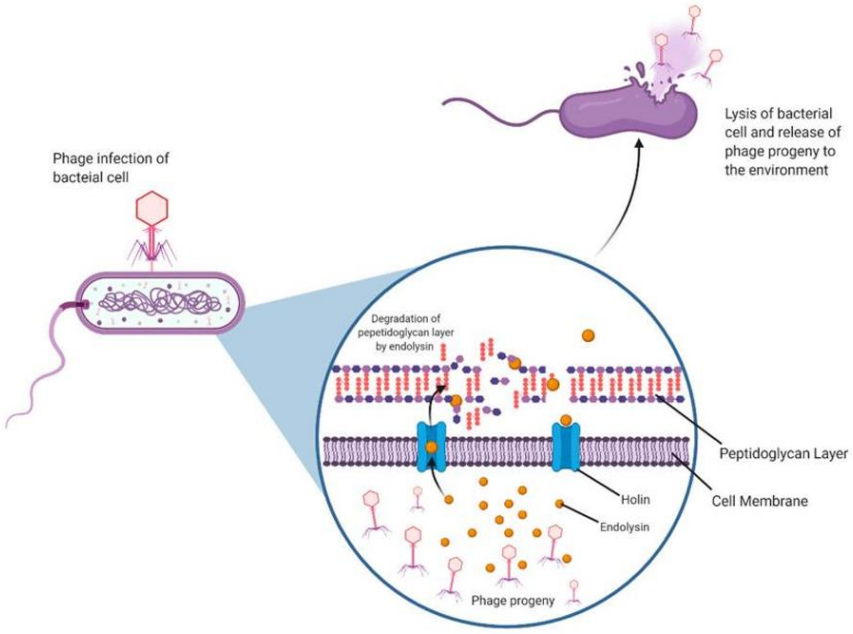


Figure 1.1. Lysis of bacterial cell due to the peptidoglycan degradation by a phage endolysin (from Murray et al, 2021, "© 2021 by the authors; licensed under CC BY 4.0, unmodified.

At a protein level, the structure of the endolysins differs between those targeting Gram-negative and Gram-positive bacteria, given the differences in cell-wall architecture of the two groups [45]. Generally, endolysins of Gram-positive bacteria consist of a modular structure, comprising a functional domain for cell wall recognition at the C-terminus (Cell Binding Domain, CBD) and a catalytic domain at the N-terminus (Enzymatically Active Domain, EADs). In phages of Gram-negative bacteria, in contrast, these endolysins are small single-domain proteins, acting as EAD. However, the architecture is not limited to these structures, and many exceptions to these rules have been described [48]. One example of the modular structure is LysK endolysin, consisting of 2

enzymatically active domains (CHAP and amidase-2) and one bacterial SH3 (SH3b) cell wall-binding domain, as depicted in figure 1.2 [49].

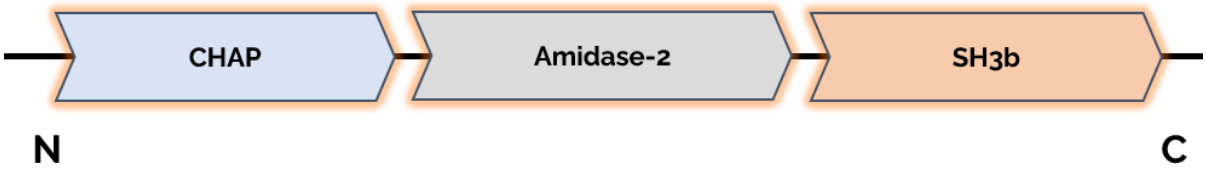


Figure 1.2. Modular structure of the LysK endolysin, consisting of 2 enzymatically active domains (CHAP and amidase-2) and one cell wall binding domain (SH3b), reproduced according to Kashani et al 2017. N: N-terminal, C: C-terminal.

Protein engineering has been effectively applied for improving endolysins, mainly to increase their lytic activity or reduce their immunogenicity. The techniques followed to achieve this improvement were domain recombination, site-directed mutagenesis, directed evolution and fusion to cationic peptides or outer membrane permeabilizers [45],[50],[51]. The latter was used to increase the chances of targeting Gram-negative bacteria, since the cell wall is surrounded by an outer membrane (not present in Gram-positive bacteria) which prevents the endolysin to reach the peptidoglycan when it is applied from the outside of the cell. In the scope of this thesis, however, only Gram-positive targets were explored.

Their modular architecture, with separate catalytic and binding domains, allows for high specificity toward target species without affecting beneficial commensals. This microbiome-sparing property is particularly valuable in the context of the previously described gut pathogens, where loss of microbial diversity is known to increase susceptibility to infection. As highlighted in previous sections, disruptions to the gut microbiome—especially due to antibiotics—are central to the pathogenesis and recurrence of certain infections. Therefore, therapies that eliminate pathogens without disturbing the broader microbial ecosystem are highly desirable.

Endolysins also show low propensity for resistance development, often retain activity under harsh conditions, and can be engineered to improve therapeutic properties. In this thesis, we focus on three relevant endolysins—Ply511 (against *L. monocytogenes*), CD27L (against *C. difficile*), and LysSA11 (against *S. aureus*)—for their proven specificity and lytic activity.

1.5. Characterization and potential of selected endolysins

Throughout this thesis, we focused on three endolysins: Ply511, CD27L, and LysSA11—targeting *Listeria monocytogenes*, *Clostridioides difficile*, and *Staphylococcus aureus*, respectively.Ply511 is an N-acetylmuramoyl-L-alanine amidase originally derived from *Listeria* bacteriophage A511 [52]. The endolysin recognizes and lyses all tested *L. monocytogenes* serovars, despite structural differences in wall teichoic acids. Ply511´s cell binding domain (CBD), exhibits high specificity and has been shown not to bind to non-target Gram-positive and Gram-negative bacteria [53]. Its enzymatic activity is optimal at pH 8–9 and is enhanced by divalent cations such as Co^{2+} and Mn^{2+}, with substantial stability maintained even after heating to 90°C [53]. Interestingly, the truncation of its C-terminal domain has been shown to enhance its lytic activity, suggesting that structural modifications can further optimize its function [54] . Ply511 has been previously expressed in lactic acid bacteria in an active form [55] however, none of the strains secreting Ply511 were able to inhibit the growth of *L. monocytogenes*.

CD27L, an endolysin targeting *C. difficile*, has shown high lytic activity across 30 tested strains, including hypervirulent ribotype 027. Its action is effective under a wide range of pH conditions (4.5–8.3) and remains active at various NaCl concentrations [56]. CD27L demonstrated a dose-dependent reduction of *C. difficile* viability, with complete bacterial elimination observed at lower inoculum levels (10^4 CFU or fewer). Interestingly, truncated versions of CD27L lacking the cell wall binding domain (residues 1–179) retained or even exceeded the lytic efficiency of the full-length enzyme against different strains.[57] Further engineering, such as a single point mutation in residue 98 (L98W mutation), enhanced the lytic spectrum of this enzyme allowing the lysis of different serovars of *Listeria monocytogenes* [57]. Mayer et al., (2008) attempted to express CD27L in *L. lactis* as a delivery method to the GI tract and proved *in vitro* that crude protein extracts from *L. lactis* expressing CD27L lysed fresh cells of *C. difficile*. Recently, [58] proved that CD27L_EAD could selectively target *C. difficile* without disturbing the faecal microbiota composition. However, no further studies have been carried out to prove its action in other environments closer to the human gut.

LysSA11, derived from a *Staphylococcus aureus* phage, contains a cysteine histidine-dependent amidohydrolase/peptidase (CHAP) domain and a cell wall binding region [59]. It is active at physiological temperatures (37°C), pH 8, and under moderate salt concentrations (200 mM NaCl), retaining approximately 50% activity within pH 6–8 and 25–35°C. LysSA11 has demonstrated potent activity in food matrices and on

abiotic surfaces [59]. Furthermore, this endolysin has previously been expressed in yeast, showing that yeast could effectively express and display LysSA11 on its surface. When yeast was put in contact with *S. aureus in vitro* it showed a potent activity and eradication without the need for extensive purification methods [60].

1.6. Challenges and attempts to deliver endolysins to the GI tract

The main advantage of endolysins over antibiotics, which is their ability to avoid disrupting the host microbiome, has been examined in several studies. While specific bacterial strains are often used for specificity testing, there are only a couple of in-human studies where human microbiome has been studied during endolysin treatment: one study assessed the skin microbiome in different models [61] and another one tested the effect of the endolysin in the vaginal microbiome [50]. Recently, [58] proved that anti-*C. difficile* CD27L_EAD could reduce the pathogen in combination with vancomycin, with slight effects in the faecal microbiota composition, proving efficacy, the selective treatment of the endolysin and superiority over antibiotics. Other lysins have been tested in different animal models, aiming to evaluate its impact on the gut microbiome, such as Pal or Cpl-1, targeting *Streptococcus pneumoniae,* reporting no significant changes in mice gut microbiota [62]. A more thoroughout analysis was carried out by [63], evaluating the impact of anti-enterococcal lysin, LysEF-P10, showing a recovery of the disrupted microbiota after enterococcal infection with low doses (5μM) and a broader action with high doses (100 μM). Another study of four different broad-range anti-gram-negatives endolysins, reported a moderate effect in mice microbiota *in vivo* [64], which corresponds with the wide spectrum of action of these endolysins. The differences between studies have been attributed to the different nature of each lysin, considering that each protein should be studied case by case, since it can have a different impact on the microbiome [65].

There are few studies where endolysins have been expressed in probiotics for delivery to the GIT. For example, *Lactobacillus johnsonii*, was engineered to express and secrete the CP25L endolysin directly in the GIT, showing activity against *Clostridium perfringens*[66]. However, this report focuses mainly on the *in vitro* activity of the protein, proving its expression, and there is no study on the delivery of the endolysin to the GIT neither *in vivo* nor *in vitro*. Similarly, studies on the anti-*Listeria* Ply511 [55] or anti-*C. difficile* CD27L in its different versions [56],[58] have evaluated expression and lytic activity, but the delivery to the GIT remains unaddressed.

Other strategies to deliver endolysins to the GIT are microcapsules and nanoparticles, which can protect proteins from stomach acidity and enzymatic degradation, potentially enabling controlled release in the intestine. While encapsulation of whole phages and some antimicrobial proteins has succeeded in reaching the intestinal tract [67], encapsulation specifically for endolysins has not been tested in the GIT.

Endolysins must retain activity in the harsh gastrointestinal environment, which includes a low pH, digestive enzymes (including several proteases), the mucus barrier which surrounds intestinal tissues, and finally the target pathogen is likely to be stablished within a complex microbial ecosystem in the gut. Therefore, after a careful evaluation of the endolysin lytic activity, the key challenge when targeting these pathogens in the gut, is the efficient delivery of endolysins to their site of action in the GIT. During the work carried out in this thesis, we explore the delivery of endolysins to the GIT using yeast.

1.7. Yeast and yeast probiotics as delivery platform

1.7.1. *Saccharomyces cerevisiae*

Traditionally, yeast has been used for fermentations in what is considered the first step of human kind towards biotechnology. Nowadays, yeast, unicellular eukaryotic microbes, play a major role in modern biotechnology, especially in three major fields: metabolite production (e.g. bioethanol production), recombinant protein production (e.g. vaccine production) and *in vivo* bio-transformations (e.g. cephalosporin conversion), as reviewed in [68].

Yeasts are easy to manipulate genetically, relatively cheap to grow due to their minimal media requirements. Their growth to high cell densities is fast and robust, which also facilitates the scale-up process, and produce high protein yields [69]–[72]. Yeast can be used to clone DNA and as expression vector for fragments larger than 100Kbp and up to 2000kbp, in yeast artificial chromosomes (YACs)[73] but also allows the integration of DNA into their genome, in a very precise manner, due to its efficient homology-directed repair mechanism [74].

The process of protein production in yeast, similar to higher eukaryotes, follows three major steps: transcription, mRNA editing and translation, sometimes followed by post-translational modifications. The use of yeast as protein production system is especially relevant for eukaryotic proteins, since yeasts are able to carry out many of the post-translational modifications, which normally occur in higher eukaryotic organisms.

Many yeasts are generally recognized as safe, and present low risks of endotoxin contamination, pathogenicity or viral inclusions in protein production [69]–[72].

The yeast host strain selection is the first step of the recombinant production process: *Saccharomyces cerevisiae* is the most studied and frequently used yeast for protein expression, although other yeast species such as *Komagataella phaffii* (formely *Pichia pastoris*), *Hansenula polymorpha* and *Yarrowia lipolytica* (among others) are being used to improve some of the characteristics and limitations of *S. cerevisiae* [70].

1.7.2. Yeast surface display

Cell surface display of proteins is a technique that has been used for directed evolution of proteins [75], for antibody engineering and maturation [76], as prophylactic vaccine vehicles [77], for new protein-protein interaction discoveries [78]. Only lately, this technique is being exploited for biotechnological and biomedical applications, where functional yeasts are coupled with different proteins of interest. Examples are the display of hydrolytic enzymes like cellulases and β-galactosidase in industrial strains, for improvement of the economic viability of lignocellulosic fermentations [79], or as antimicrobial agents by displaying phage-encoded endolysins against *Staphylococcus aureus* [60]. In a molecular level, yeast cells are transformed with a vector encoding the protein of interest, which is genetically fused to a cell-surface anchor protein. Once it is expressed, the fusion protein is immobilized to the cell, but remains in the extracellular space [75],[78]. This system is regarded as being cost-effective, since one copy of the yeast *S. cerevisiae* can yield at least 105 copies of the protein in its surface [75],[76]. This system has been used in different yeast such as *K. phaffii* and *Saccharomyces boulardii* [80],[81].

Several different anchor proteins can be used for expression, Aga2p being the most commonly used [82]. The construct design for this system includes two epitope tags for detection and quantification using fluorescently labelled antibodies: a hemagglutinin (HA) tag between Aga2p and the N-terminus of the protein of interest, and a C-terminal c-myc tag. A scheme of the construct is shown in Figure 1.3 [78],[83] . Alternative GPI-anchored proteins such as Sed1p and Sag1p have also been described aiming to optimize the display system towards different applications. Sed1p, a major cell-wall protein, projects the fused protein outwards; enzymes displayed via Sed1p often exhibit higher activity and have been described as more accessible to substrates than those displayed via the a-agglutinin [84] . In contrast, Sag1p is part of the mating adhesion system; proteins fused to Sag1p tend to localise inside the cell wall or in vacuoles, reducing their exposure [85]. These differences

mean that Sed1p-based displays are generally better for enzymes that require direct substrate contact, while Sag1p can be useful when shielding the displayed protein is advantageous.

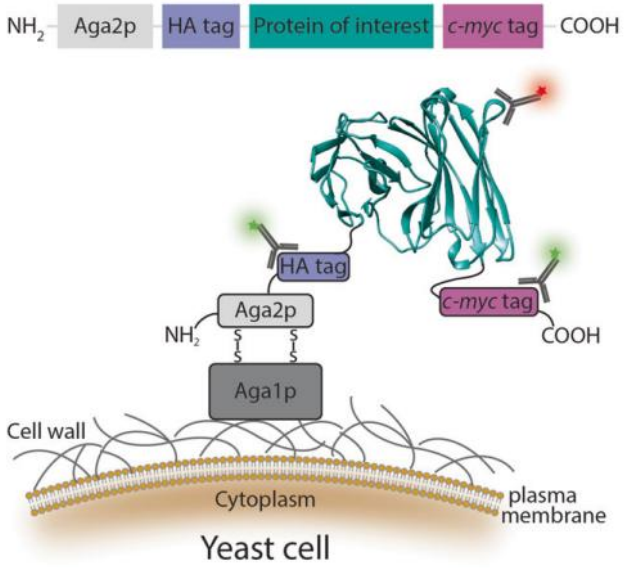


Figure 1.3. Yeast surface display technique, genetic construct and cellular representation [78],[83]. © 2015 and 2024, by the authors; Mustafa et al., 2024 and Cochran et al., 2015. Licensed under CC BY 4.0, unmodified.

1.7.3. Yeast protein secretion

In the context of heterologous protein production, another advantage of yeast is their ability to secrete proteins in their native, biologically functional form. Their fast growth in inexpensive media make the yeast a good candidate for protein production in large scale industrial processes. Moreover, secretion allows for simplification of the downstream purification process, in terms of time and costs [86]. *K. phaffii* is the yeast most frequently used for recombinant protein secretion since it has been optimized towards improvement of this ability [70],[87]. It is worth highlighting that the yeast secretion pathway involves the correct protein folding and quality control system in the Endoplasmic Reticulum (ER), where normally the proteins are subject to different covalent modifications, such as glycosylation. Glycosylation is especially relevant for glycoprotein production, but not always aimed, since it can influence protein activity. After proper folding and assembly in the ER, the proteins are exported to the Golgi apparatus, before secretion into the intracellular space [86].

The protein secretion process has been engineered in different strains to improve its efficiency towards different applications [88]. In yeast, signal peptides (also known as leader sequences or secretion signals), are

short, hydrophobic sequences fused to the N-terminus of a nascent protein that guide it into the secretory pathway. They direct the polypeptide to the ER and Golgi apparatus and are then removed, once the protein enters the secretory vesicles. The mating-factor α (MFα) pre-pro signal is the most widely used leader sequence in yeast [88]. Other signals, including those derived from yeast cell-wall proteins such as Sed1, Sta1 or Clp1, have been explored to improve secretion or surface display. Inokuma demonstrated that the Sed1 secretion signal increased secretion of cellulolytic enzymes in *S. cerevisiae* compared with the commonly used MFα signal [89],[90]. Jin et al., 2021 extended this to *S. boulardii*, showing that Sed1 enabled the highest secretion of Bp GH16A among four tested signals, doubling the quantity of target protein (NeoDP4) obtained (Jin et al., 2021). However, a recent study found that the MFα signal improved display efficiency of Alexa 594, over the native Sed1 signal in *S. boulardii.* This difference in expression between hosts and proteins, indicates that each combination of secretion signal and target protein should be tested and compared in different hosts to determine the best combination.

1.8. Yeast as a platform for production of phages or phage proteins

Yeast versatility allows for a wide range of applications, and bacteriophages and bacteriophage-encoded proteins are promising tools for combating antimicrobial resistance. We aimed during the work of this thesis, to develop a platform, combining both yeast and phage technologies for different applications, such as (i) yeast production of bacteriophages, which was discontinued after a thorough analysis of the state of the art, and (ii) production of a yeast with specific antimicrobial effects, encoding and expressing endolysins against gut pathogens, which was the main focus of this thesis.

Production of phages for phage therapy needs a cost-effective standardization. The ability of yeast as expression systems to accommodate big inserts of DNA and express them can make them interesting as a platform for phage production and has already been used for virus-like particle (VLP) production [92]. Since most genomes described for tailed phages are generally between 30Kbp and 180Kbp in size, we hypothesized that this genome is able to be expressed in yeast, as a first step towards a viable phage production.

The yeast machinery for transcription and translation is different from the bacterial one, which is used for phage replication. In yeast, gene transcription is carried out by RNA polymerase II (RNA pol II), which assembles together with transcription initiation factors at the TATA box element in the promoter [93]. After

production, this mRNA is processed by enzymes that bind to the yeast RNA Pol II. They cap the transcript in its 5´end, add a poly-A tail in the 3´end and remove intronic sequences. Then the transcript is recognized and exported from the nucleus to the cytoplasm, for translation (Jensen et al., 2003). In the phage genome, however, these yeast promoters are not present, therefore, in order to avoid edition or additions in the phage genes, a phage polymerase should be used instead of RNA pol II. Previous studies have already explored this idea, aiming to optimize protein production in yeast, since RNA pol II has lower processivity power than the phage T7 RNA polymerase (T7 RNAP).

It has been demonstrated that T7 polymerase can be integrated in the yeast genome, expressed and directed to the yeast nucleus for transcription of DNA into mRNA, with a nuclear location signal from simian virus SV-40 (SV-40 NLS) [95]–[99]. These studies demonstrated that T7RNAP can recognize and transcribe the T7 promoters, however, translation of the gene of interest was never achieved. The authors hypothesized that the mRNA produced is not exported from the nucleus to the ribosomes, which are located in the cytoplasm. This is explained by the lack of capping and poly A tail in the transcripts, which are necessary for nuclear export, and following protein expression. Only recently [100] claimed to have achieved T7RNAP dependent expression in yeast, by using an HIV viroporin to permeabilize the nuclear membrane, and allow transcripts to reach the ribosomes. Interestingly, their approach is based on the presence of an internal ribosomal entry site (IRES) and an eukaryotic termination signal in the expression constructs. IRESs are viral proteins that allow the binding of the translation machinery to mRNA independently of 5'-cap recognitions, enabling translational initiation to proceed [101],[102]. Previously, IRES elements have been reported to have promotor capacity for RNA polymerase II [103]. However, this work lacks appropriate negative controls for T7-polymerase or RNA polymerase II. Therefore, it is possible that the observed transcription was driven by IRES signals via RNA polymerase II, rather than by T7RNAP with HIV viroporin, as claimed by the authors. In conclusion, there remains room for further optimization of this process.

With our work we aimed to answer the question of whether a virion (phage structural particle) could be created, by inserting the phage genome and expressing it with the T7RNAP, using the yeast ribosomes. In a later stage, phage DNA should be packaged into the viable particle, potentially by coupling it with terminase and packaging signals.

This was explored and explained in a review article: Sáez Moreno D, Qimron U, Azeredo J, Domingues L. Towards T7 RNA polymerase (T7RNAP)-based expression system in yeast: challenges and opportunities. Bioengineered. 2022 Jul-Dec;13(7-12):14947-14959. doi: 10.1080/21655979.2023.2180579. PMID: 37105766; PMCID: PMC10234217 [104]

1.9. Towards T7 RNA polymerase (T7RNAP)-based expression system in yeast: challenges and opportunities

Back in 1987, Chen et al. demonstrated that T7RNAP could be expressed in the yeast *S. cerevisiae*, where it could initiate transcription from a T7 promoter in the yeast nucleus. However, the mRNA produced from the transcription of T7RNAP was not translated into protein. Since then, a handful of researchers have tried to improve this system to make T7RNAP more efficient in yeast without success.

The first reported study [105] showed that T7RNAP could access yeast chromosomal DNA, and that its transcription could be stimulated by providing a better access to chromatin. In fact, T7RNAP transcription is less effective in nuclear chromatin of higher eukaryotes, contrary to lower eukaryotes [106]–[108]

Benton et al., (1990) improved this system by introducing a nuclear location signal (NLS) to direct the phage polymerase to the yeast nucleus, resulting in 5-10-fold of the mRNA quantity produced from a chromosomal target gene. They also introduced a T7 terminator in the construction of their target DNA, which was correctly recognized by T7RNAP, reducing the size of the long transcripts that were being produced. However, also those transcripts were not translated into protein.

In an extensive study, Dower and Rosbash (2002) [95] identified two key points for the failed T7RNAP-transcript translation, (i) the export of mRNA from the nucleus to the cytoplasm and (ii) the capping of the transcript for ribosomal recognition. They showed that transcripts were polyadenylated if they added an eukaryotic terminator (with its polyadenylation signal) and this promoted their export to the cytoplasm. The transcripts remained nuclear when the T7 terminator, lacking the polyadenylation signal was used, highlighting the need for polyadenylation for nuclear export. To overcome the need for capping the T7RNAP transcripts, they designed a stem-loop in the target RNA and co-expressed the corresponding RNA binding protein fused to a Cbp20p (cap-binding protein). These efforts, however, did not lead to success in protein translation. However,

they strongly suggest the need for both capping and polyadenylation of the mRNA to achieve translation from the T7RNAP-based system.

In a more recent study, Hobl et al., (2013) aimed to establish the first T7RNAP-based expression system in *K. phaffii*. They showed the nuclear location of T7RNAP in *K. phaffii* and the ability of the enzyme to recognize its promoter and generate mRNAs. To tackle first the nuclear export of the transcripts, they inserted the AOX1 terminator sequence downstream of the reporter gene, carrying a polyadenylation signal. Second, to work around the need for capping the transcripts, they aimed to drive translation in the absence of cap by using a viral IRES upstream of the reporter gene. This led to protein expression apparently driven by T7RNAP. However, the expression is not T7RNAP-dependent; the negative control, a yeast not expressing T7RNAP but carrying the reporter gene construction also showed transcript expression. Follow-up Rapid Amplification of cDNA Ends (RACE) experiments revealed that the IRES used upstream of their reporter gene, from the encephalomyocarditis virus (EMCV) and hepatitis C virus (HCV) had promoter activity, thus recruiting RNA pol II and driving T7-independent expression.

Finally, the last report to date involving T7RNAP transcription in yeast [111], aimed to construct a stable expression system. In the first instance, their T7RNAP-based system indicates successful protein translation. However, to achieve cap-independent translation, they also used a viral IRES. A critical analysis of the study reveals that the IRES sequence used upstream of their target gene (EMCV IRES) was the one previously identified by [110] to have promoter activity. Moreover, the fact that an eukaryotic terminator was present downstream of the target gene makes it a possibility that the expression was not driven by T7RNAP, but by the yeast RNA Pol II. Contrary to Hobl et al., (2013), the study fails to use a yeast deficient in T7RNAP as a negative control to demonstrate that the expression is driven by the bacteriophage enzyme. It is thus tempting to think that the translation occurs because it is the yeast RNA pol II transcribing and we believe it is not possible to conclude that the construction of a stable T7 expression system in yeast was achieved, without more data. The authors, however, were successful in increasing the luminescence of their reporter gene by using a viral viroporin, which supports their hypothesis of increasing the membrane permeabilization for enhancing protein production.

Therefore, the challenge for synthetic biology to create a T7RNAP-based expression in yeast remains open; can we direct the yeast protein production from a bacteriophage polymerase?

1.9.1. Strategies to overcome the need for capping T7RNAP transcripts

1.9.1.1. Fusion protein T7RNAP and capping enzymes

As discussed previously, mammalian capping enzymes are recruited by the CTD of RNA pol II. Trying to engineer this process, some authors fused the CTD to T7RNAP in an attempt to achieve capping in hosts different from yeast. Surprisingly, none of the studies report protein translation.

Kaneko and co-authors (2007), showed that it was possible to create a fusion protein of T7RNAP with the phosphorylated mouse pol II CTD. While a C-terminal fusion rendered the T7RNAP transcription inactive, an N-terminal fusion allowed the catalytic domain to transcribe. They reported the formation of R-loops (nascent transcripts are hybridized into the template DNA during transcription). However, there is no reference in their study to whether the fusion protein was able to generate protein translation. Natalizio and co-authors (2009) tested another fusion protein in a similar approach, where they linked the phosphorylated mammalian CTD to T7RNAP. They show that this fusion is not sufficient to achieve the capping of transcripts when compared to the T7RNAP alone, *in vivo*. This suggests that the transcripts will likely not be translated into protein.

These studies suggest the possibility that the CTD is not able to solely recruit the capping enzymes to catalyze the capping of the T7RNAP transcripts [113]. Although the interaction between the CTD of RNA pol II and the capping enzymes is well documented [114]–[117], previous cryo-electron microscopy (cryo-EM) showed that the CTD is not the only structure interacting with the capping enzymes [116], thus the CTD might not be enough for directing the capping activity to T7RNAP.

In recent years, research focus has increased in mammalian cells, both for gene regulation studies (e.g., for gene therapy) and for eukaryotic protein production. Considering such interest, the creation of a T7RNAP-based expression system in mammalian cells was achieved [118],[119]. The system, called C3P3, is based on two components: first, a DNA template with the gene of interest and artificial polyadenylation. Second, a bacteriophage RNA polymerase driving transcription, fused with NP868R, an mRNA capping enzyme from the African swine fever virus (ASFV). This system has proven successful in generating capped and polyadenylated transcripts and achieving protein expression from a T7 promoter.

Although there are differences between mammalian (higher eukaryote) and yeast (lower eukaryote) translation, it is tempting to think that a parallel system in yeast could be the next step to follow and that a similar approach could be utilized for T7RNAP transcripts to be recognized by the yeast ribosomes for protein production (Figure 1.4A).

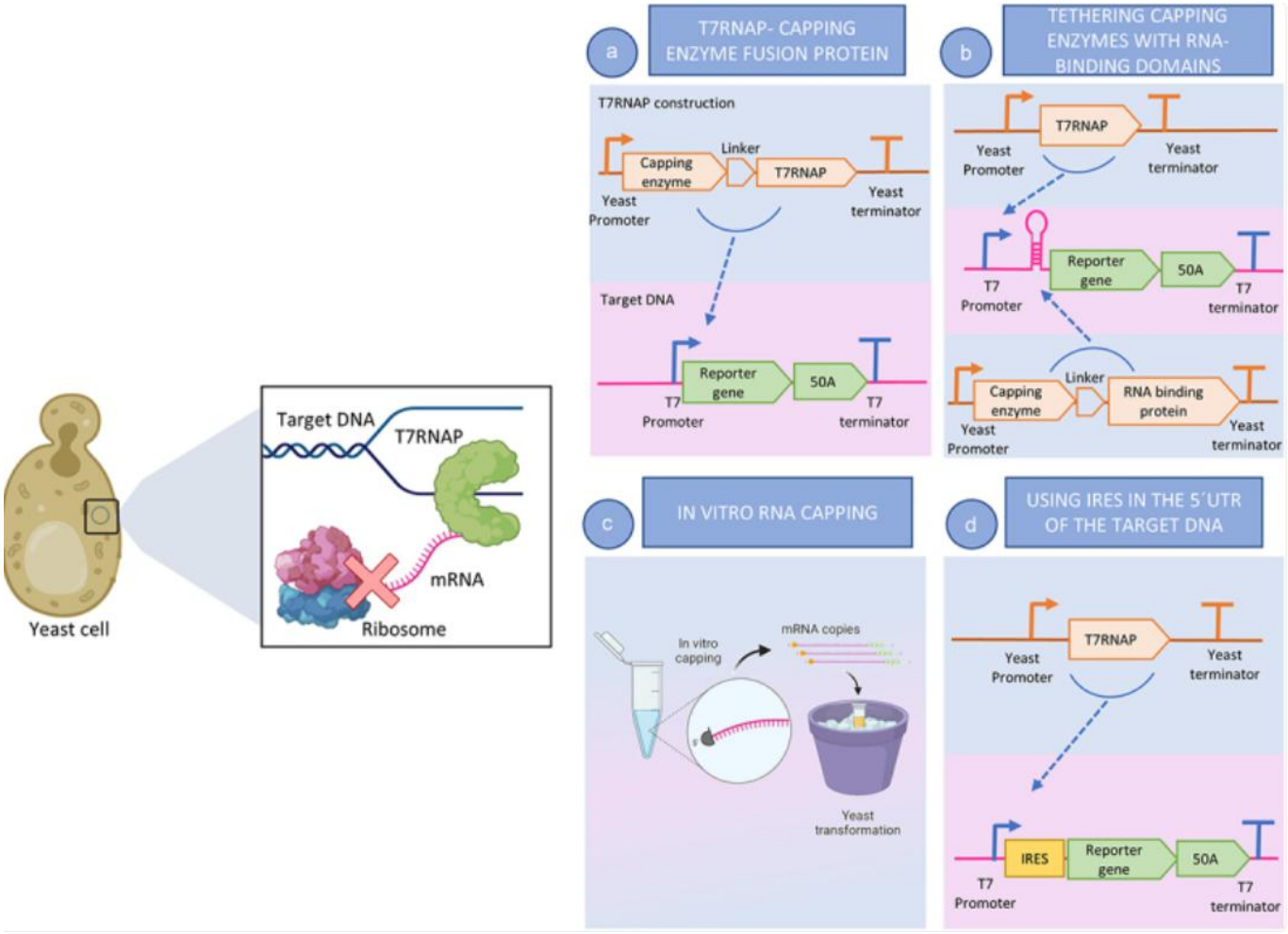


Figure 1.4. Graphical overview of the different strategies proposed or used in literature to achieve capping of the T7RNAP transcripts. In the graphic on top, nascent RNA is represented in pink (single strand) from T7RNAP, represented in green (as a single subunit), from© 2022 by the authors; licensed under CC BY 4.0, unmodified. Saez Moreno et al., (2022) [104]

1.9.1.2. Tethering of capping enzymes with RNA binding proteins

Dower and Rosbash (2002) proved by a-cap antibody binding assay that the T7 transcripts are not capped when T7RNAP drives the transcription. They hypothesize this to be the reason for the lack of translation in their study. To improve the system, they engineered a 5´stem loop in the transcript and by expressing a

fusion protein (a cap binding protein with an RNA-binding protein), they could achieve better splicing efficiency. However, the capping status was not reported, therefore it is unknown if their efforts were effective.

This strategy was later adopted in mammalian cells to achieve protein expression by Jäis et al. (2019) for the creation of a tethering system by fusing viral capping enzymes to RNA binding domains to efficiently create a capping mechanism that allows the translation of T7RNAP transcripts (Figure 1.4B). It is unclear if this mechanism would also work in yeast.

1.9.1.3. In vitro capping of T7RNAP transcripts

Another strategy used to cap T7RNAP transcripts is the *in vitro* capping. This technology is applied to produce mRNA for therapeutic purposes, which recently resulted in the lightspeed development of mRNA-based vaccines against SARS-CoV-2. *In vitro* capping by CleanCap technology [120] was used for one of the developed vaccines [121]. This technology is based on the initiation of transcription by T7RNAP after a T7 promoter, aided by 5'-Cap-containing oligonucleotide primers that resemble the desired cap structure and are complementary to the template DNA sequence at the transcription initiation site. For further information on different *in vitro* cap synthesis refer to the review [122].

Russell, Hambidge and Kirkegarrd, (1991) used *S. cerevisiae* spheroplasts to introduce mRNA for transient expression of luciferase. They used capped and non-capped transcripts generated by T7RNAP to induce expression and found that the non-capped transcripts were able to generate luminescence, albeit around 10 times lower than the capped ones. Figure 1C represents a possible strategy for generating capped mRNA in vitro, followed by a yeast transformation with the capped mRNA. The study also suggests the need for high quantities of RNA for protein production [123], which *in vivo* might be dependent not only on the production but also on the degradation rate.

1.9.1.4. Use of internal ribosome entry sites

Using IRES to achieve translation without the need for capping, was a strategy used by [110],[111], who included an IRES in the 5´UTR of the target gene, downstream of the T7 promoter. While this approach (Figure 1.4D), is promising since it might allow for bypassing the need for capping, strict controls must be provided, and the results must be carefully interpreted. Overall, it seems reasonable to think that the capping is at least an enhancer, if not a necessary element, for the creation of a stable expression system in yeast. A plausible

strategy thus, could be, a fusion protein comprised of T7RNAP and capping enzymes that would be able to cap the transcripts and solve the first step towards protein translation in yeast.

1.9.2. Strategies to poly-adenylate T7RNAP transcripts

In eukaryotes, the fate of the transcript depends not only on the cap but also on the polyadenylation of the mRNA tail; the 3´-end formation of mRNA seems to influence nuclear export [124],[125] and de-adenylation plays an important role in mRNA degradation [126]. Therefore, the proper 3´-end formation should be accounted for when designing the T7RNAP-expression system.

Dower and Rosbash (2002) studied the 3´end formation of T7RNAP-generated transcripts in yeast. When the transcripts are terminated by a T7 terminator, they are not polyadenylated and remain nuclear. However, when they tested the transcripts terminated by a polyadenylation signal, they found that transcripts are exported to the cytoplasm and are polyadenylated. This suggests that 3´-end processing might be necessary for nuclear export. This is supported by other studies that correlate proper 3´end formation with nuclear export [127],[128]

The same group [129] later demonstrated that self-cleaving hammerhead ribozymes in the 3´end of RNA pol II transcripts resulted in the accumulation of nuclear mRNAs, even if some transcripts were able to reach the cytoplasm. They added a synthetic tail of 48 adenosines upstream of the ribozyme which prevented the nuclear accumulation of mRNAs (Figure 1.5). This demonstrates that nuclear export can be independent of cleavage and polyadenylation, potentially through Pab1-mediated transport [129]. A similar strategy was used by Jäis et al., (2019),where a 40-adenosine track was followed by a self-cleaving ribozyme (Figure 1.5) to achieve the artificial polyadenylation of the reporter gene in a T7RNAP-based mammalian expression system.

The cap and the poly(A) tail act synergistically *in vivo* to promote translation, but their function individually is rather inefficient [130]. This inefficiency is notable, especially in the presence of other mRNAs with 5´cap and 3´poly(A) tail competing for translation [131] which would be the case in any context where yeast are growing while producing the intended protein.

These findings suggest that a synthetic poly(A) tail upstream of a ribozyme element in the DNA template might suffice for the nuclear export of the transcripts. Even if the association with nuclear export is not clear,

and some of the transcripts might reach the cytoplasm, the interactions between the poly(A) tail of the transcripts and Pab1 in translation, strongly suggest the need for a synthetic poly(A) tail, especially if the target translation will be via "closed-loop model" (cap-dependent).

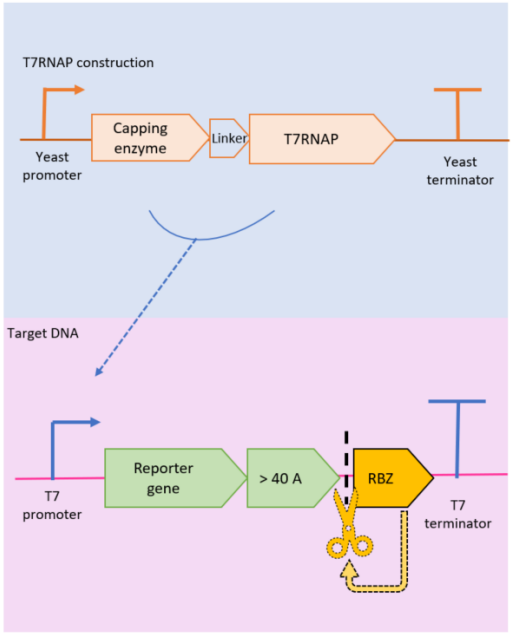


Figure 1.5. Graphical overview of the genomic construction of a T7RNAP-Capping Enzyme fusion protein (blue background) and the target gene (pink background) downstream of the T7 promoter. The reporter gene is followed by an artificial poly(A) tail of at least 40 adenosines and a self-cleaving ribozyme (RBZ). From© 2022 by the authors; licensed under CC BY 4.0, unmodified. Saez Moreno et al., (2022) [104]

1.9.3. Expression system design: nuclear or cytoplasmic location?

It has been demonstrated that an NLS incorporated in the N-terminal of the protein directs the T7RNAP into the yeast cytoplasm both in *S. cerevisiae* and in *K. phaffii* [109],[110]. However, it should be noted that there might be advantages and disadvantages in placing the target gene in the nuclear chromosome instead of in a plasmid.

The chromosomal integration of the target gene downstream of the T7 promoter offers a stable and controllable system [132], however, chromatin accessibility in the nucleus could interfere with T7RNAP efficiency [105],[108].

The nuclear export of the transcripts to the cytoplasm might be another limiting step in the protein expression process. Processes of quality control and degradation, assure that mRNA exported to the cytoplasm is capped,

polyadenylated and spliced. Several mechanisms prevent aberrant mRNA from reaching the cytoplasm, being discarded during early steps of their biogenesis [124]. In fact, Yan et al., (2021) demonstrate that an increase in protein production is possible by increasing the nuclear membrane permeabilization in *S. cerevisiae*.

Following the genomic location, the promoter of choice to express the T7RNAP will also differ depending on whether the pathway must be active during cell growth (constitutive promoter) or if a certain degree of control is needed for the expression (inducible promoter). The integration of the T7RNAP in the yeast genome, and the introduction of the target gene in a vector (analogous to the pET expression system in *E.coli* [133]) could allow for a more efficient expression system since the nuclear export of the transcript would not have to be accounted for. In contrast, using a vector for expression could mean more variability in protein expression levels, depending on the plasmid copy number and a possible less stable system [132].

The choice of yeast for the design of the protein expression system should also be considered. To date, *S. cerevisiae* and *K. phaffii* have been used for the design of such system. While *S. cerevisiae* offers the advantage of being a well-studied organism, the use of *K. phaffii* as a protein expression system is steadily increasing and is already a key host for industrial production of biopharmaceuticals [134].

In prokaryotes the interplay between the T7RNAP and its promoter has been well described [133],[135],[136]. In yeast, processes such as transcription and replication are regulated by chromatin structure, where the condensed heterochromatin can inhibit gene expression [137]. T7RNAP can access its promoter in *S. cerevisiae* chromatin [105]. In *K. phaffii*, T7RNAP recognized its promoter present in a vector [110], thus it is still unclear if the enzyme can access and transcribe effectively from *K. phaffii* chromatin. Moreover, *K. phaffii* centromere structure differs from *S. cerevisiae* [138]. Previous studies have shown that chromatin structure can affect T7RNAP transcription, which is less effective in nuclear chromatin of higher eukaryotes, contrary to lower eukaryotes [106]–[108]. Thus, considering the interplay between the polymerase and the promoter should also be considered when choosing between the integration of the target gene or the use of a plasmid for the expression.

1.9.4. Is a T7RNAP feasible in yeast?

The studies highlighted here show the progress made, but also show the unresolved challenges towards a T7RNAP-based expression system in yeast. Nevertheless, we believe that identifying the challenges is a way forward to solving them, and we are thus certain that, as with the mammalian T7-based system, a T7-yeast

based expression system will also be established. We expect the future progression of this system to further develop with the exploitation of genome editing tools such as CRISPR/Cas [139]–[141]. Providing the benefits of a host-independent transcription to yeast protein expression, will not only be a useful tool for synthetic biology, but it will also contribute to understanding the genetic regulation in eukaryotic systems and will ultimately increase the versatility of yeast in the sustainable production of different metabolites.

During the course of this thesis, Kar *et al.* (2025) [142] developed a T7RNAP expression system in *S. cerevisiae*, which they described as "an orthogonal transcription engine for programmable gene expression in eukaryotes". Their approach involved engineering a fusion enzyme that combined T7RNAP with the single subunit capping enzyme from the African swine fever virus, using *S. cerevisiae* as the host. This system enabled programmable control of gene expression through T7RNAP-based genetic circuits in yeast, and its performance was further validated in mammalian cells Implications in the workplan of this thesis.

In developing the workplan for this thesis, several limitations led us to discontinue the experimental part: i) T7RNAP is specific to T7 phage. Although it shares similarities with other phage polymerases, in principle, phage genomes require the T7 promoter to be recognized and transcribed. This implies that any phage produced in this system would require extensive engineering to replace its native promoters with T7 promoters. ii) For yeast ribosomes to efficiently recognize and translate transcripts produced by T7RNAP, the entire phage genome would need to be codon-optimized, resulting in a very high cost for each phage. iii) While the assembly of phage T7 has been well described and most of its protein interactions are known, extending these findings to other less-studied phages is a risky approach, especially given that many phage coding sequences encode proteins of unknown function.

Considering the high risk and costs of the project, the experimental work was deprioritized, and the focus shifted towards the expression of phage proteins, endolysins.

1.10. *Saccharomyces boulardii*: probiotic properties and engineering

Apart from valuable tools for biotechnology, yeasts are an important part of the human gut microbiome; therefore, they have been exploited as probiotics. By definition, a probiotic is microorganism-based product that can benefit the host. In order to be marketed as such, it needs to follow the 2002 FAO/WHO Working Group Report: the potential probiotic strain needs to be identified, its functionality needs to be characterized

(e.g. tolerance to gastrointestinal conditions (pH, temperature, enzymes)) and its effectiveness tested in clinical trials.

Probiotics are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [143]. Although most currently used probiotics are lactic acid bacteria (LAB) (Lactobacillus spp., Bifidobacterium spp., Bacillus spp., etc.) [144], *Saccharomyces boulardii*, stands out as the only probiotic yeast approved by FDA [145]. Recently, LAB and non-conventional probiotics were reviewed in detail elsewhere [146]. This section aims to focus exclusively on *S. boulardii*, providing an in-depth discussion on the most effective genetic tools currently available and compiling the modifications made to date to enhance its therapeutic and probiotic properties.

S. boulardii was firstly isolated from peels of tropical fruits [147] and its probiotic activities have been studied in several clinical and non-clinical trials in the last few decades (McFarland, 2017; Moré and Vandenplas, 2018). This yeast shows high tolerance to acidic conditions and grows well at 37°C (the human host's internal temperature) [150], both key factors for surviving the harsh conditions of the GI tract. It is considered Generally Regarded as Safe (GRAS) by the FDA (US Food and Drug Administration) [151] and is recommended for treating acute gastroenteritis, for prevention of antibiotic-associated diarrhoea and has proven to have positive effects in traveller's diarrhoea, acute infectious diarrhoea, Crohn's disease, ulcerative colitis [152]–[154] due to the inhibition of pathogens and the increase of beneficial probiotic species in the gut [155]–[157]. A recent meta-analysis highlights the efficacy of *S. boulardii* for the treatment of paediatric acute diarrhoea, showing that the administration of this yeast reduced its duration and more participants were cured when compared with the control group [158]. Moreover, the use of *S. boulardii* as an adjuvant therapy for combating *Helicobacter pylori* is supported by another recent meta-analysis comprising 19 studies and over 5,000 cases, showing both a higher the eradication rate and the reduction of adverse effects when *S. boulardii* was used to complement the standard of care [159].

While the positive effects of this yeast have been proven, the exact health effects of the consumption of *S. boulardii* are under constant investigation and have been attributed to several mechanisms of action. This probiotic yeast exerts a broad range of health-promoting activities, attributed to its antimicrobial, immunomodulatory, and gut-support properties. One key mechanism involves the secretion of antimicrobial

peptides capable of neutralizing pathogenic toxins (i.e., toxins A and B from *Clostridium difficile*, cholera toxin and pathogenic *Escherichia coli* surface endotoxins) [160],[161]. Additionally, *S. boulardii* can directly bind to pathogens microbes (a process facilitated by mannose residues on its cell wall), bind to toxins and reduce the availability of pathogens binding sites in intestinal surfaces, preventing their adhesion [161].

The mechanisms of action of *S. boulardii* extend beyond pathogen neutralisation. *S. boulardii* produces short chain fatty acids (SCFA's) (acetate, propionate, and butyrate) that not only inhibit pathogen proliferation but also contribute positively for the microbiome modulation. This yeast also has shown immune modulation properties and reduced levels of pro-inflammatory cytokines such as TNF-α and IL-8 in paediatric acute diarrhoea patients [162]. It has been shown to stimulate the production of anti-inflammatory cytokines Il-10 and to supress pathways associated with inflammatory response (e.g., NF-κB-mediated signal transduction pathways)[160]. *S. boulardii* also aids in maintaining the integrity of the intestinal mucosa through stimulation of mucin production, secretion of polyamines such as spermidine and spermine and production of SCFA's [161],[163],[164]. Moreover, antioxidant effects of *S. boulardii* were shown to protect against oxidative stress in rats, something hypothesised to be related to the enhancement of the blood-brain barrier integrity [165] animal trials [166],[167] and a case-report [160],[167] suggest its positive influence in mental disorders, via modulation of the gut microbiome. The probiotic yeast also exerts other trophic effects like stimulating the production of brush border membrane digestive enzymes and favouring nutrient absorption [160],[168]. For a more detailed view of the proven health effects and the ongoing investigations on molecular mechanisms driving those, we recommend the reader to turn to [160],[169],[170]. *S. boulardii* is not naturally part of human gut microbiota, and competes with the endogenous microorganisms, which leads to low residence times in the gut (3-5 days after stopping dosing) [171],[172]. This limitation can be addressed through repeated dosing, co-administration with antibiotics or with genetic engineering, as will be further discussed. In fact, in the clinic, *S. boulardii* is often used to mitigate the side effects of antibiotic therapy related to gut dysbiosis or *C. difficile* infection [173]. *S. boulardii* unlike bacterial probiotics, is not susceptible to antibiotics and can be administered concurrently [147],[157],[174]. Recently, a randomized controlled trial showed that *S. boulardii* as adjunct therapy with vancomycin resulted in reduced *C. difficile* infection recurrence without any adverse effects [175]. This fact can also enable a more accurate dosage since it allows it to be effectively cleared from the gut [176],[177], which is another potential advantage for the use of *S. boulardii* as a transient drug delivery vessel. There is a growing scientific and clinical interest in *S. boulardii*, Figure 1.6 illustrates the increase in the number of publications referring to this probiotic.

Moreover, according to Clinicaltrials.gov there are 45 completed clinical trials including the use of *S. boulardii*, and there are several *S. boulardii* probiotic products already in the market.

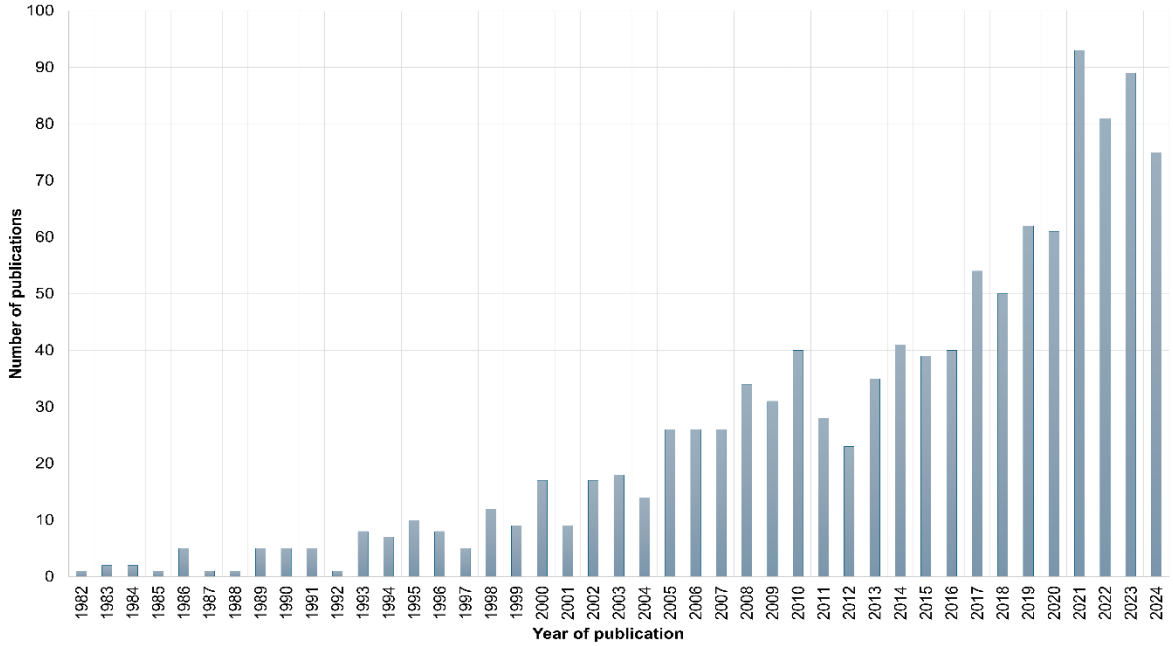


Figure 1.6. Number of scientific papers published per year on *Saccharomyces boulardii*, according to PubMed® Total number of publications contemplating *S. boulardii* in the last 42 years (1982–2024). Data were obtained from PubMed® using the queries: "Saccharomyces boulardii" and filtered by year (1982 to 2024). https://pubmed.ncbi.nlm.nih.gov Accessed in April 2025.
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Despite the interest and efficacy of *S. boulardii*, the global probiotic market is in fact dominated by LAB, particularly Lactobacillus and Bifidobacterium species, being among the most clinically evaluated probiotics, widely incorporated into yogurt, dairy, and capsule-based formulations. These strains are more commonly used in food and supplement formulations due to their historical association with fermented foods. In fact, over 1000 completed clinical trials have involved LAB strains, compared to only 13 for *S. boulardii* as of 2025 (according to ClinicalTrials.gov) and the number of strains in the market is much superior. In contrast, *S. boulardii* offers certain advantages, such as resistance to antibiotics, lower optimal pH, and auto-aggregation capacity [178] and its transient behavior which can also be seen as an advantage, for short-term therapeutic actions, such as prevention of antibiotic-associated diarrhea or traveler´s diarrhea. However, this limited gut colonization can also be seen as a disadvantage, since it requires multiple dosing. This, together with its less diverse strain library (only a few strains have been studied) and lower use than LAB in food fermentations have impacted its use as a probiotic in humans.

However, in recent decades, advances in genetic and genome engineering have unlocked new opportunities for the modification and enhancement of *S. boulardii*. Over the past few years, substantial knowledge has been accumulated on the most effective genetic elements and tools, paving the way for developing *S. boulardii* strains with improved probiotic properties. These enhancements include the ability to deliver molecules of interest, extend residence times in the GI tract, expand substrate utilization, and implement biocontainment strategies. Genetic engineering has made it possible to exploit one of the key advantages of *S. boulardii* over LAB as a probiotic: its ability to express more complex proteins and even entire pathways. This is due to the yeast's capacity for post-translational modifications and its tolerance for the insertion of longer DNA fragments, which significantly expands the range of compounds it can produce, something not achievable with LAB.

1.10.1. Heterologous gene expression systems and metabolic engineering

S. boulardii shares more than 99% of nucleotide identity along its genome with *S. cerevisiae* [179] and, even though they have different metabolic and physiological characteristics, they both can produce and secrete heterologous proteins. The capacity of *S. boulardii* to survive and colonize the gut, together with the ability to secrete heterologous proteins, offers a strong potential for *S. boulardii* to deliver molecules of interest to the gut by genetic engineering. There are a few sequenced strains of *S. boulardii* (Table 1.1) among which, the most used strain is *S. boulardii* ATCC-MYA-796.

Table 1.1. Publicly available genome sequences of S. boulardii strains. **(a)-** Reference genome; **(b)-** This strain is also referred as "*CNCM I-745*"; **(c)-** Genome not available.

Strain	GenBank Accession number	Assembly level	Has been genetically engineered?
KCTC 13826BP [(a)]	GCA_026225675.1	Contig	No
BIOCODEX [(b)]	GCA_001298375.2	Chromosome	Yes
ATCC MYA-796	GCA_000769245.1	Chromosome	Yes
ATCC MYA-797	GCA_001625055.1	Scaffold	Yes
lyo (DiarSafe)	n/a [(c)]	-	Yes

strain 17	GCA_000734875.3	Chromosome	No
EDRL	GCA_000442675.2	Contig	No
kirkman	GCA_001545905.1	Contig	No
unique28	GCA_001413975.1	Chromosome	No
Unisankyo	GCA_001545975.1	Scaffold	No
CLPY01	GCA_021216695.1	Scaffold	No

Yeast can carry out post-translational modifications that, apart from glycosylation, closely resemble those performed by mammalian cells [180]. This is a major advantage of using *S. boulardii* as a potential delivery vessel over commonly used probiotic bacteria, which are unable to express such complex proteins. In fact, many recombinant proteins such as insulin and the Hepatitis B antigens are produced industrially by *S. cerevisiae* [181]. Moreover, *S. cerevisiae* and *K. phaffii* are widely used expression systems for the development of recombinant vaccines, protozoal proteins and tumour antigens [182] highlighting the potential of engineering probiotic yeast.

1.10.2. Plasmid expression systems in *S. boulardii*

The first work related to yeast transformation was reported in 1978 where a *S. cerevisiae* strain with *leu2-* double mutation was transformed with a plasmid and, the resulting strain, was able to express beta-isopropylmalate dehydrogenase; the enzyme coded by the *LEU2* gene [183]. Since then, the expression of heterologous proteins has evolved, and non-conventional yeast can also be transformed and express heterologous proteins. Plasmid transformation is the easiest and simplest way to express foreign DNA in microorganisms.

With the exception of some studies that employed electroporation, the transformation method used in *S. boulardii* is the PEG/LiAc method (or some variation) [184]. This method is widely used to transform *S. cerevisiae*. Although this method is proven to work in *S. boulardii,* the transformation efficiency can be up to 100 times lower when compared with *S. cerevisiae* [185]. Plasmids, have three major elements that can impact gene expression: the expression cassette, the selective marker and the origin of replication.

The expression cassette is what defines which protein will be overexpressed and the basic structure consists of a promotor, the gene of interest and a terminator [186].

The selection marker ensures that plasmids are maintained in the cell, otherwise they can be lost after cell replication [187]. Yeast plasmids normally contain a selective marker for bacteria (for plasmid construction and propagation) and a selective marker for yeast (for transformant selection and plasmid maintenance). This kind of vectors are referred as "shuttle vectors", as they can replicate in two different host species [188]. Dominant selective markers like antibiotic resistance genes are widely used but they are not the only type. Auxotrophic markers are also frequently used for selection of positive transformants. These markers eliminate the need of adding extra chemicals to the selective media that can affect cellular function [189],[190]. The downside of using this kind of markers is the need of generating auxotrophic strains. This is done by disrupting the function of a gene that is essential for the survival of the microorganism, i.e., enzymes that participate in the synthesis of amino acids or pyrimidines [190]. However, the auxothrophy may impact metabolism, as reported for the uracil mutation (Δ*ura3*) [190],[191]. Furthermore, the selection of positive transformants requires the absence of specific nutrients that enable the growth of the mutant strains, thus selecting for the strains that have genetically complemented the mutation.

Regarding types of plasmids and their correspondent replication origins, if the desired objective is to achieve high expression levels, the go-to is a yeast episomal plasmid (YEp) with a 2μ origin [192]. This kind of plasmids is also referred to as high-copy number plasmids since each cell can harbour between 28-58 copies (in *S. cerevisiae*) [193]. However, the metabolic burden can impact cell fitness (Gomes et al., 2018; Rouches et al., 2022; He et al., 2024). Alternatively, there are yeast centromeric plasmids (YCp) whose origin is a combination of an autonomously replicating sequence (ARS) and yeast centromeric sequence (CEN) [192]. With a CEN/ARS origin, the balance between gene expression and cell growth is maintained, since only 4-8 copies are harboured per cell in *S. cerevisiae* [193]. There are also yeast-replicating plasmids (YRp) that do not have CEN elements, lacking plasmid stability conferred by CEN, which explains the emergence of YCp [192]. Finally, yeast-integrating plasmids (YIp) can also be used for heterologous protein expression. In this case, the plasmids must be integrated into a chromosome via homologous recombination since they have no origin of replication [188].

There are several examples of heterologous protein expression via plasmid in *S. boulardii* (Table A1.1). A detailed discussion on the applications of engineered yeast is provided in the second part of this section. The yeast selection markers that are most frequently used are *G418/ KanMX* (dominant) and *URA3* (auxotrophic). Plasmid selection markers used for genetic engineering of *S. boulardii* are described in Table A1.1. Durmusoglu, et al. (2021) demonstrated that the choice of the selective marker had an impact in protein expression. They studied the expression of *mRuby* in a 2μ plasmid and found that the use of auxotrophic markers (*URA3*, *HIS3*, *TRP1*) results in higher expression when comparing with dominant markers (*KanMX*, *ZeoR*, *NatR*, *HygR*) with the 2μ/ *URA3* pairing achieving the highest overall expression. This study highlights the differences in gene expression resulting from the interaction between origins of replication with auxotrophic and dominant markers [196]. The same study also compared the expression using the CEN/ARS origin in the same conditions, demonstrating that the expression was higher when 2μ plasmids were used. In another study, the expression of Erythromycin destructase (EreA) was found to be 17-fold higher when plasmid-mediated, compared to a chromosomal integration. This difference in expression levels was attributed to the high-copy nature (2μ) of the plasmid used Table A1.1[197].

Apart from the replication origin and the selection marker in a plasmid, the promoter and terminator driving the expression of the gene of interest play a key role in the expression. Durmusoglu et al. (2021), to optimize the expression of *mRuby*, evaluated 18 different *S. cerevisiae* constitutive promotors (combined with the *TDH1* terminator) and 7 terminators (combined with 3 promotors of different strength). Their results show that most of the promotors from *S. cerevisiae* maintain their relative expression levels in *S. boulardii,* and the terminator choice impacts the expression in a promotor-dependent way [196]. Previously, several *S. cerevisiae* promotors were confirmed to be present in *S. boulardii* genome with high homology percentages (97-100% nucleotide identity) indicating that there were probably more examples of promotors shared by the two yeasts [198]. Another example of the use of fluorescent proteins in *S. boulardii* is the expression of *mCherry,* which was used to compare expression levels between a 2μ plasmids and a single locus integration, indicating that only 8.6% cells carrying the 2μ plasmids expressed *mCherry*, versus 97.3% for the single integration [199]. Some strains of *S. boulardii* have shown the presence of the native 2μ plasmid with high similarity to the one found in *S. cerevisiae* [179]. This plasmid harbours 4 genes (*FLP*, *REP1*, *REP2* and *RAF1*) that allows self-replication and maintenance through generations. This makes it a selfish genetic element, as its replication is independent of the host yeast cells. Typically, this plasmid is maintained at a high copy number with

chromosome like stability and has shown to have some impact in cell fitness [200]. Curing the native 2µ plasmid can improve stability of subsequent transformation with plasmids containing the 2µ origin [201]. Conversely, in *S. cerevisiae*, the endogenous 2µ plasmid has been directly edited to express the dihydroartemisinic acid metabolic pathway and the *TPI1* gene [202]. Thus, an auxotroph for *TPI1* was used, showing increased production of dihydroartemisinic acid and higher plasmid stability in a culture with no selective pressure. Curing the endogenous plasmid or using it for genetic engineering have not been explored in *S. boulardii* but it might open new possibilities for genetic engineering in the probiotic yeast.

Beyond being used to quantify expression, fluorescent proteins have a key role in molecular biology since they are reporters for protein localization [203]. The fluorescent protein *mRuby* was also expressed in *S. boulardii* using a plasmid that harboured a mitochondrial targeting sequence. Despite being originally designed for *S. cerevisiae,* the results show that it can also be used in *S. boulardii* (Liu et al., 2016). Green fluorescent protein (GFP) was successfully expressed using *S. boulardii*, delivering it to the immune tissue of the GI tract of mice (Peyer's patches) proving that this yeast can possibly deliver molecules of interest to the gut [204]. In *S. boulardii* enhanced GFP (EGFP) was also used as a proof of concept for yeast surface display, again demonstrating the viability of this technology in *S. boulardii,* before going on to express other recombinant proteins [205],[206]. Other reporter genes like lacZ or resistance markers (Ura3p and Hygromycin B), have also been used in *S. boulardii* (Table A1.1) [198],[207],[208].

1.10.3. Conventional genome engineering in *S. boulardii*

Although plasmid expression can be used for achieving high levels of expression, there are some drawbacks associated with these expression systems. Plasmids are not suitable for all applications due to high metabolic burden (associated with high copy number plasmids), plasmid instability without selective pressure and possible antibiotic resistance transmission [187],[194],[195],[209],[210]. Gene integration methods like YIp, Cre-loxP, Ty (transposons of yeast) elements or the PiggyBac transposon system, offer a stable genome integration without the drawbacks associated with plasmids expression systems. In this section, examples of the application of these engineering strategies in *S. boulardii* will be discussed (Table A1.2).

As referenced above, YIp vectors lack an origin of replication and rely on integration into the genome via homologous recombination to be stably maintained in recipient cells. Table A1.2 provides examples of YIp modification in *S. boulardii*. An example of the use of YIp's in *S. boulardii* was the expression of the fusion

protein formed by the chicken ovalbumin and *C. perfringens* enterotoxin. Interestingly, authors found that *GAL-1* (one of the 3 promotors tested, Table A1.2) was not able to drive the expression of this fusion protein, due to the inability of *S. boulardii* to utilize galactose [211]. Another example of YIp gene integration in *S. boulardii* is the expression of the monomeric streptavidin (mSA) [212].

Not only single expression cassettes, but also metabolic pathways have been introduced to *S. boulardii* via integrative plasmids. The xylose pathway (*XYL1*, *XYL2*, and *XYL3* from *Scheffersomyces stipitis*) was introduced in the *URA3* locus. When compared to *S. cerevisiae* with the same xylose fermentation ability, the modified probiotic yeast achieved higher rates of xylose fermentation [207]. One other strategy for chromosomal integration of a pathway was used by Ling et al. (2023), where two copies of a medium chain fatty acid pathway were integrated into the *URA3 locus*. Due to its large size, this pathway was subdivided in three fragments and assembled using the DNA assembler method (Shao et al., 2009). The 5'-end of the first fragment had a homology region to the targeted *locus*, while the 3'-end had a homology region that overlapped with the second fragment 5'-end. The third fragment was designed to have a 5'-end that overlapped with the 3'-end of the second fragment and a 3'-end that was homologous to the targeted *locus*. This allowed for the pathway to be assembled in the right order and to be integrated into the targeted *locus* (Ling et al., 2023). This strategy has the potential to be utilised for assembly of more than three expression cassettes, provided that each fragment has ends that overlap the two flanking expression cassettes and that the first and last cassette have homology for the targeted locus (Shao et al., 2009).

Cre-loxP is another method for gene integration or disruption and it is based on the recognition and binding of specific loxP sites by the Cre Recombinase from the *E. coli* bacteriophage P1. These sites are 34 base pairs (bp) long in which two 13 bp palindromic sequences are separated by 8 bp asymmetric core sequence. Depending on the orientation of the loxP sites, Cre Recombinase can induce a deletion, inversion or translocation. The loxP sites can be added to the genome by flanking them with homologous sequences that will be integrated by homologous recombination. After that, Cre Recombinase is transformed into the cells and the recombination of loxP site occurs. Everything in between the loxP sites is excised from the genome [213]–[216]. Thus, this system allows for a more targeted engineering approach compared to YIps, as only the intended modification remains in the genome after the selective marker is excised. An added advantage is the ability to perform multiple gene disruptions [217] and/or integrations. However, one loxP sequence remains in the genome as a result.

Although gene integration is also possible by using Cre-loxP, in *S. boulardii* this system was used for gene disruption, generating *URA3* auxotrophic strains (K. Chen et al., 2020; Wang et al., 2015) as well as disrupting the *ADE2* gene (Table A1.2). The disruption of this gene makes yeast colonies turn red instead of white and it was used to construct a strain capable of sensing doxycycline [219].

Genetic engineering tools can also derive from innate mechanisms rather than being adapted from other microorganisms. Ty is a type of Class I elements that exist in substantial amount throughout the eukaryotic genome. Ty elements are mobile and can contribute for chromosomal rearrangement, gene duplication or variations in genome size [220]–[222]. The transposition mechanism of Class I elements is known as copy-paste [223]. The corresponding mRNA is reverse transcribed back to DNA before transposing in another locus of the genome [224]. There are 5 sub-families of known Ty elements (Ty1 to Ty5) in *S. cerevisiae* [225], and *S. boulardii* shares some of them [199]. When comparing the consensus Ty elements utilized in the EasyCloneMulti vectors [222] and the genome of *S. boulardii* MYA-796, Liu et al. (2020) reported that the Ty elements were highly conserved in *S. boulardii*, finding 140 Ty elements distributed across the Ty1.1, Ty1.2, Ty2, and Ty3 sub-families.

Genome integration using Ty elements allows for multiple copy integration achieving enzymatic activity levels comparable to the ones attained with plasmids [226]. In *S. boulardii*, Ty elements were used to express Interleukin-10 (IL-10), tumour necrosis factor receptor 1 extracellular domain, alkaline phosphatase, and atrial natriuretic peptide (ANP) [199]. The authors developed this method due to the low efficiency achieved using single-locus integration in *S. boulardii* ATCC MYA-796; which is a common problem encountered and reported for *S. boulardii* in comparison to *S. cerevisiae* (Douradinha et al., 2014; Liu et al., 2020). Using Ty elements, Liu et al. (2020) improved the number of colonies recovered after transformations and show an enhanced expression of EGFP. However, the number of copies integrated was not determined in that study.

Other transposons-based strategies have also been implemented in *S. boulardii* namely, to produce l-Ergothioneine (Tang et al., 2024b). The system used was the PiggyBac transposon, which allows for a stable genome integration with the possibility of footprint-free excision. This system relies on the enzyme PB transposase, which recognises transposon-specific inverted terminal repeat sequences located on both ends of the transposon vector. Then, the fragment is moved from the vector and integrated in TTAA chromosomal sites. This mechanism is referred as “cut and paste” and, after the integration, the fragment can be precisely

excised from the chromosome, with the gap being repaired by ligation. This footprint-free excision is valuable due to the reversibility of the genetic modifications performed with this system (Chen et al., 2020; Zhao et al., 2016).

1.10.4. CRIPSR-CAS genome editing strategies in *S. boulardii*

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated endonuclease 9) is a genetic engineering tool that, in the last few years, has been exhaustively studied and developed. Like Cre-loxP, CRISPR-Cas systems are based on mechanisms observed in bacteriophages that were adapted for genetic engineering. CRISPR is the bacterial equivalent of the human immune system, providing defence against phage infection and plasmid transfer [230],[231].

This tool is based on a double-strand break performed by the nuclease Cas9, which is guided to a specific location of the genome by a guide RNA (gRNA). The gRNA is a 20-base pair sequence that hybridizes next to a Protospacer Adjacent Motif (PAM) sequence [232]. The specificity of this method relies on the fact that the DNA cleavage is strictly dependent on the presence of a PAM immediately downstream of the target site and, the identity between gRNA and the DNA of the targeted site, must be near perfect [233]. The recognized PAM sequence depends on the origin of the nuclease. *Streptococcus pyogenes* Cas9 (SpCas9) is the most used endonuclease in CRISPR-Cas systems. The preferred PAM for this endonuclease is 5'-NGG-3' (N= A, T, C, G), which is common in most of the organisms resulting in easier application of the CRIPSR-Cas9 tool [234],[235]. After this cut, negative selection takes place: only the yeast able to repair the nuclease cut survives. For this repair to be successful, using homologous recombination, two homologous DNA sequence flanking of the cut site must be given, one upstream and one downstream. Between these homologous regions, the desired DNA sequence is inserted, allowing it to be integrated precisely into the chromosome at the target location.

CRISPR-Cas systems offer several advantages over other integration methods. Using Cre-loxP, along with the integration of the gene of interest, extra genetic material will also be integrated into the genome because one loxP site is always left behind [213],[215]. Multiple integrations in the genome using this tool can be harmful as each modification accumulates more undesired scars in the genome. In addition, when integrating genes with Cre-loxP, there is a need to integrate a selective marker in the genome for selection that will be excised after [215]. CRISPR-Cas is a marker-less tool that allows multiple integrations and eliminates the need for integrating selective markers. In addition to its significance in the development of industrial bio-based production

processes [236], this is also a crucial point for health applications as antibiotic-selective markers are not accepted in clinical trials due to the possibility of spreading the resistance to other microorganisms in the gut microbiome [237]. One of the most used toolkits for CRISPR-Cas9 editing is the EasyClone-MarkerFree (Jessop-Fabre et al., 2016; Stovicek et al., 2015), which despite being designed specifically for *S. cerevisiae*, has been used to effectively edit some of the available chromosomal sites [199],[240]. These sites must share identical nucleotide bases in the cut site/PAM sequence between both yeasts. The lower efficiency of *S. boulardii* transformation using this method [199] comparing to *S. cerevisiae*, could be related to the higher thickness of the probiotic cell-wall or to the double-strand break repair mechanisms not being as efficient.

Durmusoglu et al. (2021), used mTurqouise2 and mRuby2 to compare the expression in different sites of integration and to compare different methods of integration (Table A1.3). Although, the expression locus showed to have influence (up to 3.4-fold difference) in the expression it had a minor effect when compared with promotor choice (up to 15.3-fold difference). To compare different integration methods, two different Cas endonucleases were used (SpCas9 and LbCas12a) as well as a method without the assistance of the cut performed by the endonuclease. LbCas12a from Lachnospiraceae bacterium ND2006 (PAM- 5′ TTTN-3′ (N= A, C, G)) was the most efficient followed by SpCas9 and unassisted DNA [196].

To expand the genetic engineering tools available for *S. boulardii*, in addition to identifying optimal integration sites, it is also essential to characterize promoter strength. Recently, Sands et al. (2024) utilized CRISPR-Cas engineering to express yeast-enhanced green fluorescent protein (yEGFP) and mKate2 for promotor characterization. The 12 promotors were characterized in media supplemented with different carbon sources (glucose, acetate, fructose, sucrose and inulin) under aerobic and anaerobic conditions. *In vivo* promotor characterization was also performed and showed high correlation with *in vitro* activity [240]. yEGFP genomic integration was also used as reporter for monitoring rtTA expression. This integration is part of the previously mentioned doxycycline biosensing system. Two promoters (TDH3 and MYO2) were tested and, although MYO2 achieved lower expression levels when compared with TDH3, it was chosen to drive rtTA expression due to lower levels of false positives (i.e. red yeast when doxycycline was not present) [219]. Another biosensing yeast was developed to detect ATP (Adenosine triphosphate) levels in mice with DSS-induced colitis using CRISPR-Cas9 to introduce the Firefly Luciferase expression cassette into an intergenic region between HIP1 and a gene coding for tRNA in chromosome VII of *S. boulardii* [241]. Lastly, Kim et al. 2023 used a double integration strategy to enhance expression of human lysozyme with a chicken lysozyme secretion

signal in *S. boulardii* [242]. A previous study also expressed this protein but only a single copy was integrated [207].

CRISPR-Cas is a versatile tool that can be used with other genetic integration tools like Cre-loxP [219] but integration is not the only utility for this tool. Previously, the generation of *S. boulardii* auxotrophic strains has been done via UV mutagenesis [204],[208] and Cre-loxP (Table S1.3). However, effective UV mutagenesis can induce undesired mutations [243] and Cre-loxP is not a marker-less tool [213],[215]. On the contrary, CRISPR-Cas9 can be used for generating auxotrophic strains by only disrupting the function of a specific gene while not leaving scars in the genome. In *S. boulardii*, several auxotrophic strains have been generated by CRISPR-Cas some of them being auxotroph for multiple compounds (Table A1.3).

Auxotrophic *S. boulardii* strains developed by CRISPR-Cas9 have been used to develop a biocontainment strategy so that the engineered strain would not be able to proliferate in the environment after being excreted. In this regard, 7 auxotroph genes (URA3, HIS3, TRP1, SNO1, SNZ1, THI2 and THI6) and 2 genes related to temperature tolerance (REI1 and BTS1) were disrupted. The knockout (KO) of SNO1 and SNZ1 showed no significant growth defect in media lacking the correspondent nutrients. The simultaneous KO of URA3, THI6 and BTS1 resulted in a strain that had a growth defect at temperatures lower than 20°C and in media without uracil and thiamine. This strain could also produce a glucagon-like peptide-1 receptor agonist Exendin-4 at similar levels compared to *S. boulardii* uracil auxotroph meaning that the biocontainment strategy did not impact the target expression [244].

Gene KO can also be useful to improve the expression of a targeted molecule. In *S. boulardii*, CRISPR-Cas9 was used for the knockout of 14 different proteins involved in secretory pathways. The results showed that KO of some of the genes responsible for vacuolar /extracellular degradation (YPS1, PRB1 and PEP4) had the most positive impact on expression of peptide NPA, Table A1.3. The combination of the KO of YPS1, PRB1, PEP4 and APE1 resulted in a 1004% increased production of NPA when compared to the strain without these genes disrupted. These tests were done using a 2μ plasmid expression system but, when combining the quadruple KO strain with NPA genome integration, the expression increased by 13.2-fold when compared with a strain with only NPA integrated [245].

CRISPR-Cas9 can also be used for correction of mutations as demonstrated by the correction of the PGM2 mutation in *S. boulardii* [246],[247]. This gene is an example of the differences between *S. cerevisiae* and the

probiotic yeast. In the latter, PGM2 transcription is terminated earlier resulting in a truncation that negatively impacts galactose metabolism. In *S. cerevisiae*, the galactose metabolism is more efficient as the PGM2 is correctly terminated. The truncation of PGM2 was firstly reported by Liu et al. (2018) and the correction of the mutation resulted in a *S. boulardii* able to utilize galactose [246]. Durmusoglu et al. (2024), went further and on top of the correction of the PGM2 mutation, tested several inducible promotors including GAL, that was previously reported not to drive any protein expression in the probiotic yeast [211]. The GAL1 promotor was able to direct expression in *S. boulardii* with a corrected PGM2 and was then used for the surface display of SA1 (protein that binds and inhibits the activity of toxin A from *C. difficile*). Besides, it was also used to control expression of nanoluciferase and to build a genetic logic gate where this protein would only be produced when galactose and anhydrotetracycline where simultaneously present [247].

1.11. Applications of genetically engineered *S. boulardii*

Regardless of the genetic engineering strategy, improving the probiotic effects of *S. boulardii*, towards a health-related application has been the end goal in all studies in literature. The studies that take on a more methodological/characterization approach are essential to better understand how genetic engineering tools can be applied to enhance *S. boulardii* probiotic potential. From the 38 studies related to *S. boulardii* genetic engineering discussed, 29 are directly focused on engineering a probiotic aimed to further improve human health. The applications can be sub-divided in immune system response, pathogen neutralization, retention time improvement, anti-obesity effects, vitamin precursor production, biocontainment, use of alternative carbon sources, use as biosensor and others (Figure 1.7). Enhancing the capacity of *S. boulardii* to use alternative carbon sources is the subcategory that attracted more focus, followed by the potentiation of its neutralizing ability by secreting antimicrobial proteins, and the enhancement of the gut residence time.

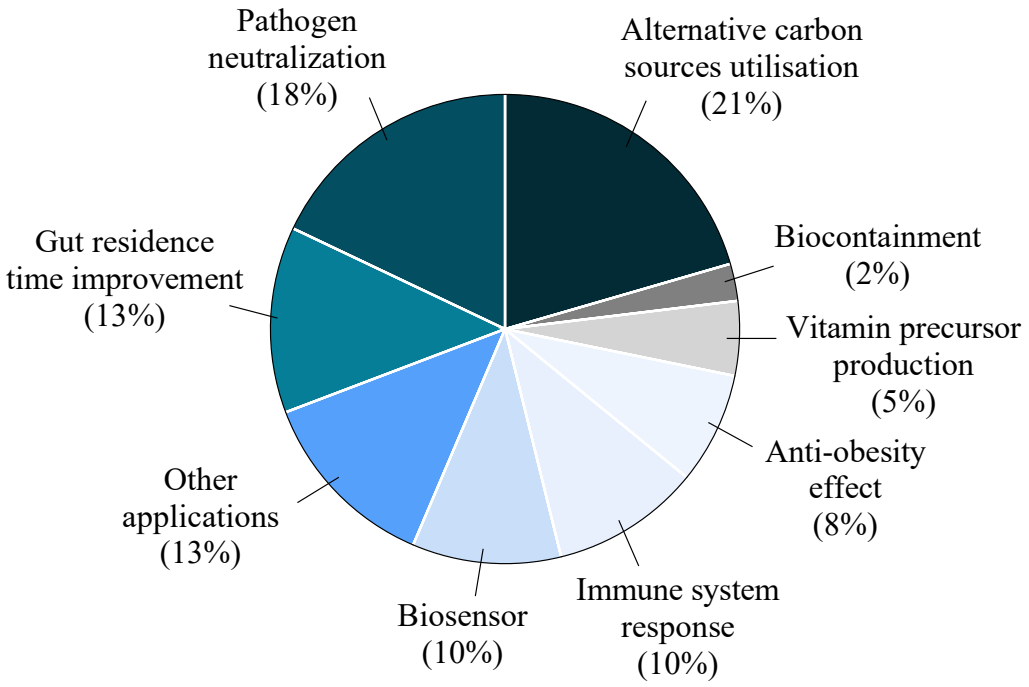


Figure 1.7. Overview from the health-related applications of genetically engineered *S. boulardii* (38 studies total published between the years 2013 and 2025). © 2025 by the authors; Carvalho et al., 2025. licensed under CC BY 4.0, unmodified.

1.12. Genetically enhanced probiotic: what are the regulations?

There are 45 completed clinical trials involving *S. boulardii* (clinicaltrials.gov), and several probiotic products containing this yeast are already in the market. However, no engineered strains of *S. boulardii* have yet reached the clinical trial stage. Most of the studies reviewed here focus on *in vitro* and preclinical data from animal models. Several key factors may explain the absence of clinical data. First, the strict regulatory framework surrounding GMOs, which varies depending on whether the product is classified as a drug or as a supplement, poses significant hurdles. Although the *in vitro* results discussed here are promising and point to a range of potential applications, there is still a lack of data of safety, scalability and biocontainment. This gap likely limits investment in clinical trials for engineered *S. boulardii*. Despite a strong market potential and clear clinical needs for some areas, regulatory approval remains uncertain.

Another factor influencing the market are public perception and concerns over genetically modified probiotics despite the accumulation of data suggesting the solution for other growing concerns like obesity, malnutrition, inefficient therapies for GI disorders [248] and the ever-growing antibiotic resistance from gut pathogens [249],[250].

The regulation of genetically modified probiotics is reviewed elsewhere [251],[252]. These regulations differ significantly across regions of the world and lead to different regulatory paths depending on the intended use and claims of the product. Table 1.2 describes the different regulatory frameworks found in different countries around the world as well as the products marketed to date related to genetically modified probiotics.

Table 1.2. Regulatory agencies responsible in different countries around the world for the regulation of genetically modified probiotics. [253],[254]

Region	Primary Regulator	Relevant Regulation(s)	Products in the market	Ref.
US	FDA, USDA	- Dietary Supplement Health and Education Act (1994) - National Bioengineered Food Disclosure Standard (2016)	ZBiotics® Sugar-to-Fiber, ZBiotics® Pre-Alcohol	[253],[254]
EU	EFSA	- Regulation (EC) No 1829/2003 (GMO food and feed) - Directive 2001/18/EC (environmental release) - Regulation (EC) No 1924/2006 (health claims)	None	[255],[256]
Canada	Health Canada, CFIA	- Natural Health Products Regulations (2004) - Canadian Environmental Protection Act (1999)	None	[257],[258]
Australia & New Zealand	FSANZ, TGA	- Food Standards Code, Standard 1.5.2 (GMOs and novel foods)	None	[259]
Japan	CAA, MHLW	- Food Sanitation Law; - Standards for Genetically Modified Foods	None	[260]
China	NMPA, MARA	- Agricultural Genetically Modified Organisms Safety Regulation (2001)	None	[261]
India	FSSAI, GEAC	- Environmental Protection Act (1986) - Food Safety and Standards Regulations (2011)	None	[262]
Brazil	ANVISA, CTNBio	- Biosafety Law (Law No. 11.105, 2005)	None	[263]

Interestingly, in the US, the company Zbiotics, got approval to market a genetically modified nutritional supplement that prevents hangover by breaking down acetaldehydes produced from alcohol, consisting of *B. subtilis* strain expressing acetaldehyde dehydrogenase [255],[256] (ZBiotics® Pre-Alcohol) and recently, a second product containing an engineered *B. subtilis strain* producing levansucrase, which catalyzes the conversion of sucrose to levan, a prebiotic (ZBiotics® Sugar-to-Fiber) [257]. These products adhere to the dietary supplement standards, must comply with Good Manufacturing Practices and its claims focus on structure and function. In regulatory terms, it operates within the framework for probiotics and GMOs but avoids claims that could classify it as a drug or therapeutic product.

1.13. Can *S. boulardii* be engineered to improve its therapeutic properties? Conclusions and future perspectives

Advancing the research on how to improve probiotic effects via genetic engineering tools offers the opportunity to use *S. boulardii* as a platform for the delivery of different molecules directly into the human gut. The characterization of genetic parts is of utterly importance to understand the basis and translate those to applications; from improving gut modulation to the targeted delivery of therapeutic proteins, the possibilities for the use of this probiotic have been demonstrated in different studies. There is an increasing interest of the scientific community in this probiotic, as demonstrated by the increasing number of publications. Since not all *S. boulardii* strains that have been sequenced have also been genetically modified (36%, Table 1) it is expected that variations occur between strains. However, the genomic data available and the characterization of several genomic parts already provides a strong foundation for future genetic modifications.

The genomic engineering tools from *S. cerevisiae* have been successfully applied in *S. boulardii*, showing effective outcomes for both plasmid-based expression systems and genome integration techniques. For the use of this probiotic, the development of CRISPR-Cas-based techniques, has been of great importance, since it allows the scarless and marker-free integration of the desired modifications, which will likely address safety and regulatory requirements. Ethical concerns, potential off-targets and careful genetic designs are key aspects that must be addressed when developing such products and will ensure that advances in biotechnology can be translated to improvements in human well-being.

Altogether we expect that as knowledge of the gut microbiota advances, as well as effective genetic engineering tools, further genetic modification of probiotics to deliver molecules of interest to the gut will be of utmost importance for improved human therapies.

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Chapter II.

CRISPR-Cas9 engineered *Saccharomyces cerevisiae* for endolysin delivery to combat *Listeria monocytogenes*

This chapter focuses on the engineering of *S. cerevisiae* strains, using CRISPR-Cas9, to express Ply511, an anti-Listeria endolysin. We prove that the surface display of the protein does not lead to a reduction in *Listeria* numbers and we demonstrate that secreting the endolysin to the extracellular media, did lead to a reduction in *Listeria*, using the whole cells, the supernatant and the cell extracts.

Part of the tasks related to surface display were performed in the EGBCR - Engineering Biology Research Centre in Kobe University (Kobe, Japan) under the supervision of Prof. Tomohisa Hasunuma.

This chapter is based on the following original research article:

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Abstract

Listeriosis is an infection caused by the consumption of food contaminated with *Listeria monocytogene*s. It leads to febrile gastroenteritis, central nervous system infections and even death in risk populations. Bacteriophage endolysins selectively kill bacteria hydrolyzing their cell walls and have emerged as a potential tool for listeriosis control. Ply511 is an anti-listeria endolysin that has activity against all serovars of *L. monocytogenes.* The yeast *Saccharomyces cerevisiae* has been used to produce endolysins for biocontrol, but prior efforts relied on plasmids, which can lead to gene loss and include selection markers unsuitable for therapeutic use. Integration of endolysins in its genome has also been previously demonstrated, relying however, on selection markers for selection and maintenance of the modifications. This study explores *S. cerevisiae* as a generally regarded as safe (GRAS) platform for producing and displaying Ply511 through CRISPR-Cas9 integration, offering a marker-free and stable solution for *Listeria* biocontrol. Our results demonstrate that the surface display of Ply511 does not lead to bacterial reduction. In contrast, we show that yeast secreting endolysin significantly reduces *L. monocytogenes* in cells, supernatants, and cell extracts. The strongest effect was observed with concentrated spent supernatant and cell extract, which reduced *L. monocytogenes* below the lower limit of quantification. Additionally, the spent supernatant exhibited active anti-*Listeria* activity in milk. This study highlights yeast-secreted endolysins as a promising platform for listeriosis control and demonstrates the yeast secretion of endolysins can be used for the biocontrol of pathogenic bacteria.

Keywords: *Listeria monocytogenes*, endolysin, CRISPR-Cas9, biocontrol, engineered *Saccharomyces cerevisiae*, probiotics.

2.1. Introduction

Listeria monocytogenes is a Gram-positive pathogen causative of human and animal infections, normally acquired through ingestion of contaminated food. The range of foods linked to listeriosis goes from ready-to-eat products to fresh produce and dairy [2]–[4]. Listeriosis can lead to severe systemic infections including meningitis, septicemia, and abortion in risk groups, with a high (20%) case-fatality rate [5].

The food industry has established strict food safety protocols, such as pasteurization, refrigeration and/or the use of preservatives. However, *L. monocytogenes* is able to survive in refrigerated temperatures and can be found in processing plants [6]. As a result, there is a need to develop alternative strategies for control, that can be applied in situ, both during food production and storage, to minimize contamination risk. There is a growing interest in the incorporation of yeast as probiotic supplement, especially in dairy products [7]. Moreover, symptoms of listeriosis in humans are often treated with antibiotics [8],[9]. However, antibiotic-resistant strains of *Listeria* have already been reported [10], underscoring the urgent need for alternative treatments.

Bacteriophage endolysins, also known as enzybiotics, are enzymes that degrade bacterial peptidoglycan and have been proposed as an alternative to antibiotics to treat bacterial infections [11]. Endolysins are highly specific [12],[13], not harming beneficial microbes, which is a common concern for the food industry and for human health. Endolysin Ply511 has shown to be a promising tool for biocontrol since it is active against all serovars of *Listeria* spp. [14],[15]. The use of endolysins against *Listeria* could serve as a treatment or as a way of prevention either in a food matrix, or in the human intestine, e.g. as probiotic or postbiotic, as it has been proposed before for endolysins [12]. These strategies, however, depend on the production and the delivery of an active endolysin against the pathogen of interest.

The yeast *Saccharomyces cerevisiae* is a generally recognized as safe (GRAS) organism and is widely used for the display and secretion of heterologous proteins. The use of *S. cerevisiae* for heterologous protein display on its surfaces has been shown to enhance protein stability [16], which can be beneficial in low pH environments, such as food matrices, or for gastrointestinal delivery. In fact, *S. cerevisiae* has previously been used to produce endolysins LysA and LysA2 against *Limosilactobacillus fermentum* and *Levilactobacillus brevis* [17] and to display endolysin LysKB317 against *L. fermentum* [18] or LysSA11 against *Staphylococcus aureus* [16]. These studies showed that *S. cerevisiae* can be used for endolysin production, however, they rely

on the use of plasmids (and their selection markers) or induction strategies, which would make application and commercialization challenging. In contrast, the use of CRISPR-Cas9 to integrate the genetic modification assures a marker-free, stable and scarless chromosomal integration [19].

In this study, we report for the first time the use of CRISPR-Cas9 to engineer *S. cerevisiae* strains for the expression of the anti-*Listeria* endolysin Ply511 in both surface-displayed and secreted forms. We explore the potential of these engineered yeast strains as a platform for biocontrol applications, evaluating their activity against different serovars of *L. monocytogenes* and assessing their performance in a food matrix. This study lays the groundwork for the development of yeast-based systems for endolysin production, highlighting their potential in food safety and pathogen management strategies. By expressing endolysin, yeast can also be utilized as an innovative food additive, using its antimicrobial properties to target and lyse harmful bacteria, thereby enhancing food preservation and safety.

2.2. Materials and Methods

2.2.1. *Escherichia coli* transformation and maintenance

E. coli DH5/NZY5α (Nzytech, Lisbon, Portugal) was used for plasmid construction and propagation. *E. coli* NZY5α transformation was conducted according to NZY5α competent cells protocol (Nzytech, Lisbon, Portugal). Briefly, 5 µL of assembled plasmids (2 to 20 ng/µL) were added to *E. coli* competent cells. After 30 minutes of incubation on ice, cells were heat shocked at 42 °C for 40 seconds. Then, cells were incubated on ice for 2 minutes and 150 µL of SOC medium (Nzytech, Lisbon, Portugal) was added. After growing for 1 hour (37 °C, 200 revolutions per minute (rpm)), cells were plated on LB agar supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Positive transformants were confirmed by colony PCR and were propagated. All the plasmids were purified using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Burlington, MA, USA). The correct cloning was verified using Sanger sequencing by Eurofins Genomics (Ebersberg, Germany). A list of primers used for colony PCR are provided in Table A2.1.

2.2.2. Plasmids

Table 2.1 lists all plasmids used in this study. Plasmids and primers utilized in cloning steps are shown in Appendix (Table A2.1). The In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) was used for plasmid

assembling. The integrative plasmid pCfB3035-Ply511-Sed1_SD, pCfB2904-Ply511-Sed1_SD and pCfB2909-Ply511-Sed1_SD for cell surface display of endolysin Ply511 were constructed from the plasmid pCfB3035, pCfB2904 or pCfB2909 (Jessop-Fabre et al. 2016), for integration into *S. cerevisiae* chromosomes X-4, XI-3 and XII-5, respectively.

Surface display cassette was obtained from pI2-EG-kanMX [20] by substitution of *Trichoderma reesei* endoglucanase for the codon optimized cassette synthesized by GenScript (Rijswijk, Netherlands) containing DNA sequences for*:* Ply511 (EC:3.5.1.28) gene for *S. cerevisiae* followed by a GS-based linker (GSSGGS) – epitope tag from *simian virus 5* (V5-tag) - GS-based linker$(G_4S)_3$ and the recognition and cleavage site for human rhinovirus 3C and PreScission proteases (HRV3C cut site).

The integrative plasmids pCfB3035-Ply511_SEC, pCfB2904-Ply511_SEC and pCfB2909-Ply511_SEC were obtained from their surface display counterparts described above (Table 2.1), using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan). Gene maps with annotated sequences are provided in Fig. A2.1 and Fig. A2.2.

Table 2.1. Plasmids used in this study

Plasmid name	Description	ntegration site	eference
CfB3035-Ply511-Sed1_SD	smid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, GS-based linker, *V5 epitope*, GS-based linker, HRV 3C cut site, *Sed1* anchor, *Sag*1 terminator and homology arms for site X-4	X-4	his study
CfB2904-Ply511-Sed1_SD	smid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, GS-based linker, *V5 epitope*, GS-based linker, HRV 3C cut site, *Sed1* anchor, *Sag*1 terminator and homology arms for site XI-3	XI-3	his study
CfB2909-Ply511-Sed1_SD	smid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, GS-based linker, *V5 epitope*, GS-based linker, HRV 3C cut site, *Sed1* anchor, *Sag*1 terminator and homology arms for site XII-5	XII-5	his study
3035-Ply511_SEC	smid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, *Sag1* terminator and homology arms for site X-4	X-4	his study
2904-Ply511_SEC	smid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, *Sag1* terminator and homology arms for site XI-3.	XI-3	his study

32909-Ply511_SEC	ısmid containing DNA sequences for *Sed1* promoter, *Sed1*SS, *Ply511*, *Sag1* terminator and homology arms for site XII-5	XII-5	his study
CfB2312 (Cas9)	:as9 expression plasmid for marker-free integration	-	[19]
B3042 (gRNA-X-4)	Guide RNA plasmid targeting site X-4	X-4	[19]
33045 (gRNA-XI-3)	Guide RNA plasmid targeting site XI-3	XI-3	[19]
33050 (gRNA-XII-5)	Guide RNA plasmid targeting site XII-5	XII-5	[19]
pI2-EG-kanMX	:e display cassette containing DNA sequences encoding *Sed1* promoter, *Sed1*SS, *Sed1* anchor and *Sag1* terminator	-	[20]

2.2.3. Yeast transformation and maintenance

The yeast *S. cerevisiae* CEN.PK113-7D [21] was used for transformation and is referred to as *S. cerevisiae* or yeast throughout the text.

S. cerevisiae was transformed using the PEG/Lithium acetate method [22]. To generate the engineered strains, first, yeast was transformed using a Cas9-expressing plasmid (pCf2312). Following that transformation, the guide RNA plasmid (pCFB3050, targeting to XII-5, X-4 or XI-3 integration site, respectively) was transformed into the Cas9-expressing strains together with the corresponding linearized integrative vector constructed. For multiple integrations, the yeast was simply curated from the plasmid carrying the guide RNA before the subsequent transformations targeting the other two integration sites. Before activity testing, all yeast were curated from both plasmids by serial passaging in liquid or agar without antibiotics.

Yeast strains were propagated at 30 °C and maintained at 4 °C on YPD plates (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar). For plasmid-carrying yeast strains, YPD media was supplemented with 200 µg/mL of geneticin G418 (Cas9-expressing plasmid, pCf2312) or/and 100 µg/mL nourseothricin sulfate (guide RNA-containing plasmids).

For yeast colony PCR, a colony was picked with a sterile toothpick and transferred to a fresh plate. The rest of the colony was then diluted in 50 µL of NaOH 20mM. After incubation at 95 °C for 5 minutes, 1 µL was

added to a microtube containing NZYTaq II 2x Green Master Mix (NZYtech, Lisbon, Portugal) and the primers to verify the correct transformations (Table 2.2).

Table 2.2. Primers used in this study for the verification of the chromosomal insertion.

Primer ID	Sequence (5'–3')	Target locus	Reference
905	CTCACAAAGGGACGAATCCT	X-4	[19]
906	GACGGTACGTTGACCAGAG	X-4	[19]
911	GTGCTTGATTTGCGTCATTC	XI-3	[19]
912	CACATTGAGCGAATGAAACG	XI-3	[19]
899	CCACCGAAGTTGATTTGCTT	XII-5	[19]
900	GTGGGAGTAAGGGATCCTGT	XII-5	[19]

2.2.4. Fluorescence microscopy analysis

Yeast wild-type or *Scv*-Ply511-Sed1-XII-5 were cultured for 96 h at 30 °C and 200 rpm. After incubation, it was centrifuged for 5 min at 2000 g and washed three to five times with phosphate buffered saline at pH 7.5 (PBS). The washed yeast suspension was added to 270 µL of a solution of PBS containing different concentration of V5 Tag Monoclonal Antibody (TCM5), PE-Cyanine7 (Thermo Fisher Scientific, Waltham, MA, USA) and incubated 30 min at 4 °C in the dark.

After incubation, the labeled yeast cells were visualized using fluorescence microscopy Olympus (Tokyo, Japan) BX51 epifluorescence microscope with fluorescence illuminator, equipped with a U-RFL-T mercury lamp light source and fluorescence filter cubes. Imaging was performed using the filter cube with excitation 545–580 nm and emission 610 nm, which was selected as the closest match for PE-Cyanine7's fluorescence properties. Exposure time varied from 3-5s upon excitation. Bright-field images were taken as controls. Data was acquired with Olympus (Tokyo, Japan) cellSens software.

2.2.5. *Listeria monocytogenes* strains

L. monocytogenes strains used for this study were obtained from CECT (Valencia, Spain): CECT 911 (Serovar 1/2c), CECT 937 (Serovar 3b), CECT 938 (Serovar 3c), CECT 5672 (Serovar 4b), CECT 939 (Serovar 4c). Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA) was used for their growth. Tryptic Soy Broth with 1.5% agar or Oxford Selective Agar (Sigma-Aldrich, Burlington, MA, USA) was used for their maintenance and enumeration.

2.2.6. Evaluation of enzymatic activity

To evaluate the enzymatic activity of yeast-expressed endolysin, we prepared a lawn of heat-killed *L. monocytogenes* on YPD-agar. *L. monocytogenes* strains were grown overnight in Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA*)* at 37°C. The cell suspension was heat-killed by autoclaving at 121°C for 20 minutes. The heat-killed bacterial cells were then concentrated 50-100x by centrifugation and resuspended in YPD-agar (1.5% agar) to achieve an opaque, homogeneous mixture. Yeast were either spotted (5 µL) or spread (100 µL) onto the YPD-agar plates containing the heat-killed *L. monocytogenes* suspension. Control plates included spots of wild-type yeast (WT) and cell extract containing the endolysin alone as a positive control. Plates were incubated at 30°C for 48 hours. Peptidoglycan degradation was assessed by observing zones of clearing around yeast colonies or spots, indicating enzymatic activity.

To produce the cell extracts containing the endolysin Ply511, *E. coli BL21* (DE3) cells (ThermoFisher Scientific, Waltham, MA, USA), containing the recombinant plasmid (pQE-30_pHPL511,[23]) were grown at 37 °C in LB medium supplemented with ampicillin at 100 µg/mL until an optical density at 600 of 0.5–0.6 was reached. Protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG, Sigma-Aldrich, Burlington, MA, USA), followed by incubation for 24 h at 16 °C, 120 rpm. The disruption of cells was performed as described previously, through thaw–freezing cycles and sonication [24]. The crude cell extract resulting from the sonication was filtered and used as positive control.

2.2.7. Evaluation of anti-*Listeria* activity of yeast cells

To evaluate the killing activity of yeast against *L. monocytogenes*, co-culture assays were conducted. Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 20

mL of YPD, in a 100 mL Erlenmeyer flask and grown at 30 °C and 200 rpm for 96 h. Yeast cells were then centrifuged at 5,000 x g for 10 min and washed three times with Tris buffer (50 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of ~10^9 colony forming units (CFU)/mL. *L. monocytogenes* cells were grown overnight, centrifuged at 9,000 x g for 10 min, washed, and resuspended and diluted in the same Tris buffer, to a concentration of ~10^4 CFU/mL.

Yeast and *L. monocytogenes* cells were mixed (900 µL with 100 µL, respectively) in a 24-well plate and incubated at 150 rpm and 30 °C. Co-cultures were sampled at 3 h, 6 h, and 24 h. Viable *L. monocytogenes* cells were quantified by plating serial dilutions onto Oxford Selective Agar (Sigma-Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated.

2.2.8. Evaluation of anti-*Listeria* activity of yeast supernatants

To evaluate the killing activity of yeast against *L. monocytogenes*, co-culture assays were conducted. Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 20 mL of YPD, in a 100 mL Erlenmeyer flask and grown at 30 °C and 200 rpm for 96 h. Yeast cells were then centrifuged at 5,000 g for 10 min and supernatants were recovered. This supernatant was then filtered through a 0.22 µm sterile polyethersulfone membrane filter to remove any residual yeast cells. The expected molecular weight of Ply511 is 36 kDa, therefore, to concentrate the protein, filtered supernatants were concentrated 10-fold using Amicon Ultra-15 Centrifugal Filter Units with a 30 kDa molecular weight cut-off (MilliporeSigma, Burlington, MA, USA) by centrifugation at 5,000 × g until the desired volume was reached. Concentrated supernatants were collected and stored at 4 °C if used on the same day, or at -20 °C until further use.

L. monocytogenes cells were grown overnight, centrifuged at 9,000 x g for 10 min, washed, and resuspended and diluted in Tris buffer (as mentioned above), to a concentration of ~10^4 CFU/mL.

Concentrated supernatants (900 µL) and *L. monocytogenes* cells (100 µL) were mixed in a 24-well plate and incubated at 150 rpm and 30 °C. Co-cultures were sampled at 3 , 6 , and 24 h. Viable *L. monocytogenes* cells were quantified by plating serial dilutions onto Tryptic Soy Broth with 1.5 % agar (Sigma-Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h, and colony-forming units (CFU/mL) were calculated.

For the evaluation of the anti-Listeria activity in milk, concentrated supernatants (400 µL) and *L. monocytogenes* cells diluted in semi-skimmed milk (50 µL) were mixed with semi-skimmed milk (350 µL) in a 24-well plate and incubated at 150 rpm and 4 °C. Semi-skimmed milk was previously pasteurized by heating the product to 90 °C for 30 min. Samples were taken at 6, 24 and 48 h. Viable *L. monocytogenes* cells were quantified by plating serial dilutions onto Oxford Selective Agar (Sigma-Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated.

2.2.9. Evaluation of anti-*Listeria* activity of yeast extracts

To evaluate the killing activity of yeast against *L. monocytogenes*, co-culture assays were conducted. Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 20 mL of YPD, in a 100 mL Erlenmeyer flask and grown at 30 °C and 200 rpm for 96 h. Yeast cells were then centrifuged at 5,000 g for 10 min and washed with Tris buffer (50 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of ~10^9 CFU/mL.

To prepare the sonicated yeast extract, cell suspensions were subjected to sonication using a Cole-Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA). Sonication was carried out in three cycles of 5 minutes each, with 10 intervals of 30 seconds ON and 30 seconds OFF at 40% amplitude. The sonicated extract was then centrifuged at 10,000 × g for 10 minutes to remove cell debris, and the supernatant was filtered through a 0.22 µm sterile filter (Millipore-Sigma, Burlington, MA, USA) to ensure sterility. The filtered sonicated extract was stored at 4 °C if used on the same day, or at –20 °C until further use.

L. monocytogenes cells were grown overnight, centrifuged at 9,000 g for 10 min, washed, and resuspended and diluted in the same Tris buffer, to a concentration of ~10^4 CFU/ml.

Yeast extracts (900 µL) and *L. monocytogenes* cells (100 µL) were mixed in a 24-well plate and incubated at 150 rpm and 30 °C. Co-cultures were sampled at 3 , 6 , and 24 h. Viable *L. monocytogenes* cells were quantified by plating serial dilutions onto Tryptic Soy Broth with 1.5 % agar (Sigma-Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h, and colony-forming units (CFU/mL) were calculated.

2.2.10. Yeast growth monitoring

To assess yeast growth, cultures were monitored in a 96-well plate using an automated system for real-time biomass evaluation. Overnight cultures were initially grown in YPD broth at 30 °C and subsequently diluted 1:100 in fresh YPD to a final working volume of 200 µL per well. Each well was inoculated with a standardized yeast suspension, and plates were incubated in a BioLector XT microbioreactor (Beckman Coulter, Brea, CA, USA) at 30 °C with shaking at 800 rpm. Optical density (OD) measurements (biomass) were recorded every 15 minutes to capture growth kinetics over time.

Yeast growth was also quantified by plating serial dilutions on YPD agar. Samples from the 96-well plate were serially diluted in saline (0.9 % NaCl solution) and plated onto YPD agar to assess colony-forming units which were counted and photographed.

2.2.11. Statistics

All data analysis was performed in GraphPad (version 9.0.0). Unpaired *t*-test were used to evaluate statistical significance. The upper threshold for statistical significance for all experiments was set at $p < 0.05$.

2.3. Results

2.3.1. Construction of various novel Ply511-displaying yeast strains

To test the yeast surface display of the endolysin Ply511, i.e., a fusion protein between a protein of the yeast cell wall (Sed1) and a protein of interest (endolysin Ply511), *S. cerevisiae* CEN.PK113-7D was used. Using CRISPR-Cas 9, the endolysin-expressing cassette was integrated in one, two or three different loci (chromosomes X, XI and XII, Table 2.3), previously reported to lead to stable insertions [19]. The endolysin expression cassette included the sequences of the *Sed1* promoter, for the Sed1 secretion signal peptide (Sed1SS), endolysin Ply511 fused with a flexible linker, a V5 epitope tag, an additional GS-based linker, the Sed1 anchor protein, and the *Sag1* terminator (Figure 2.1). The choice of promoter, anchor and terminator pair was done according to previous optimization (Inokuma et al. 2016).



Figure 2.1. Endolysin display cassette integrated into *S. cerevisiae*. The coding sequence is highlighted with orange (*Sed1* secretion signal (*Sed1*SS)-*Ply511*-GS-based linker (GSSGGS)-*V5*tag-GS-based linker$(G_4S)_3$-*HRV3C* cut site-*Sed1* anchor protein), preceded by the *Sed1* promoter and followed by the *Sag1* terminator. *V5*tag refers to epitope tag from *simian virus 5*.

The constructs were integrated in their respective locus and the correct integration was confirmed by diagnosis PCR (Fig. A2.3). The strains constructed are listed in Table 2.3.

Table 2.3. Yeast strains constructed in this study from *S. cerevisiae* CEN.PK113-7D

Strain designation	Modified locus	Donor plasmid features	Number of copies
Scv-Ply511-Sed1-XII-5	XII-5	Sequences encoding for *Sed1* promoter, Sed1 secretion signal, Ply511, GS-based linker (GSSGGS)-, V5 epitope, GS-based linker$(G_4S)_3$, HRV 3C cut site, Sed1 anchor, *Sag1* terminator	1
Scv-Ply511-Sed1-XII-5-XI-3	XII-5; XI-3	Same as above	2
Scv-Ply511-Sed1-X-4-XI-3-XII-5	X-4; XI-3; XII-5	Same as above	3

We observed no difference in growth between the wild-type (WT) strain and the constructed strains, except for the strain with the triple integration (*Scv*-Ply511-Sed1-X-4-XI-3-XII-5), which showed significantly impaired growth over a 24-hour period, as shown in Fig. A2.4.

2.3.2. V5 epitope can be detected and used as indicator of protein expression in the yeast surface

To confirm the efficient display of the Ply511 endolysin fused to the Sed1 anchor protein, a V5 epitope tag was added to the linker (Fig. 2.1). Using an anti-V5 tag fluorescent antibody the labelled cells were visualized using fluorescent microscopy, showing the display of the protein of interest uniformly on the cell-wall of the yeast (Fig. 2.2).

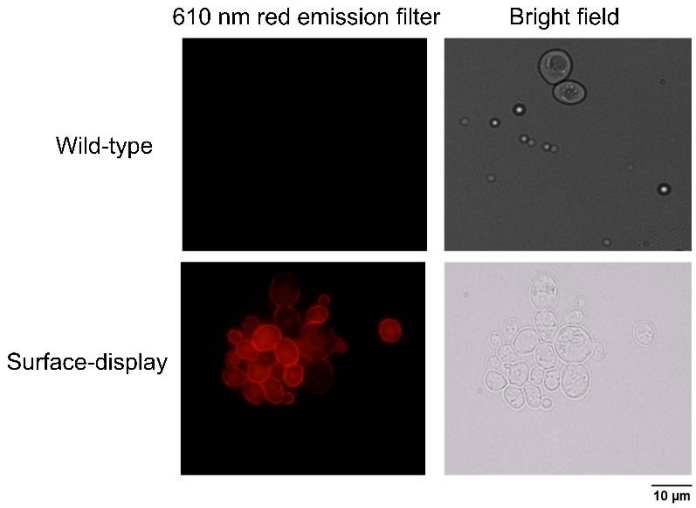


Figure 2.2. Microscopic images of wild-type yeast and yeast displaying Ply511 tagged with an anti-V5 antibody, captured under bright field and a 610 nm red emission filter, as indicated in the legend.

2.3.3. *S. cerevisiae* displaying endolysin Ply511 shows enzymatic activity

To confirm the enzymatic activity of the endolysin, we tested the yeast's ability to degrade the peptidoglycan in a lawn of heat-killed bacteria. Recombinant yeast was spotted or spread over an opaque YPD-agar mixture containing heat-killed *L. monocytogenes.* We observed that after 48 h of incubation, the recombinant yeast cleared the opaque surface around its growth by degrading the *L. monocytogenes* peptidoglycan layer (Fig.2.3), similar to the activity of free endolysin and in contrast to WT yeast (Fig. 2.3). This activity was consistent across all *Listeria* spp. serovars tested (Table A2.2), indicating that the enzyme, when fused to the yeast cell wall, retains its peptidoglycan degradation function; it is also likely that Ply511 is being liberated from the yeast cell and manages to diffuse and degrade bacterial peptidoglycan.

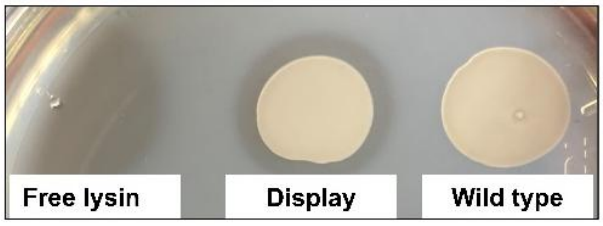


Figure 2.3. Enzymatic activity of *S. cerevisiae* displaying endolysin Ply511. Effect of the free endolysin Ply511, the yeast displaying Ply511 and the wild-type yeast over a heat-killed layer of *L. monocytogenes* CECT 939, serovar 4c.

2.3.4. *S. cerevisiae* displaying endolysin Ply511 does not have antimicrobial activity and its growth is impaired

To assess whether the recombinant yeast could effectively reduce *L. monocytogenes* in co-culture, we mixed the two species in buffer (Tris Buffer 50 mM, 200 mM NaCl at pH 8.0) and monitored viable *L. monocytogenes* cells over time. As shown in Table A2.3**,** no significant difference in *L. monocytogenes* $\log_{10}$ (CFU/mL) was observed at 3, 6 or 24 hours, even at varying yeast concentrations. Interestingly, *L. monocytogenes* continued to grow in presence of yeast over the 24-hour period. This lack of killing activity was consistent regardless of the cassette copy number (1, 2 or 3) integrated into the yeast chromosome, the buffer type (20mM Citrate, pH 5.0; Tris, pH 8.0; or PBS, pH 7.4), the *L. monocytogenes* growth phase (exponential or stationary), or the anchor protein used (Sed1 or Sag1) (data not shown).

2.3.5. *S. cerevisiae* triple integration of the Ply511 secretion cassette does not affect yeast growth

We hypothesized that the growth impairment of the yeast carrying three copies of the display of Ply511 could be due to the over-expression and saturation of the Sed1 protein in the yeast surface. Additionally, the lack of killing activity might be due to the endolysin being immobilized on the yeast cell wall, which may prevent it from freely accessing and reaching the bacterial peptidoglycan. To enhance *S. cerevisiae*'s anti-*Listeria* activity, we removed the sequences for the *Sed1* anchor from our construct, designing it to secrete Ply511 into the extracellular medium. The secretion cassette included the sequences for the *Sed1* promoter, the *Sed1* secretion signal peptide, the *Ply511* endolysin, and the *Sag1* terminator (Fig. 2.4). Using CRISPR-Cas9, we integrated the endolysin-expressing cassette into three loci on chromosomes X, XI and XII (Fig. A2.3) creating the strain *Scv*-Ply511-X-4-XI-3-XII-5.



Figure 2.4. Endolysin secretion cassette integrated into S. cerevisiae. The coding sequence is highlighted with orange (Sed1 secretion signal-Ply511), preceded by the Sed1 promoter and followed by the Sag1 terminator.

Unlike the yeast with triple integration for surface display, the yeast secreting endolysin showed no growth impairment, achieving a biomass growth rate similar to the WT in both broth and on plate (Fig. A2.5a and A2.5b).

2.3.6. S. cerevisiae secreting Ply511 is active against L. monocytogenes in co-culture

To assess if the recombinant yeast could effectively reduce *L. monocytogenes* serovar 4c in co-culture, we mixed bacterial and yeast cells in Tris Buffer and monitored viable *L. monocytogenes* cells over time. As shown in Fig. 2.5, the recombinant yeast secreting endolysin significantly reduced *L. monocytogenes* levels after 24 hours of contact.

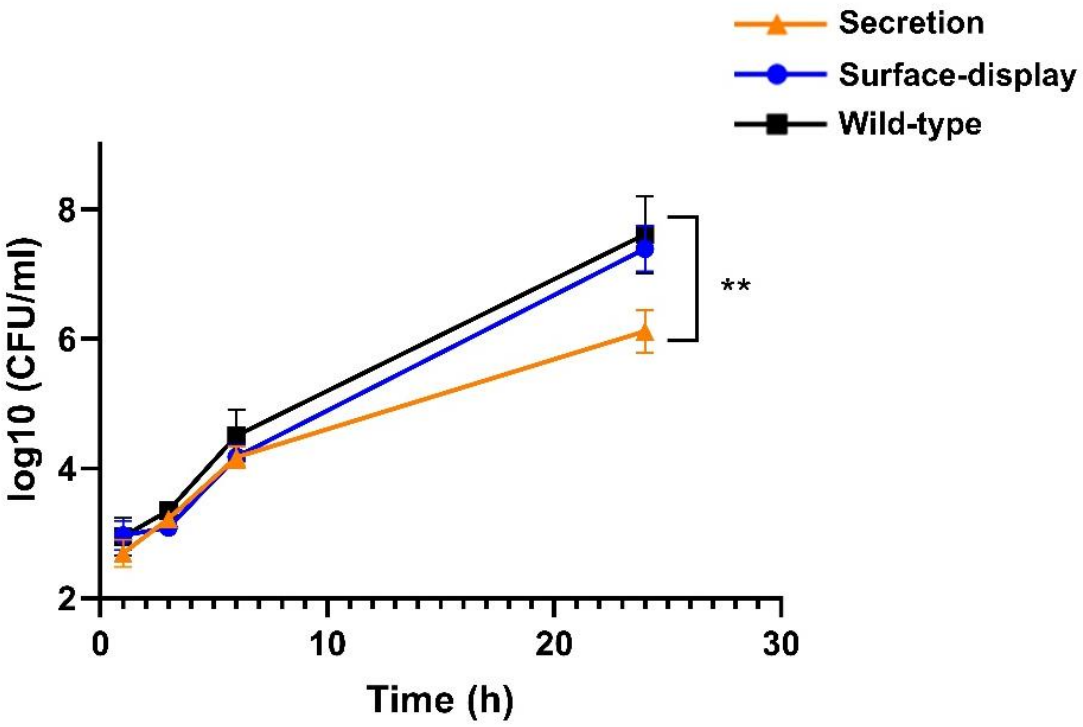


Figure 2.5. Log10 (CFU/mL) of *L. monocytogenes* serovar 4c upon mixture with *S. cerevisiae* as indicated in the legend. The figure shows the log10 (CFU/mL) mean of three replicates, standard deviation is represented as error bars. The unpaired t test comparison between the WT yeast and the yeast secreting Ply511 at 24 h, is indicated as follows: ****p ≤ 0.0001, ***p ≤ 0.001, **p ≤ 0.01, and *p ≤ 0.05.

2.3.7. Spent supernatant from S. cerevisiae secreting Ply511 shows anti-Listeria killing activity

To confirm that the yeast was effectively secreting an active protein, we tested the antimicrobial activity of the spent supernatants against three different *L. monocytogenes* serovars (1/2c, 4b and 4c). As shown in Fig. 2.6a, after 6 hours, the concentrated (10x) supernatant reduced 1.3 log_{10} (CFU/mL) in serovar 1/2c (Fig. 6a), 4.2 log_{10} (CFU/mL) in serovar 4b (Fig. 2.6b) and 1.7 log_{10} (CFU/mL) in serovar 4c (Fig. 2.6c), compared to the concentrated supernatant from the WT yeast.

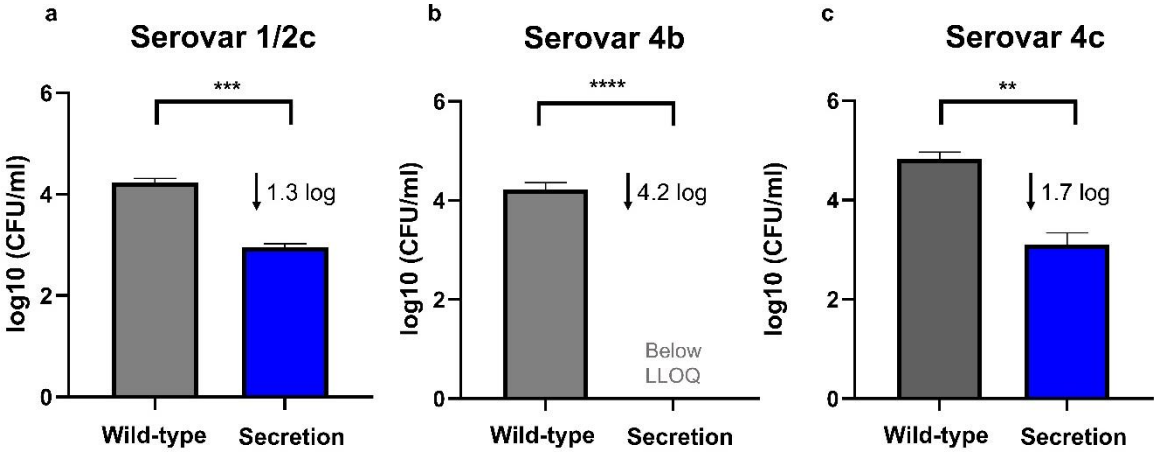


Figure 2.6. Log_{10} (CFU/mL) of *L. monocytogenes* upon mixture with *S. cerevisiae* spent supernatant concentrated (10x) from WT or SEC (secreting Ply511) after a period of 6 h. (a) serovar 1/2c (b) serovar 4b and (c) serovar 4c. The figure shows the log_{10} (CFU/mL) mean of three replicates, standard deviation is represented as error bars and the unpaired t test comparison between the two groups, indicated as follows: ***$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$. The lower limit of quantification (LLOQ) in this assay was 2.69 log10 (CFU/mL).

2.3.8. Spent supernatant shows anti-Listeria killing activity in milk

After confirming the activity of the supernatants, we investigated whether they could effectively reduce *L. monocytogenes* in milk- a food product highly susceptible to contamination with this bacterium. We mixed *L. monocytogenes* serovar 4b with 10x concentrated spent supernatant at 4°C in a 1:1 supernatant-to-milk ratio. As shown in Fig. 2.7, both mixtures allowed *L. monocytogenes* to grow over 48 hours, which may result from the nutrients left in the spent yeast supernatants or from the ability of *L. monocytogenes* to grow in milk. However, at 6, 24 and 48 hours, the supernatant from yeast secreting Ply511 reduced *L. monocytogenes* CFU/mL by 40 %, 50 % and 80 %, respectively, compared to WT supernatant. Only the reduction at 48 hours was statistically significant, corresponding to a decrease of 0.7 log_{10} (CFU/mL).

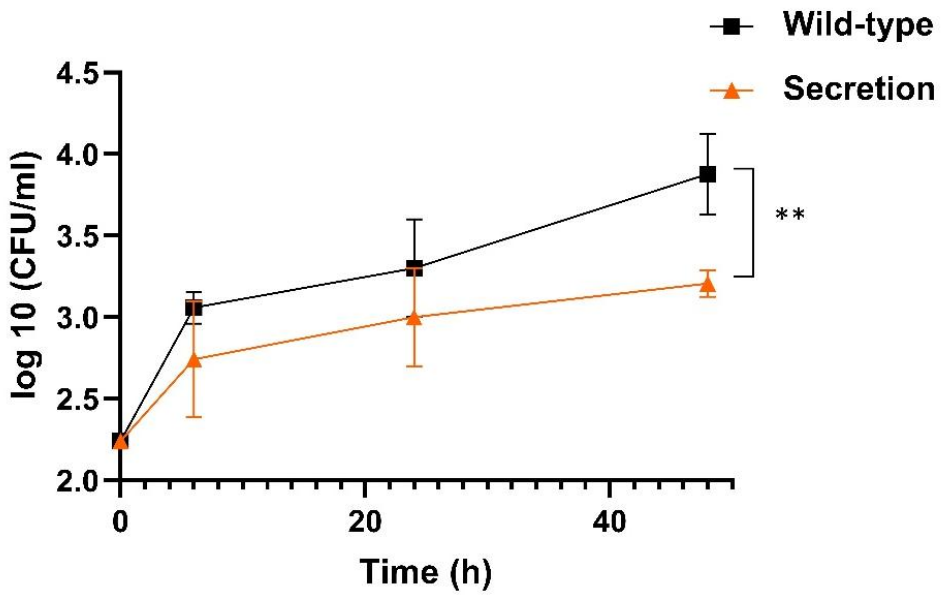


Figure 2.7. Activity of supernatants in milk. The figure represents the time-course (6 h, 24 h and 48 h of $\log_{10}$ (CFU/mL) of *L. monocytogenes* Serovar 4b upon mixture with *S. cerevisiae* cell extract from WT or SEC (secreting Ply511). The figure shows the $\log_{10}$ (CFU/mL) mean of three replicates and standard deviation is represented as error bars. The unpaired t test comparison between the two groups at 48 h, is indicated as follows: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, and *$p \leq 0.05$.

2.3.9. Improved anti-*Listeria* killing activity in yeast cell extracts

Since both yeast cells and supernatant were found to be active individually, we hypothesized that the yeast fraction might still contain some protein. To test this, we mechanically lysed the yeast cells and recovered the soluble fraction, referred to as the yeast extract. When tested against *L. monocytogenes,* we observed that while the WT yeast extract supported *L. monocytogenes* growth, the extract from engineered yeast significantly reduced *L. monocytogenes* cells to below the lower limit of quantification for serovars 1/2c (Fig. 2.8a) and 4b (Fig. 2.8b). Specifically, WT yeast extract supported growth to 8.74 and 8.42 $\log_{10}$(CFU/mL) for serovars 1/2c and 4b, respectively, after 24 hours. In contrast, no viable cells were recovered at 6 or 24 hours with the engineered yeast extract. Against serovar 4c, the engineered yeast extract reduced *L. monocytogenes* by an average of 6.5 $\log_{10}$(CFU/mL) after 24 hours (Fig. 2.8c).

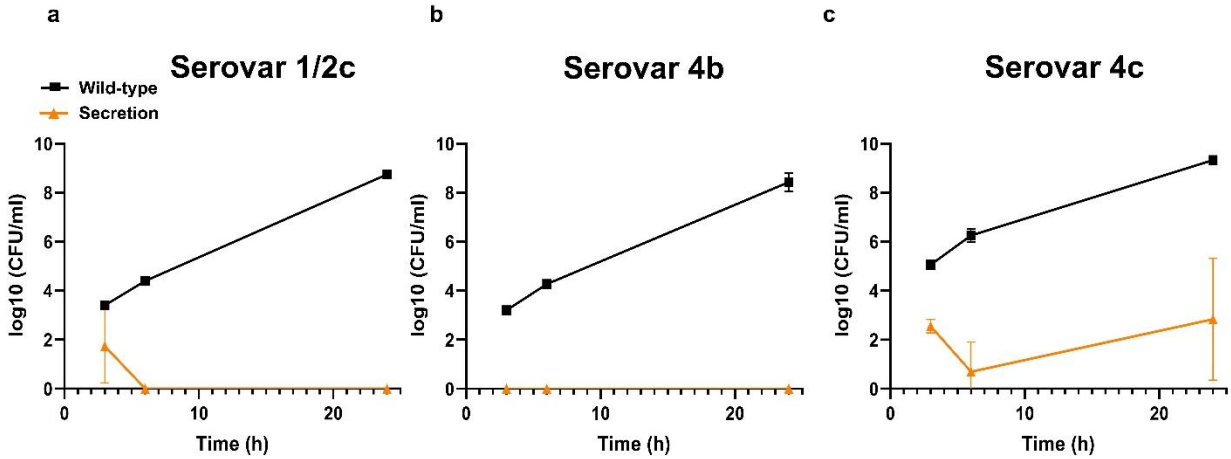


Figure 2. 8. Time-course of log10 (CFU/mL) of *L. monocytogenes* upon mixture with *S. cerevisiae* cell extract from WT or SEC (secreting Ply511) (a) Serovar 1/2c (c) Serovar 4b and (c) Serovar 4c. Values represent the mean of three replicates and standard deviation is represented as error bars. The lower limit of quantification in this assay was 2.69 $\log_{10}$(CFU/mL). SEC stands for yeast secreting Ply511 and WT stands for wild-type yeast.

2.4. Discussion

In this study, we analyzed the capacity of *S. cerevisiae* to display or secrete the anti-*Listeria* endolysin Ply511, demonstrating the potential of a yeast-based platform for endolysins expression to control listeriosis, with potential applications in food biopreservation and pathogen biocontrol.

First, we demonstrate that our engineered yeast strain, which features triple integration of the expression cassette at three different chromosomal sites, results in the production of an enzymatically active protein through both display and secretion. Since listeriosis is frequently acquired through contaminated food, this modified yeast has the potential to contribute to in-situ bio-preservation of certain food products. For applications in humans, food or feed, the genetic engineering strategy must avoid antibiotic resistance markers and ensure the stability of the modification for effective protein delivery. CRISPR-Cas9 allows for multiple integrations, enables scarless insertion and provides greater stability compared to plasmid-driven expression, while also eliminating the need for selection markers [19]. This approach not only reduces production costs but may also offer regulatory advantages for applications in both food safety and human therapy

Surprisingly, we found that displaying the endolysin Ply511 on the surface of *S. cerevisiae* did not inhibit the growth of *L. monocytogenes* compared with the WT strain. The failure of the surface displaying yeasts to lyse and kill suspended viable cells can be explained by the continuing growth of the bacteria and inability of the surface bound lysin to affect this. These negative results led us to conclude that the activity of the displayed endolysin is highly protein or host dependent. For instance, [16] reported strong activity for the endolysin LysSA11 against *S. aureus*, while [18] demonstrated the activity of yeast surface display of LysKB317 against *Limosilactobacillus fermentum.* The lack of activity observed in our study may stem from the endolysin 's mode of action, which requires diffusion through the bacterial peptidoglycan to access its substrate. This diffusion is significantly hindered when the protein is anchored to the yeast cell wall and might not be enough to achieve *L. monocytogenes* killing. Our results suggest that this effect is protein or host dependent, however, both, Chun et al. (2020) and Lu et al. (2023) used a different yeast host (EBY100) and a different anchor protein for the display (Aga2p) both factors that influence protein activity. Sed1 and Sag1 anchors allow only for an N-terminal fused anchor, we tested this configuration since Lu et al. (2023) showed that N- or C-terminal configuration did not influence activity. However, this was not tested for Ply511 endolysin. Also, further increasing linker length to avoid steric hindrance may have a positive influence in activity [20],[26].

To circumvent the lack of activity from the surface display, we decided to use the yeast for secreting the endolysin. Our results show that both the yeast cells and their spent supernatant effectively reduced *L. monocytogenes* levels. Furthermore, we improved this activity by subjecting the yeast cells to a simple mechanical lysis process to obtain a yeast extract. This approach offers an interesting application of this technology, allowing the yeast to deliver the protein in food matrices or within the gut, acting as a probiotic, or to release the protein at the site of interest without the need to keep cells alive, thereby acting as postbiotic. Such strategies may be particularly relevant for controlling *L. monocytogenes,* a foodborne pathogen that first interacts with the gut before potentially entering the bloodstream [27].

We observed that the Sed1 secretion signal peptide could effectively drive the secretion of Ply511 into the extracellular media, as previously shown with other proteins [28]. However, we also found that yeast cells, even after extensive wash and without supernatant, were still capable of reducing *L. monocytogenes* levels. This suggests that the secretion signal may be directing some of the protein to the cell membrane. We have previously observed this phenomenon with other proteins, further supporting this hypothesis (data not shown) and with a different secretion signal [29]. The fact that yeast cells reduce *L. monocytogenes* after 24hs but not

at earlier time-points, as shown in Fig. 2.5, suggests that yeast might have some metabolic activity after several hours in buffer, thus releasing active protein and/or that there is some yeast cell lysis, which might cause the release of protein. This causes the activity not to be immediate, contrary to what would happen with purified endolysins.

The endolysin Ply511 was previously expressed by *Lactococcus lactis* [30] and later in 4 different lactic acid bacteria [31]. Gaeng et al [30] showed the active expression of Ply511 in an active form by a bacterial probiotic. This report is the first one to demonstrate that yeast can express this endolysin and lead to *L. monocytogenes* reduction. The activity of the endolysin produced in yeast supernatants might be different from that of the protein being produced in bacteria, due to yeast ability to glycosylate proteins. Although the glycosylation of Ply511 was not directly verified in this study, the observed activity of yeast supernatants suggests that the potential glycosylation involved in the secretion does not abolish the activity of this endolysin.

Strategies such as promoter engineering, secretion signal engineering, genetic modifications of native genes involved in the secretory pathway or protease knockouts have been shown to improve *S. cerevisiae* secretion of heterologous proteins [32]. These strategies and better understanding of the *Sed1*SS-driven secretory pathway would allow to further improve the secretion of the endolysin, as evidenced by the fact that some protein is retained inside of the cells, ultimately leading to a better anti-listeria activity.

The engineered yeast supernatants and extracts demonstrate efficacy against *L. monocytogenes* serovars 1/2c, 4b and 4c, underscoring the broad host range of this endolysin [14],[15]. Serovar 4b exhibited the highest susceptibility to Ply511, which is significant given its prevalence in foodborne outbreaks [33]–[35]. Moreover, serovar 4b is linked to a high fatality rate and is commonly found in dairy products [34],[35]. Thus, the activity of these spent supernatants in milk is particularly promising for the potential application of this modified yeast in food preservation.

2.5. Conclusion

This study demonstrates the innovative delivery of phage endolysins via yeast, showing the potential of this technology to effectively combat *L. monocytogenes*. Future *in vivo* tests and assessment of susceptibility to digestion are crucial next steps in advancing this promising application. The vision for this yeast-based

platform extends beyond just *L. monocytogenes;* and holds significant potential targeting a variety of gut-related pathogens offering a versatile and effective approach to enhancing food safety and public health.

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Chapter III.

Evaluation of the delivery of an anti-Listeria endolysin via CRISPR-Cas9 engineered probiotic *Saccharomyces boulardii*.

This chapter is dedicated to our efforts towards creating a platform for endolysin delivery, engineering *S. boulardii* strains, adapting the *S. cerevisiae* CRISPR-Cas9 tools. We demonstrate the effects of the delivery of the endolysin Ply511 into the human gut and its microbiota. We show the activity of the yeast extract in a defined bacterial consortium (SIHUMI) and evaluated the delivery of Ply511 under gastrointestinal conditions, and its effect in two different scenarios, as whole-cell and as yeast-extract, studying its impact against *Listeria* against different simulations resembling the human gut environment.

Part of the tasks described in this chapter were performed by JP Carvalho under my co-supervision and by myself but at APC Microbiome Ireland in University College Cork (Cork, Ireland) under the supervision of Prof. Colin Hill and part at the University of Santiago de Compostela under the supervision of Prof. Alejandra Cardelle.

This chapter is based on the following original research articles:

Moreno DS, Carvalho, JP, Murray E., Rios, NS, Lamas, A, Cobas, AC, Hill, C, Azeredo J, Domingues L. Evaluation of the delivery of an anti-*Listeri*a endolysin via CRISPR-Cas9 engineered probiotic *Saccharomyces boulardii*; **Under review.**

Abstract

Listeriosis is a foodborne infection caused by *Listeria monocytogene*s that causes febrile gastroenteritis, central nervous system infections and that can often lead to fatality. Upon consumption of contaminated food, *Listeria* is able to survive a number of gastro-intestinal stressors, including competition with the host microbiota. The emergence of antibiotic-resistant clones of *L. monocytogene*s together with the side-effects of antibiotic treatment highlights the need for alternatives or additives for its treatment and prevention. *Saccharomyces boulardii* is a probiotic yeast that is often used alongside antibiotics to minimize side effects since it is not affected by them as a result of its eukaryotic nature. Furthermore, it can be engineered to produce a wide range of molecules.

We previously engineered *S. cerevisiae* through CRISPR-Cas9 integration to produce Ply511, a bacteriophage endolysin active against *L. monocytogene*s, showing the potential of engineered yeast to produce endolysins for biocontrol. In this study, we extended this approach to the probiotic yeast *S. boulardii* and directly compared the two yeasts as secretion hosts for Ply511. Using a simulated human gastrointestinal environment, we evaluated their ability to retain endolysin activity and reduce *L. monocytogenes* levels. We then tested the cell extracts from both yeasts in a bacterial consortium termed SImplified HUman intestinal MIcrobiota (SIHUMI), confirming a specificity for *Listeria*. Finally, we evaluated their activity in a simulated intestinal fermentation using fecal samples from human donors. Overall, this study demonstrates the potential of delivering endolysins to the gut via engineered probiotic *S. boulardii.*

Keywords: *Listeria monocytogenes*, endolysin, CRISPR-Cas9, biocontrol, engineered probiotics.

3.1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, a disease producing a range of symptoms from febrile gastroenteritis to meningitis, often resulting in fatalities [1],[2]. Upon consumption of contaminated food, the bacterium can survive gastrointestinal (GI) stressors such as mechanical digestion, saliva, the low stomach pH, enzymes and bile acids [3],[4]. Once in the intestine it competes with the commensal microbiota. *L. monocytogenes* may survive these interactions and translocate across the GI barrier to initiate its infection cycle, reaching the bloodstream [5]. Moreover, it has been found in human feces which indicates that, apart from its transient colonization for the initiation of infection, it can also reside for long periods of time in the gut [6]. The abundance of *L. monocytogenes* in human fecal samples has been correlated with a low α-diversity, indicating the importance of a varied microbiome for protection [6]. Antibiotic treatment has been shown to decrease α-diversity in the gut microbiota [7], which combined with the worldwide appearance of antibiotic-resistant clones of *L. monocytogenes* [8],[9], highlights the need for the development of alternatives to antibiotics for the treatment and prevention of *L. monocytogenes* infections.

Probiotics can confer health benefits upon ingestion, including enhancing protection against pathogens. An example of this protection is the production of nisin, a bacteriocin produced by *Lactococcus lactis* affecting *L. monocytogenes* [10]. The interest in modifying human host-microbiota systems for therapeutic purposes has led to the development of engineered live biotherapeutic products, or engineered probiotics, to enhance probiotic properties [11]. *Saccharomyces boulardii* is a probiotic that is often used alongside antibiotics to minimize posterior gut dysbiosis [12] since, due to its eukaryotic nature, it is not affected by antibiotics and it can be engineered to produce a wide range of molecules [13].

Endolysins, phage-derived antibacterials, also referred to as enzybiotics [14] have been proposed as an alternative or adjunct to antibiotics. Their main advantage over antibiotics to treat bacterial infections is the fact that they would be expected to have minimal impact on the host microbiome, because of their high specificity [15]. Endolysin Ply511 is active against all serovars of *Listeria spp.* [16],[17] and could be suitable for the prevention or treatment of listeriosis. To date, several studies have shown the production of different endolysins by different hosts, specifically *S. cerevisiae*, and by bacterial probiotic strain (reviewed elsewhere: [15]). However, we are not aware of any previous reports of engineering *S. boulardii* to produce endolysins, nor of directly assessing the effect of these endolysins on the gut microbiome.

We previously engineered *S. cerevisiae* to express Ply511, an endolysin effective against *L. monocytogenes* [18]. In this study, we engineered *S. boulardii* to produce Ply511 and we evaluate its performance under simulation of GI digestion. We also evaluated its effect in a SImplified HUman intestinal MIcrobiota (SIHUMI) model, a bacterial consortium composed of culturable and fully sequenced human-derived enteric strains that can be individually tracked using qPCR [19]–[21], and finally we assessed their effect in a model of the fecal microbiome, upon simulated intestinal fermentation using samples from human donors.

3.2. Materials and Methods

3.2.1. *Escherichia coli* transformation and maintenance

E. coli DH5/NZY5α (Nzytech, Lisbon, Portugal) was used for plasmid construction and propagation. *E. coli* NZY5α transformation was conducted according to NZY5α competent cell protocol (Nzytech, Lisbon, Portugal). Briefly, 5 µL of assembled plasmids (2 to 20 ng/µL) were added to *E. coli* competent cells. After 30 min of incubation on ice, cells were heat shocked at 42 °C for 40 s. Then, cells were incubated on ice for 2 min and 150 µL of SOC medium (Nzytech, Lisbon, Portugal) was added. After growing for 1 h (37 °C, 200 revolutions per minute (rpm)), cells were plated on LB agar supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Positive transformants were confirmed by colony PCR and were propagated. All the plasmids were purified using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Burlington, MA, USA). The correct cloning was verified using Sanger sequencing by Eurofins Genomics (Ebersberg, Germany).

3.2.2. Plasmids

Table 3.1 lists all plasmids used in this study. Plasmids maps and primers utilized in cloning steps are shown in [18]. The In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) was used for plasmid assembling. The integrative plasmid pCfB3035-Ply511_SEC, pCfB2904-Ply511_SEC and pCfB2909-Ply511_SEC for secretion of endolysin Ply511 were constructed from the plasmid pCfB3035, pCfB2904 or pCfB2909 [18],[22] for integration into *S. cerevisiae* or *S. boulardii* chromosomes X-4, XI-3 and XII-5, respectively.

Table 3.1. Plasmids used in this study.

Plasmid Name	Description	Integration Site	Reference
pCfB3035-Ply511_SEC	Plasmid containing DNA sequences encoding for SED1 promoter, SED1SS, Ply511, SAG1 terminator and homology arms for site X-4	X-4	[18]
pCfB2904-Ply511_SEC	Plasmid containing DNA sequences encoding for SED1 promoter, SED1SS, Ply511, SAG1 terminator and homology arms for site XI-3	XI-3	[18]
pCfB2909-Ply511_SEC	Plasmid containing DNA sequences encoding for SED1 promoter, SED1SS, Ply511, SAG1 terminator and homology arms for site XII-5	XII-5	[18]
pCfB2312 (Cas9)	Cas9 expression plasmid for marker-free integration	-	[22]
pCfB3042 (gRNA-X-4)	Guide RNA plasmid targeting site X-4	X-4	[22]
pCfB3045 (gRNA-XI-3)	Guide RNA plasmid targeting site XI-3	XI-3	[22]
pCfB3050 (gRNA-XII-5)	Guide RNA plasmid targeting site XII-5	XII-5	[22]

3.2.3. Yeast transformation and maintenance

The yeast *S. cerevisiae* CEN.PK113-7D [23] or *S. boulardii* CNCM I-745 (UL250, Biocodex, France) was used for transformation and is referred to as *S. cerevisiae or Scv* and *S. boulardii* or *Sb*, respectively, throughout the text.

Yeast transformation was performed using the PEG/Lithium acetate method [24]. To generate the engineered strains, yeast cells were first transformed with a Cas9-expressing plasmid (pCf2312). In a second step, a guide RNA plasmid (pCFB3050, targeting integration sites XII-5, X-4 or XI-3, was co-transformed into the Cas9-expressing strains along with the corresponding linearized integrative vector. For multiple integrations,

the guide RNA was removed between transformations by curing the yeast before targeting the next integration site. Prior to activity testing, all strains were curated of both plasmids through serial passaging in liquid or solid medium without antibiotics.

Yeast strains were propagated at 30 °C (*Scv*) or 37°C (*Sb*) and maintained at 4 °C on YPD plates (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar). For plasmid-carrying yeast strains, YPD media was supplemented with 200 µg/mL of geneticin G418 (Cas9-expressing plasmid, pCf2312) or/and 100 µg/mL nourseothricin sulfate (guide RNA-containing plasmids). Half of the concentration of each antibiotic was used for *Sb,* to allow for a faster recovery after the transformation protocol.

For yeast colony PCR, a colony was picked with a sterile toothpick and transferred to a fresh plate. The rest of the colony was then diluted in 50 µL of NaOH 20mM. After incubation at 95 °C for 15 minutes, 1 µL was added to a microtube containing NZYTaq II 2x Green Master Mix (NZYtech, Lisbon, Portugal) and the primers to verify the correct transformations (Table 3.2).

Table 3.2. Primers used in this study for the verification of the chromosomal insertion in *S. cerevisiae* or *S. boulardii.*

Primer ID	Sequence (5'–3')	Target locus	Reference
905	CTCACAAAGGGACGAATCCT	X-4	[25]
906	GACGGTACGTTGACCAGAG	X-4	[25]
911	GTGCTTGATTTGCGTCATTC	XI-3	[25]
912	CACATTGAGCGAATGAAACG	XI-3	[25]
899	CCACCGAAGTTGATTTGCTT	XII-5	[25]
900	GTGGGAGTAAGGGATCCTGT	XII-5	[25]

3.2.4. *Listeria monocytogenes* strains

L. monocytogenes strain used for this study were obtained from CECT: CECT 5672 (Serovar 4b). Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA) was used for their growth. Tryptic Soy Broth with 1.5% agar or Oxford Selective Agar (Sigma-Aldrich, Burlington, MA, USA) was used for their maintenance and enumeration.

3.2.5. Evaluation of enzymatic activity

To evaluate the enzymatic activity of yeast-expressed endolysin, we prepared a lawn of heat-killed *L. monocytogenes* on YPD-agar. *L. monocytogenes* strains were grown overnight in Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA) at 37 °C. The cell suspension was heat-killed by autoclaving at 121 °C for 20 minutes. The heat-killed bacterial cells were then concentrated 50-100x by centrifugation and resuspended in YPD-agar (1.5% agar) to achieve an opaque, homogeneous mixture. Yeast strains were spotted (5 µL) onto the YPD-agar plates containing the heat-killed *L. monocytogenes* suspension. Control plates included spots of wild-type yeast (WT) as a positive control. Plates were incubated at 30°C for several days and pictures were taken at different timepoints throughout incubation. Peptidoglycan degradation was assessed by observing zones of clearance around yeast colonies or spots, indicating enzymatic activity.

3.2.6. Yeast growth kinetics

To assess yeast growth, cultures were monitored in a 96-well plate using an automated system for real-time OD_{600} evaluation. Overnight cultures were initially grown in YPD broth at 30 °C and subsequently diluted 1:100 in fresh YPD to a final working volume of 200 µL per well. Each well was inoculated with a standardized yeast suspension, and plates were incubated in a Microplate reader (ThermoFisher, Waltham, MA, USA) at 37 °C with shaking. Optical density (OD) measurements were recorded every 15 min to capture growth kinetics over time. This assay was done in triplicate and under aerobic and anaerobic conditions.

3.2.7. Preparation of Yeast Extracts

Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 20 mL of YPD, in a 100mL Erlenmeyer flask and grown at 30 °C and 200 rpm for 96 h. Yeast cells were then centrifuged at 5,000 x g for 10 min and washed three times with Tris buffer (50 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of ~$5x10^9$ colony forming units (CFU)/mL.

To prepare the sonicated yeast extract, cell suspensions were subjected to sonication using a Cole-Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA). Sonication was carried out in two cycles of 10 min each, with 10 intervals of 30 s ON and 30 s OFF at 40% amplitude. The sonicated extract was then centrifuged at 10,000 × *g* for 10 min to remove cell debris, and the supernatant was filtered through a 0.22-µm sterile

filter (Millipore-Sigma, Burlington, MA, USA) to ensure sterility. The filtered sonicated extract was stored at 4 °C if used on the same day, or at – 20 °C until further use.

3.2.8. Effect of Yeast Extracts on SIHUMI Community Composition

The SIHUMI consortium consists of a set of fully sequenced human-derived intestinal bacteria. In this work, we used a modified version, we named SIHUMI-L, since it includes *L. monocytogenes* CECT 5672, a strain that efficiently grows in the same culture conditions as the other SIHUMI members: *Escherichia coli LF82, Enterococcus faecalis OG1RF, Lactiplantibacillus plantarum WCFS1, Faecalibacterium duncaniae A2–165, Bifidobacterium longum ATCC 15707, Phocaeicola vulgatus DSM1447 and Mediterraneibacter gnavus (previously termed Ruminococcus gnavus) ATCC 29149* [26].

All strains were grown in solid and liquid LYHBHI medium at 37 °C in strict anaerobic conditions and were maintained as single-use glycerol stocks at –80 °C for long periods [20],[27],[28].

We followed the protocol described previously [20]. Briefly, each SIHUMI-L strain was individually grown for 24 h in 5 mL of LYHBHI at 37°C under strict anaerobic conditions. The optical density at 600 nm (OD_{600}) of each culture was measured by spectrophotometry and cultures were diluted with LYHBHI to get a final OD_{600} of 1. Ten µl of each culture was used to inoculate four tubes containing 10 mL of LYHBHI, forming four initial SIHUMI consortia. At time 0, *L. monocytogenes* was added at a final concentration of $5x\ 10^3$, simulating the dose needed for infection. Simultaneously, yeast extracts (4mL) were added to the different SIHUMI-L-containing tubes at time 0 h.

One mL samples were collected at 0, 6, 24 h and 48h after inoculation. Samples were immediately centrifuged at 7000 rpm for 2 min, supernatants were separated from the cell pellets, and both were stored at –20 °C until processing. In parallel, viable *L. monocytogenes* cells were quantified at each time point, by plating serial dilutions of the SIHUMI-L culture onto Oxford Selective Agar (Sigma Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h to count and calculate colony-forming units (CFU/mL).

Bacterial cell pellets were used for the extraction of total genomic DNA (gDNA) using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, Arklow, Ireland) following the manufacturer's instructions and using a final elution buffer volume of 200 µl per sample.

3.2.9. Quantitative real-time PCR

The genome copy number of each species within SIHUMI-L at different time points was determined from the total gDNA extracted, by qPCR using specific primers (Table 3.3). This allowed us to assess how the population of each strain in the consortium changed over time upon the addition of the yeast extracts.

Table 3.3. Selective species-specific primers for SIHUMI member strains.

Target strain	Genome size (bp)	Genome Mass (ng)	Oligonucleotide Sequence (5´ to 3´)	Amplicon size	Ref.
E. faecalis OG1RF	2739625	3.00 X 10^6	F: ACGGAGATTGTCACGCTTAGT R: TCGGCATTATCTGGGTGGTC	122 bp	[21]
E. coli LF82	4773108	5.23 X 10^6	F: CGGGTGTTGTCCTAACTGCT R: CGAGTGGTCATTGGCCTCAT	107 bp	[21]
M. gnavus ATCC 29149	3549191	3.89 X 10^6	F: GCGTGCTTGTATTCCGGATG R: GCCTGAACAGTTGCTTTCGG	115 bp	[29]
F. duncaniae A2–165	3102523	3.40 X 10^6	F: TATTGCACAATGGGGGAAAC R:CAACAGGAGTTTACAATCCGAAG	77 bp	[30]
P. vulgatus DSM1447	4773108	5.23 X 10^6	F: AAGCAGCAGGGAAATGTGGA R: CTTTCCTTACTTGCGCGTCG	142 bp	[21]
L. plantarum WCFS1	3308274	3.63 X 10^6	F: CGAAGAAGTGCATCGGAAAC R: TCACCGCTACACATGGAGTT	71 bp	[30]
B. longum ATCC 15707	2385164	2.61 X 10^6	F: GAGGCGATGGTCTGGAAGTT R: CCACATCGCCGAGAAGATTC	108 bp	[31]

3.2.10. *In Vitro* Gastrointestinal Digestion Assay

In vitro simulation of gastric, and small intestinal digestion was performed according to the INFOGEST protocol [32], adapted to use 5x10^8 CFU/mL of yeast (*S. boulardii or S. cerevisiae)* and 5x10^3 CFU/mL of *L. monocytogenes* in 5 mL of whole milk, in duplicate. All chemicals required were obtained from Sigma–Aldrich (St. Louis, MO, USA). Oral phase was not simulated since the intended use of the product would not imply mastication.

Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 20 mL of YPD, in a 100-mL Erlenmeyer flask and grown at 30 °C and 200 rpm for 96 h, to a final concentration of ~10^9 colony forming units (CFU)/mL.

Viable *L. monocytogenes* were quantified by plating serial dilutions onto Oxford Selective Agar (Sigma Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated.

3.2.11. *In Vitro* Intestinal Fermentation with Human Fecal Microbiota

Fecal samples were donated by two men and one woman between the ages of 32 and 50 years and were recruited through a trial authorized by the Regional Ethics Committee for Clinical Research (Galician Health Service, SERGAS, n° 2018/270). Based on the eligibility criteria of that trial, individuals without gastrointestinal disorders, without chronic pharmacological treatment and who had not consumed antibiotics or pre/pro/postbiotic supplements within 6 months prior to sample collection were included. The volunteers agreed to participate in the study by signing an informed consent form that detailed the utilization of their samples. They were provided with sterile containers to collect stool samples at home. Once collected at home, the sample was delivered to the laboratory within 2 h, for processing. In the laboratory, the stool samples were diluted 1:10 with phosphate-buffered saline and used immediately for the assays.

In vitro simulation of human intestinal digestion was performed based on a published study [33] to evaluate the potential use of modified and unmodified yeast as probiotic and its impact in the microbiota, in presence of a pathogen. A negative control, with pathogen and without yeast was carried out simultaneously. Conditions resembling those of the human small intestine, including a microaerophilic atmosphere, and temperature of 37 °C were replicated, using GENbox microaer Sachets (BioMerieux, Marcy-l'Étoile, France). The sterilized medium (composition indicated below) was mixed with 0.01 % (v/v) of the previously diluted feces and a final concentration of ~$5x10^4$ CFU/mL of *L. monocytogenes* and ~$5x10^6$ *S. boulardii* or *S. cerevisiae* were inoculated into the vessels. The assays were performed for 48 h; 2 mL samples were taken for analysis at 0, 6, 24, 30 and 48 h of fermentation. Total volume was 10 mL.

Viable *L. monocytogenes* was quantified by enumeration, plating serial dilutions onto Oxford Selective Agar (Sigma Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated. The estimation of yeast CFUs at different time points (0, 24 and 48 h) was determined from the total gDNA extracted, by qPCR using specific primers targeting the genomic modifications in engineered *S. cerevisiae* or *S. boulardii* secreting Ply511 (Forward: AGACAAGCTGGCCAAACAGT, Reverse: CTGGTGCTTTGTTTGTGGGG) or primers targeting *Saccharomyces*

spp. [34] (Forward: AGGAGTGCGGTTCTTTG, Reverse: TACTTACCGAGGCAAGCTACA). Medium was prepared according to previous studies [35] with slight modifications on its composition: arabinogalactan (1 g/L), pectin from apple (2 g/L), inulin (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121 °C for 15 min.

3.2.12. Statistics

All data analysis was performed in GraphPad (version 9.0.0). Unpaired *t*-test were used to evaluate statistical significance. The upper threshold for statistical significance for all experiments was set at $p < 0.05$.

3.3. Results

3.3.1. *S. boulardii* triple integration of the Ply511 secretion cassette

Previously, we proved that CRISPR-Cas9 engineered *S. cerevisiae* was able to secrete the anti-*Listeria* endolysin Ply511 and effectively reduce *L. monocytogenes* [18]. Given that *S. boulardii* is a probiotic yeast already used commercially, we aimed to integrate the genetic modification into its genome to enhance its antibacterial activity and broaden its potential health benefits.

Firstly, we analyzed *in silico* the genomic loci X, XI and XII for compatibility between the EasyClone-MarkerFree integrative vectors and *S. boulardii genome (S. boulardii Biocodex GenBank access: LIIL00000000.1 vs S. cerevisiae S288C GCF_000146045.2),* which were the sites we previously targeted in *S. cerevisiae* in [18]. Each integration plasmid comprises two homology arms of 500bp, which facilitates the recombination after the nuclease cut, guided by the gRNA. All loci analyzed contained above 97.6% nucleotide identity between *S. cerevisiae* and *S. boulardii*, showing a potential compatibility (Table A3.1). gRNA nucleotide identity and PAM sequence presence in *S. boulardii* were also analyzed and a 100% identity was obtained in X, XI and XII integration sites (Table A3.2).

To integrate the endolysin Ply511, we used a secretion cassette containing the DNA sequences for the Sed1 promoter, the Sed1 secretion signal peptide, the Ply511 endolysin, and the Sag1 terminator (Figure 3.1).

Using CRISPR-Cas9, we integrated the endolysin-expressing cassette into three loci on chromosomes X, XI and XII (Fig. A3.1) creating the strain *Sb-Ply511-X-4-XI-3-XII-5*.



Figure 3.1. Endolysin secretion cassette integrated into *S. boulardii*. The coding sequence is highlighted with orange (Sed1 secretion signal-Ply511), preceded by the Sed1 promoter and followed by the Sag1 terminator.

3.3.2. *S. boulardii* secreting Ply511 shows enzymatic activity

To confirm the enzymatic activity of *S. boulardii* expressing the endolysin, we first tested the yeast's ability to degrade the peptidoglycan in a lawn of heat-killed *L. monocytogenes*. Recombinant yeast was spotted over an opaque YPD-agar mixture containing heat-killed *L. monocytogenes*. We observed that after 48 h of incubation, the recombinant yeast cleared the opaque surface around its growth by degrading *L. monocytogenes* peptidoglycan layer, similar to the activity of *S. cerevisiae*, as previously reported in [18] and in contrast to the wild-type yeast, which did not degrade its surroundings (Figure 3.2). Although this assay is qualitative (positive or negative) and not quantitative, we did see a difference in halo diameter between both engineered yeasts over the course of 3 to 5 days (Figure 3.2), which could indicate a higher secretion rate in *S. cerevisiae* than in *S. boulardii*.

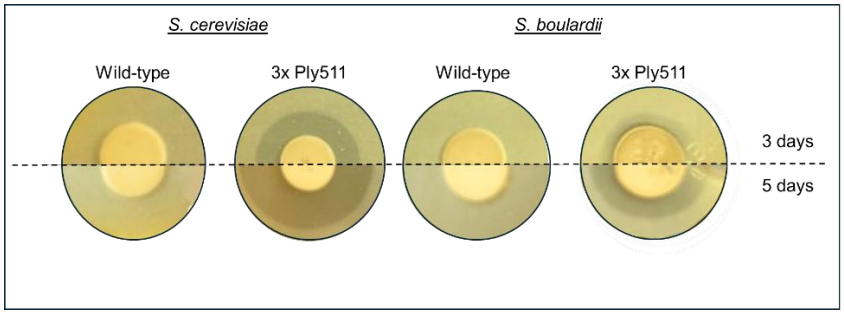


Figure 2.2. Enzymatic activity of *S. cerevisiae* or *S. boulardii* displaying endolysin Ply511 or wild type, as indicated in the legend. Effect of the yeast displaying Ply511 and the wild-type yeast over a heat-killed layer of *L. monocytogenes* Scott A.

3.3.3. *S. boulardii* secreting Ply511 growth differs depending on oxygen levels

S. boulardii secreting Ply511 showed no growth impairment aerobically and reached a similar growth (OD_{600}) to its wild-type counterpart over a period of 24h (Fig. A3.2). When its growth was tested anaerobically, *S.*

boulardii secreting Ply511 did not achieve the same growth as the wild type, after 24h the OD_{600} of the engineered yeast was 0.795, in comparison to OD_{600} of 0.999 measured for the wild type *S. boulardii*. This difference was not seen in *S. cerevisiae* strains (Fig. A3.2).

3.3.4. Yeast extracts inhibit Listeria in the SIHUMI community

We previously reported [18] that among the tested forms, yeast cells secreting endolysin and corresponding cell extracts, the cell extracts exhibited higher anti-*Listeria* activity in *S. cerevisiae* secreting the endolysin. However, it has been shown that the microbial context in which an antimicrobial acts can influence its activity [36]. Thus, to assess whether this activity would be replicated in a polymicrobial community of intestinal bacteria that would mimic intestinal conditions in a controlled manner, we used our own version of the SIHUMI community (SIHUMI-L), composed of *E. coli*, *E. faecalis, F. duncaniae, B. longum, P. vulgatus* and *M. gnavus* and *L. monocytogenes.*

We added different yeast extracts to SIHUMI-L (*S. boulardii* or *S. cerevisiae* wild-type or *S. cerevisiae* and *S. boulardii* secreting Ply511) together with a non-yeast control (containing only the SIHUMI-L community without any yeast extract).

We found that *L. monocytogenes* was able to grow within the SIHUMI consortium as indicated in Figure 4A (no extract condition), during the first 24 h, then fell after 48 h to levels below the lower limit of quantification. When in contact with all of the yeast extracts, *L. monocytogenes* levels were lower. The extract from *S. cerevisiae* Ply511 showed the highest anti-*Listeria* activity, reducing *L. monocytogenes* by 1.6 log10 (CFU) at 6 h in comparison with the wild-type extract, and could not be detected after 24 h (Fig. 3.3B). The extract from *S. boulardii* containing Ply511 only led to a slight (non-statistically significant) reduction of *L. monocytogenes* after 24 h (Fig. 3.3B). These differences in activity between *S. cerevisiae* and *S. boulardii* are in accordance with those observed on Fig. 3.2.

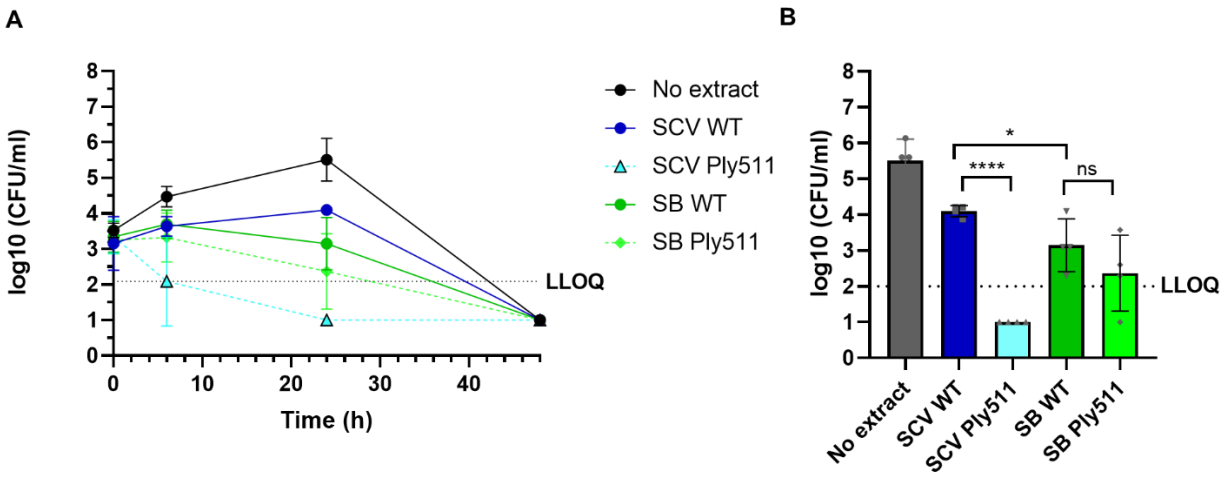


Figure 3.3. A) Log10 (CFU/mL) of *L. monocytogenes* upon mixture with the SIHUMI consortium (black), plus *S. cerevisiae* cell extract WT (dark blue), *S. cerevisiae* secreting Ply511(light blue) or *S. boulardii* cell extract WT (dark green), *S. cerevisiae* secreting Ply511(light green) after a period of 48 h. B) Log10 (CFU/mL) of *L. monocytogenes* upon mixture with the SIHUMI consortium (black), plus *S. cerevisiae* cell extract WT (dark blue), *S. cerevisiae* secreting Ply511(light blue) or *S. boulardii* cell extract WT (dark green), *S. cerevisiae* secreting Ply511(light green) at 24 h.

3.3.5. Yeast extracts do not affect the members of the SIHUMI community

Next, we evaluated the impact of this treatment on the rest of the members of the SIHUMI-L community by qPCR. No difference was observed in the outcome of the treatments with the yeast extract with or without the endolysin, which indicates that the treatment does not affect the other seven bacterial species present in the community. *E. faecalis*, *E. coli* and *B. longum* grew after 6h and dominated throughout the 48h tested. *F. duncaniae* grew slightly when mixed with *S. cerevisiae* extracts, regardless of whether those were producing Ply511 or not. When mixed with *S. boulardii* producing the endolysin, *F. duncaniae* was able to grow, but not in the presence of the extract of the wild type *S. boulardii. M. gnavus* and *P. vulgatus* were unable to grow in the SIHUMI-L community, with or without extracts. This can be the result of its interactions with L. *monocytogenes* or can be due to the cells not being viable, as depicted in Fig. 3.4. Also, *E. faecalis,* another member of the community has been reported to inhibit these strains, resulting in little to no growth in the consortium [20],[28].

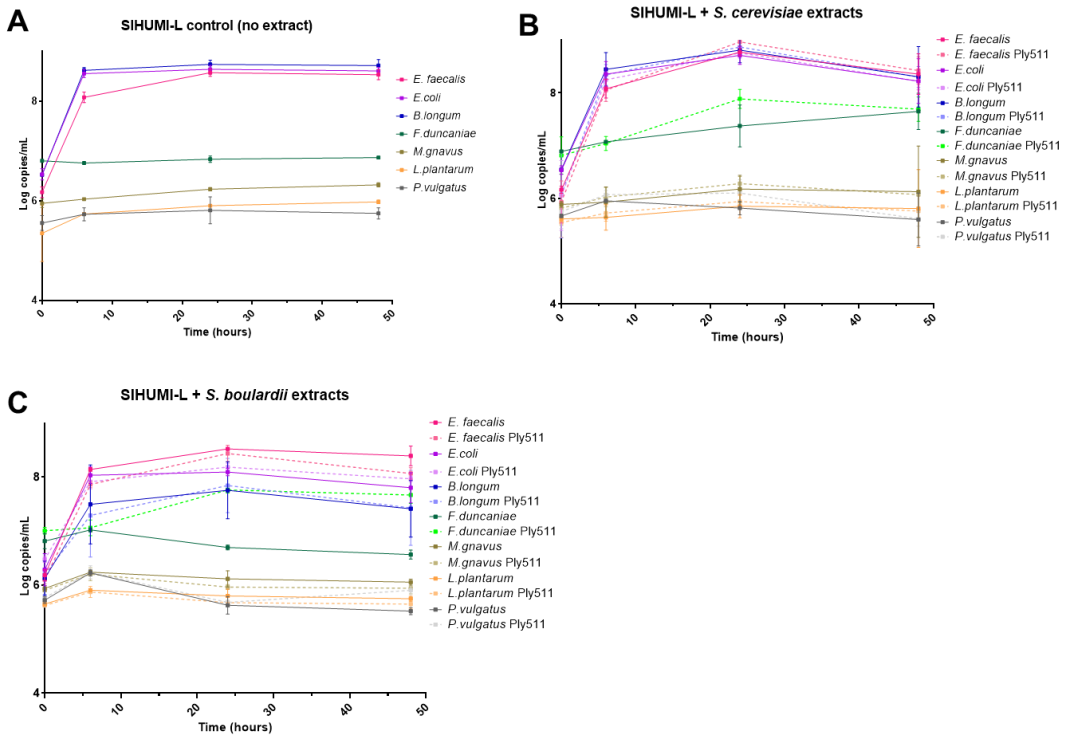


Figure. 3.4. Genome copies/mL over time (0, 6, 24 and 48 h) of members of SIHUMI consortium in LYHBHI after inoculation with *L. monocytogenes* at time 0. Each time point is represented as a mean with standard deviation of 4 replicates. A) SIHUMI-L control (no yeast extract). B) SIHUMI-L with added *S. cerevisiae* extracts containing either Ply511 or the wild-type counterpart or C) SIHUMI-L with added *S. boulardii* extracts containing either Ply511 or the wild-type counterpart.

3.3.6. *S. cerevisiae* and *S. boulardii* secreting Ply511 are active against *L. monocytogenes* in simulated gastric and intestinal fluids

After confirming the anti-*Listeria* activity of the yeast extracts, we hypothesized that yeast should better resist gastrointestinal conditions than their extracts and tested both *S. cerevisiae* and *S. boulardii* whole cells.

First, to assess if the recombinant *S. cerevisiae* or *S. boulardii* could effectively reduce *L. monocytogenes* serovar 4b in conditions resembling gastrointestinal ingestion, we mixed milk, bacterial and yeast cells in simulated gastric fluid (SGF) at pH 3 for 2 h and then for 24 extra hours in simulated intestinal fluid at pH 7. We monitored viable *L. monocytogenes* cells over time. As shown in Fig. 3.5., both recombinant *S. cerevisiae*

and *S. boulardii* secreting Ply511 significantly reduced *L. monocytogenes* levels after 24 hours compared with their wild-type counterpart. *S. cerevisiae* secreting Ply511 reduced *Listeria* levels by 1.39 ± 0.19 $\log_{10}$ (CFU/mL) in comparison with *S. cerevisiae* wild-type and *S. boulardii* secreting Ply511 reduced *Listeria* levels by 0.61 ± 0.12 $\log_{10}$ in comparison with *S. boulardii* wild-type.

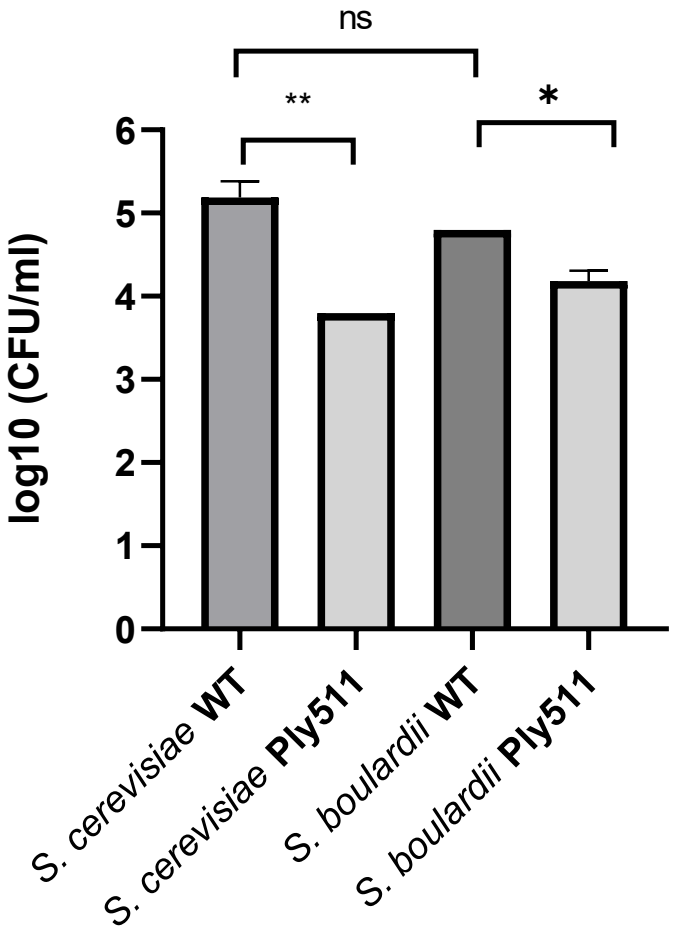


Figure 3.5. Log_{10} (CFU/mL) of *L. monocytogenes* upon mixture with *S. boulardii (SB) or S. cerevisiae* (SCV), wild-type (WT) or secreting Ply511 (Ply511), as indicated in the legend, after 24 h of contact (2 h in SGF and 22 h in SIF). The figure shows the $\log_{10}$ (CFU/mL) mean of three replicates, standard deviation is represented as error bars. The unpaired t test comparison between the WT yeast and the yeast secreting Ply511 at 24h, is indicated as follows: $^{****}p \leq 0.0001$, $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, and $^{*}p \leq 0.05$.

3.3.7. *S. boulardii* reduces *Listeria* during *in vitro* intestinal fermentation

Next, we wanted to test if recombinant yeast were able to compete and selectively reduce *L. monocytogenes* during intestinal fermentation, considering the competition against the intestinal flora of three human donors. We mixed the fecal samples with *L. monocytogenes* and the different yeast (*S. boulardii* or *S. cerevisiae* wild-type or *S. cerevisiae* and *S. boulardii* secreting Ply511) or a non-yeast control (containing only the fecal microbiota).

We observed a similar trend in all 3 donors, as depicted in Fig. 3.6:

First, when no yeast was used, *L. monocytogenes* grew in the first 24 h and decreased slightly in the 24 h to 48 h period. *L. monocytogenes* numbers were not detected after 48 h only in 1 out of 3 donors, suggesting that the fecal flora could have reduced it. Second, *L. monocytogenes* numbers grew over time when using either wild-type or recombinant *S. cerevisiae.* We observed in all 3 cases that the recombinant *S. cerevisiae* performed slightly better than the wild-type and reduced *L. monocytogenes* at 30 and 48 h, in comparison to the wild-type. The concentration of *Listeria* was, however, higher in both cases, in comparison to the control, which did not contain yeast, suggesting that *L. monocytogenes* growth was favored when introducing *S. cerevisiae* in the intestinal conditions. Third, *S. boulardii* reduced *L. monocytogenes* at all tested time-points compared to the control group (except for donor 3, time 48 h). *S. boulardii* wild-type showed a similar trend than the recombinant *S. boulardii* expressing the endolysin Ply511. However, *L. monocytogenes* numbers were generally slightly lower in the wild-type yeast. In fact, in 2 out of 3 donors, *S. boulardii* wild-type treatment was able to reduce *L. monocytogenes* below the limit of detection. The recombinant *S. boulardii* was able to reduce *L. monocytogenes* below the limit of detection in 1 out of 3 donors.

Yeast growth during the 48h fermentation was estimated via qPCR with specific primers for each strain. Both wild type and recombinant strains were detected throughout the 48h (Fig. A3.3). Estimated CFUs/mL were increased over time in all cases, as shown in Fig. A3.3 indicating that both *S. cerevisiae* and *S. boulardii* were metabolically active and growing during the tested conditions.

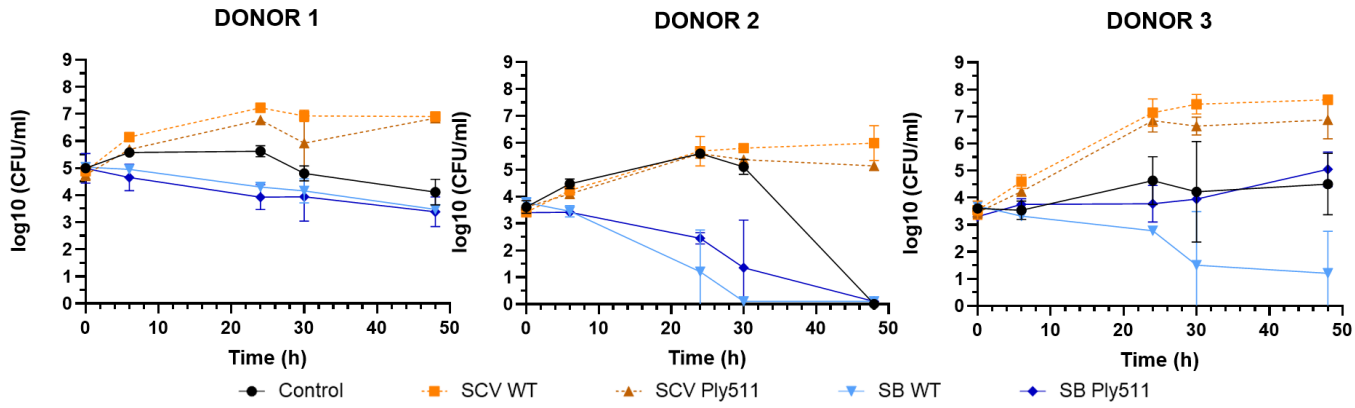


Figure 3.6. Log_{10} (CFU/mL) of *L. monocytogenes* serovar 4b upon mixture with *S. boulardii (SB) or S. cerevisiae* (SCV), wild-type (WT) or secreting Ply511 (Ply511), as indicated in the legend, during 48 h of contact with human feces from 3 different donors in anaerobic conditions. The figure shows the log_{10} (CFU/mL) mean of two replicates, standard deviation is represented as error bars.

3.4. Discussion

In this study, we show that engineered *S. boulardii* and *S. cerevisiae* can secrete endolysin Ply511 with activity against *L. monocytogenes* highlighting their potential for delivery in the gut.

Firstly, we used CRISPR-Cas9 to engineer *S. boulardii* with a scarless triple chromosomal integration free of antibiotic resistance markers and demonstrated that it can express an enzymatically active protein. The avoidance of antibiotic markers is essential in terms of regulations governing any potential therapeutic application of genetically modified organisms. When comparing *S. boulardii* strain to our previously engineered *S. cerevisiae* strain carrying the same triple chromosomal integration [18], we observed lower antibacterial activity in most assays. This reduced activity is likely to the fact that the expression cassette and secretion signal were originally optimized for *S. cerevisiae* [37] and may require further adaptation to maximize expression and/or secretion in *S. boulardii* commercial strains. While we are comparing two closely related species, it's important to note that even within a single species, *S. cerevisiae*, the same genetic modification can lead to different outcomes depending on the strain background, such as laboratory *versus* industrial strains [38],[39]. This strain-dependent variability supports our observation that the same engineering strategy may not yield identical results in *S. boulardii* and *S. cerevisiae*, highlighting the importance of host-specific optimization. An example of this is the lower protein display efficiency in *S. boulardii* vs *S. cerevisiae* [40]. In

addition, differences in glycosylation patterns between *S. cerevisiae* and *S. boulardii* may further influence the functionality of the expressed protein. We previously found engineered yeast cell extracts to have the highest activity against *L. monocytogenes*, compared to whole cells and supernatants, thus we tested both *S. cerevisiae* and *S. boulardii* extracts against *L. monocytogenes* in a controlled simplified microbiome (SIHUMI-L) under anaerobic conditions. The extracts showed an effective anti-*Listeria* activity. When we evaluated the effect of the endolysin on other members of the SIHUMI community, we found that it is specific to *Listeria*, as it did not affect the survival of the other members compared to a consortium control with no extract. The remaining four members of the community, *M. gnavus, F. duncaniae, L. plantarum* and *P. vulgatus*, were unable to grow in presence of *L. monocytogenes,* even without the addition of yeast extract. This lack of growth may be due to antagonistic interactions (such as those posed by *E. faecalis)* or due to culture viability issues, but not due to the endolysin treatment itself. *E. faecalis* and *B. longum* are among the dominant bacteria in the consortium. These are gram positive bacteria and could, in principle, be more prone to endolysin attack (unlike gram negative species such as *E. coli* or *P. vulgatus*). However, their growth was not affected by either of the cell extracts tested, which can be explained by the different peptidoglycan chemotype of these bacteria compared to *Listeria,* therefore not being targeted by Ply511. The cell-binding domain of Ply511 has a broad binding range and has been shown to bind all *Listeria* serovars [16]. The same has been observed with other species; for example, the cell-binding domains of other anti-*Listeria* endolysins have shown affinity to *Bifidobacterium* strains, suggesting some degree of similarity in their cell wall structures. However, the fact that *B. longum* was able to grow in the presence of our endolysin-containing extracts highlights the specificity of this treatment, reinforcing the potential of Ply511 as a microbiome-sparing therapeutic agent.

Since listeriosis can be fatal, particularly in vulnerable populations, we could envision the use of these yeast extracts as postbiotics, or for bio-preservation of certain food products prone to *Listeria* contamination. This approach could be especially valuable for fermented foods such as cheese, yogurts and other products where a selective anti-*Listeria* treatment would be beneficial if compatible with yeast fermentation processes. To test if yeast could serve as a delivery vehicle for endolysin in the gut, we simulated gastro-intestinal digestion by co-incubating *L. monocytogenes* with the engineered yeasts in milk. After 24 h in simulated intestinal fluid, *Listeria* counts were reduced by 1.4 log units with *S. cerevisiae* and 0.6 log units with *S. boulardii*, corresponding to a 96% or 75% reduction, respectively, compared to their wild-type counterparts. These

results suggest a positive antimicrobial effect and highlight the potential of this approach for the treatment or prevention of *Listeria* infections. To better understand the interactions within the gut environment, we simulated an intestinal fermentation by co-incubating *S. cerevisiae* or *S. boulardii* (wild-type or engineered) with a human fecal sample and inoculating *L. monocytogenes* under conditions mimicking the small intestine, where *Listeria* translocation normally occurs [41], over a 48-h period. Under these conditions, *S. boulardii* was effective in reducing the bacterial load, whereas *S. cerevisiae* appeared to promote *Listeria* growth, possibly serving as nutrient source and resulting in higher bacterial counts.

However, we did not observe a significant difference in *L. monocytogenes* reduction between the engineered and wild-type yeast strains, suggesting that endolysin secretion did not enhance the antibacterial effect under these conditions. This limited activity may be due to the competition between the yeast and the complex microbiota present in the fecal samples, potentially resulting in reduced yeast metabolic activity and, consequently, low expression of Ply511, insufficient to effectively suppress *L. monocytogenes*. These findings also underscore the contrast between testing treatments in simplified microbial communities versus in human fecal samples, the latter presenting a far more complex and diverse environment. To gain deeper insight into which microbial populations are affected and how the different yeast strains exert their effects, metagenomic sequencing should be performed.

Recently, the effect of endolysin CD27L_EAD in the gut microbiome was evaluated [42], demonstrating the specific reduction of *Clostridioides difficile* and showing differences between vancomycin and endolysin treatment. Endolysins have shown promising results in other microbiome-relevant contexts, such as the skin and vaginal tract [43],[44]. While more data is needed to conclude their microbiome-sparing effect, a growing body of evidence supports this potential.

3.5. Conclusion

This study demonstrates the capacity of yeast as a vehicle for endolysin delivery. With further improvements, this approach could lead to a platform of probiotics suitable for endolysin delivery against intestinal pathogens leading to prevention of certain infections or targeted interventions in the microbiome.

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Chapter IV.

Exploring the use of yeast as a platform for the delivery of endolysins

This chapter is dedicated to our efforts towards creating a platform for endolysin delivery, engineering *S. boulardii* strains, using the CRISPR-Cas9 tools previously optimized, to engineer *S. boulardii* strains to express anti-*S. aureus* LysSA11 and anti-*C. difficile* CD27L(1-179).

The tasks described in this chapter related to *C. difficile* testing were performed at APC Microbiome Ireland in University College Cork (Cork, Ireland) under the supervision of Prof. Colin Hill and the tasks related to *S. aureus* colonic fermentations were performed at USC in Lugo, Spain under the supervision of Alejandra Cardelle Cobas

Abstract

Staphylococcus aureus and *Clostridioides difficile* are gram positive, bacterial pathogens associated with gastrointestinal colonization and infection, with high recurrence and resistance to antibiotics. Bacteriophage endolysins are emerging as microbiome-friendly antimicrobials, providing the advantage of specificity over antibiotics against these pathogens. In this study, we engineered the probiotic yeast *Saccharomyces boulardii* to secrete two endolysins: LysSA11, targeting *S. aureus*, and CD27L(1–179), targeting *C. difficile*. We integrated several copies into its genome using CRISPR-Cas9, evaluated each strain growth kinetics, enzymatic activity, and antibacterial effect *in vitro*. Recombinant yeast strains secreting LysSA11 showed detectable enzymatic activity and a modest reduction of *S. aureus*, though expression imposed a metabolic burden and the strains failed to reduce *S. aureus* in a simulated colonic fermentation. In contrast, expression of CD27L(1–179) was not detected and we could not measure antibacterial activity under the tested conditions. These findings highlight both the potential and the limitations of yeast-based endolysin delivery platforms, underscoring the need for further optimization of secretion efficiency, stability, and activity in complex environments.

Keywords: *Clostridioides difficile*, CRISPR-Cas9, endolysin, engineered *Saccharomyces boulardii*, probiotics, *Staphylococcus aureus*

4.1. Introduction

Staphylococcus aureus is a bacterial pathogen causative of human diseases such as skin, soft tissue, prosthetic-joint, and other biofilm-related infections. It has also been found to colonize the gastrointestinal tract, particularly in hospitalized or immunocompromised individuals. Faecal carriage rates of *S. aureus* have been reported in approximately 20% of healthy adults, with typical levels reaching 10^6 CFU per 100 g of faeces, surpassing nasal carriage levels [1],[2]. The highest bacterial burdens are found in the distal gastrointestinal tract, such as the cecum and colon [3]. Gut colonization by *S. aureus* is concerning since asymptomatic carriers remain at increased risk for developing infections, especially under conditions such as immunosuppression.

Another pathogen frequently associated with gut dysbiosis and the leading cause of nosocomial gastrointestinal infections, *Clostridioides difficile* (formerly known as *Clostridium difficile*). It causes *C. difficile* infection (CDI), its symptoms range from mild diarrhea to severe colitis. CDI is often recurrent and is associated with the prior use of antibiotics, which cause the disruption of the gut microbiota allowing *C. difficile* colonization and toxin production [4],[5]. Although the initial infection can be treated with targeted antibiotics, this approach commonly leads to recurrence—20–30% of patients experience at least one relapse, and risk increases with each subsequent episode [5] since the main barrier against *C. difficile* posed by the human GIT is known as colonization resistance and is mediated by a healthy gut microbiome.

There is clinical evidence suggesting that probiotic interventions can lead to prevention and reduction of infection rates against both *S. aureus* and *C. difficile*: A clinical study demonstrated that probiotic intervention could suppress *S. aureus* gut colonization and reduce the infection rates, via oral administration of *Bacillus subtilis* [3]. *In vitro* studies using probiotic yeast, have shown that *S. cerevisiae* supernatant extracts can inhibit *S. aureus* virulence by reducing biofilm formation, hemolytic activity, and the expression of key virulence genes in MRSA strains [6]. Probiotics such as *Saccharomyces boulardii* have shown efficacy for the prevention and treatment of CDI, administered concurrently with antibiotics [5].

Thus, engineered probiotic strategies that help restore microbiota balance and deliver targeted anti-*C. difficile* or anti-*S. aureus* molecules, may be promising alternatives to traditional antibiotics in the control and prevention of these infections. Phage-derived endolysins targeting *C. difficile* (e.g. CD27L[7]–[9] and *S. aureus* (e.g. LysSA11) [10] have demonstrated strong lytic activity *in vitro* and LysSA11, in particular, has previously been expressed in a yeast surface-display system in *S. cerevisiae* [11]. The truncated version of

CD27L (residues 1 to 179) showed an enhanced lytic activity, showing a faster lysis than its full-length counterpart[9]. In this study, we aim to combine the probiotic properties of *S. boulardii* with the targeted endolysin delivery, engineering *S. boulardii* to express and produce CD27L(1-179) or LysSA11 to target *C. difficile* or *S. aureus*, respectively, enhancing its therapeutic properties, as a platform for the production and delivery of endolysins to combat gut pathogens.

4.2. Materials and Methods

4.2.1. *Escherichia coli* transformation and maintenance

E. coli DH5/NZY5α (Nzytech, Lisbon, Portugal) was used for plasmid construction and propagation. *E. coli* NZY5α transformation was conducted according to NZY5α competent cell protocol (Nzytech, Lisbon, Portugal). Briefly, 5 µL of assembled plasmids (2 to 20 ng/µL) was added to *E. coli* competent cells. After 30 min of incubation on ice, cells were heat shocked at 42 °C for 40 s. Then, cells were incubated on ice for 2 min and 150 µL of SOC medium (Nzytech, Lisbon, Portugal) was added. After growing for 1 h (37 °C, 200 revolutions per minute (rpm)), cells were plated on LB agar supplemented with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Positive transformants were confirmed by colony PCR and were propagated. All the plasmids were purified using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Burlington, MA, USA). The correct cloning was verified using Sanger sequencing by Eurofins Genomics (Ebersberg, Germany). A list of primers used for colony PCR are provided in Table 4.1.

Table 4.1. List of primers used in this study

Primer name	Sequence	Description
p1_TEF1_RV	GGTACCCTTAGATTAGATTGCTATGC (RV)	Amplification of TEF1 promoter with yeast homology arms for insertion
p2_PGK1_FW	CTCGAGTAATTCCGATAGATCAATTTTTTTC (FW)	Amplification of PGK1 terminator with yeast homology arms
p3_plySA11_FW	CAATCTAATCTAAGGGTACCATGAGATTTCCTT CAATTTTTACTGC (FW)	Amplification of PlySA11 cassette with homology arms for Infusion
p4_plySA11_RV	ATCTATCGGAATTACTCGAGTTAATGATGATGA TGATGATGATGATGATGATGCTTCCAGTTA ATTCTACCCCAG (RV)	Amplification of PlySA11 cassette with homology arms for Infusion
p5_pCfB_RV	CCTCGACTACTTTGGCCCAAATGAAGGCTTCT ATGACTAGATCT (FW)	Colony PCR for confirmation of PlySA11 into the TEF1p-PGK1t cassette

p6_pCfB_RV	TATAGGGCGAATTGGACGCCAAGCTTTAACGA (RV)	Colony PCR for confirmation of PlySA11 into the TEF1p-PGK1t cassette
p7_PGK1_FW	CTCGAGTAATTCCGATAGATCAATT (FW)	Amplification of PGK1 terminator, with homology arms
p8_LacASS_RV	GGACGCCCCAGCAGCCTGT (RV)	Amplification of TEF1 promoter and LacA secretion signal (SS), with homology arms
p9_HTCD27L_FW	GCTGCTGGGGCGTCCCATCATCATCATCATCA TCATCATCATCATCATGAAAATTTGTACTTTCA ATCTGGATCTTCTATGAAGATCTGTATCACTGT CG (FW)	Amplification of Histagged CD27L1-179, with homology arms for infusion
p10_HTCD27L_RV	GAATTACTCGAGCTAGTTGATGTTCTTGTTTAG GACACC (RV)	Amplification of Histagged CD27L(1-179), with homology arms for infusion
p11_TEF1_FW	CATAGCTTCAAAATGTTTCTACTC (FW)	Colony PCR for confirmation of CD27L(1-179) into the TEF1p-LacASS-PGK1t cassette
p12_CD27L_RV	GACCGACAGTGATACAGATCTTCAT (RV)	Colony PCR for confirmation of CD27L(1-179) into the TEF1p-LacASS-PGK1t cassette

4.2.2. Plasmids

Table 4.2 lists all plasmids used in this study. For construction of plasmid pCfB3035-LysSA11, pCfB2904-LysSA11, or pCfB2909-LysSA11 for expression of endolysin LysSA11, this procedure was followed: TEF1 promoter, pGK1 terminator and homology arms for integration into *S. cerevisiae* chromosomes X-4, XI-3, and XII-5, were obtained from plasmids pFMT_lacA_TEF1p_PGK1, pCfB3035, pCfB2904 or pCfB2909 [12],[13]. The codon optimized cassette containing DNA sequences for the entire pre-pro-α-factor secretion signal, with the downstream EAEA tetrapeptide, followed by the endolysin LysSA11, 10 Histidine residues and a stop codon, was codon optimized for expression in *S. cerevisiae* and synthesized by GenScript (Rijswijk, Netherlands). Parts were assembled using the In Fusion HD Cloning Kit (Takara Bio, Shiga, Japan), primers are provided in Table 4.1. For construction of plasmid pCfB3035-CD27L(1-179), pCfB2904- CD27L(1-179), or pCfB2909- CD27L(1-179), for expression of endolysin LysSA11, this procedure was followed: TEF1 promoter, LacA secretion signal[14] , PGK1 terminator and homology arms for integration into *S. cerevisiae* chromosomes X-4, XI-3, and XII-5, were obtained from the plasmids pFMT_lacA_TEF1p_PGK1, pCfB3035, pCfB2904 or pCfB2909 [12],[13]The codon optimized cassette containing DNA sequences for 10 histidine residues, the TEV protease cutting site residues (ENLYFQS), a flexible linker (GSS) and residues 1 to 179 of the endolysin CD27L and a stop codon, was codon optimized for expression in *S. cerevisiae* and synthesized by GenScript (Rijswijk, Netherlands). Parts were assembled

together using the In Fusion HD Cloning Kit (Takara Bio, Shiga, Japan). Gene maps with annotated sequences are provided in Figure 4.1.

Table 4.2. Plasmids used in this study

Plasmid name	Description	Reference
pCfB3035-LysSA11	Plasmid containing DNA sequences for TEF1 promoter, Alpha factor, LysSA11,10xHis, PGk1 terminator, and homology arms for site X-4.	This study
pCfB2904-LysSA11	Plasmid containing DNA sequences for TEF1 promoter, Alpha factor, LysSA11,10xHis, PGk1 terminator, and homology arms for site XI-3.	This study
pCfB2909-LysSA11	Plasmid containing DNA sequences for TEF1 promoter, Alpha factor, LysSA11,10xHis, PGk1 terminator, and homology arms for site XII-5.	This study
pCfB3035-CD27L(1-179)	Plasmid containing DNA sequences for TEF1 promoter, LacA secretion signal, 10xHis, CD27L (1-179), PGk1 terminator, and homology arms for site X-4.	This study
pCfB2904- CD27L(1-179)	Plasmid containing DNA sequences for TEF1 promoter, LacA secretion signal, 10xHis, CD27L (1-179), PGk1 terminator, and homology arms for site XI-3.	This study
pCfB2909- CD27L(1-179)	Plasmid containing DNA sequences for TEF1 promoter, LacA secretion signal, 10xHis, CD27L (1-179), PGk1 terminator, and homology arms for site XII-5.	This study
pFMT_lacA_TEF1p_PGK1	Plasmid containing DNA sequences for TEF1 promoter, LacA secretion signal, lacA gene from Aspergillus niger, PGk1 terminator, and homology arms for sites X-4, XI-3 or XII-5.	Carvalho, 2024
pCfB2312 (Cas9)	Cas9 expression plasmid for marker-free integration	Jessop Fabre et al 2016
pCfB3042 (gRNA-X-4)	Guide RNA plasmid targeting site X-4	Jessop Fabre et al 2016
pCfB3045 (gRNA-XI-3)	Guide RNA plasmid targeting site XI-3	Jessop Fabre et al 2016
pCfB3050 (gRNA-XII-5)	Guide RNA plasmid targeting site XII-5	Jessop Fabre et al 2016

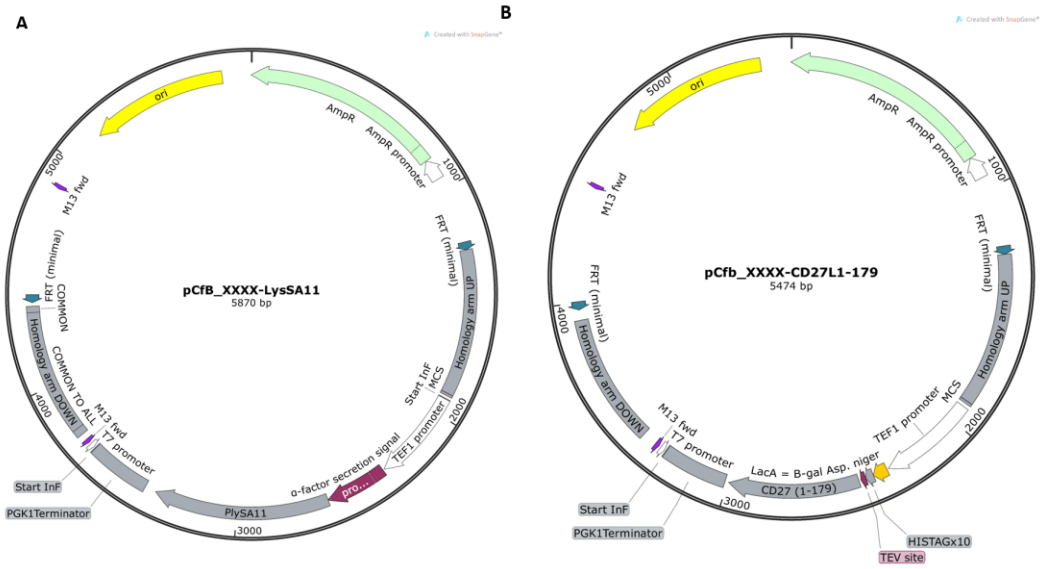


Figure 4.1.A) pCfBXXXX-LysSA11. Plasmid containing DNA sequences for TEF1 promoter, Alpha factor, LysSA11,10xHis, PGk1 terminator, and homology arms for each of the three sites (XXXX refers to each of the sites of integration, X-4, XI-3 or XII-5, marked as "homology arms up" or "down". B) pCfBXXXX- CD27L(1-179). Plasmid containing DNA sequences for TEF1 promoter, LacA secretion signal, 10xHis, CD27L (1-179), PGk1 terminator, and homology arms for each of the three sites (XXXX refers to each of the sites of integration, X-4, XI-3 or XII-5, marked as "homology arms up" or "down".

4.2.3. Yeast transformation and maintenance

The yeast *S. cerevisiae* CEN.PK113-7D [15] or *S. boulardii* CNCM I-745 (UL250, Biocodex, France) was used for transformation and is referred to as *S. cerevisiae or Scv* and *S. boulardii* or *Sb*, respectively, throughout the text.

Yeast transformation was performed using the PEG/Lithium acetate method [16]. To generate the engineered strains, yeast cells were first transformed with a Cas9-expressing plasmid (pCf2312). In a second step, a guide RNA plasmid (pCFB3050, targeting integration sites XII-5, X-4 or XI-3, was co-transformed into the Cas9-expressing strains along with the corresponding linearized integrative vector. For multiple integrations, the guide RNA was removed between transformations by curing the yeast before targeting the next integration site. Prior to activity testing, all strains were curated of both plasmids through serial passaging in liquid or solid medium without antibiotics.

Yeast strains were propagated at 30 °C (*Scv*) or 37 °C (*Sb*) and maintained at 4 °C on YPD plates (1 % yeast extract, 2 % peptone, 2 % glucose, 2 % agar). For plasmid-carrying yeast strains, YPD media was supplemented with 200 µg/mL of geneticin G418 (Cas9-expressing plasmid, pCf2312) or/and 100 µg/mL

nourseothricin sulfate (guide RNA-containing plasmids). Half of the concentration of each antibiotic was used for *Sb,* to allow for a faster recovery after the transformation protocol (Carvalho, 2024).

For yeast colony PCR, a colony was picked with a sterile toothpick and transferred to a fresh plate. The rest of the colony was then diluted in 50 µL of NaOH 20mM. After incubation at 95 °C for 15 minutes, 1 µL was added to a microtube containing NZYTaq II 2x Green Master Mix (NZYtech, Lisbon, Portugal) and the primers to verify the correct transformations (Table 4.3).

Table 4.3. Primers used in this study for the verification of the chromosomal insertion in *S. cerevisiae* or *S. boulardii*.

Primer ID	Sequence (5'–3')	Target locus	Reference
905	CTCACAAAGGGACGAATCCT	X-4	[13]
906	GACGGTACGTTGACCAGAG	X-4	[13]
911	GTGCTTGATTTGCGTCATTC	XI-3	[13]
912	CACATTGAGCGAATGAAACG	XI-3	[13]
899	CCACCGAAGTTGATTTGCTT	XII-5	[13]
900	GTGGGAGTAAGGGATCCTGT	XII-5	[13]

4.2.4. Bacterial strains

Strain *S. aureus* D329 [17] was used for this study. It was grown in Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA) or Mannitol Salt Selective Agar (Sigma-Aldrich, Burlington, MA, USA).

C. difficile APC43 and APC1412 strains used in this study were cultured under anaerobic conditions, static, in modified Brain Heart Infusion medium (Oxoid, Hampshire, England) with the addition of 0.5% yeast extract, 5 mg/L hemin (Sigma–Aldrich Burlington, MA, USA), 1 mg/ml cellobiose (Sigma–Aldrich, Burlington, MA, USA), 1 mg/ml maltose (Sigma–Aldrich, Burlington, MA, USA), and 0.5 mg/ml L-cysteine (Sigma–Aldrich, Burlington, MA, USA) – LYHBHI as described by [18].

4.2.5. Evaluation of enzymatic activity

To evaluate the enzymatic activity of yeast-expressed endolysin, a lawn of heat-killed bacteria was prepared on YPD-agar. *S. aureus* strains were grown overnight in Tryptic Soy Broth (Sigma-Aldrich, Burlington, MA, USA) at 37 °C and *C. difficile* strains were grown overnight anaerobically in LYHBHI.

The cell suspension was heat-killed by autoclaving at 121 °C for 20 min. The heat-killed bacterial cells were then concentrated 50–100 × by centrifugation and resuspended in YPD-agar (1.5% agar) to achieve an opaque, homogeneous mixture. Yeast were spotted (5 µL) onto the YPD-agar plates containing the heat-killed bacterial suspension. Control plates included spots of wild-type yeast. Plates were incubated at 30 °C until peptidoglycan degradation was visualized. Peptidoglycan degradation was assessed by observing zones of clearing around yeast colonies or spots, indicating enzymatic activity.

4.2.6. Evaluation of growth kinetics of yeast strains

To assess yeast growth, cultures were monitored in a 96-well plate using an automated system for real-time biomass evaluation. Overnight cultures were initially grown in YPD broth at 37 °C and subsequently diluted in fresh YPD to a final working volume of 200 µL per well and OD 0.05. Each well was inoculated with a standardized yeast suspension, and plates were incubated at 37 °C using a microplate reader (Thermo Fischer Scientific, Waltham, MA, USA). Optical density (OD_{600}) measurements were recorded after shaking, every 15 min to capture growth kinetics over time.

4.2.7. Evaluation of anti-Staphylococcal activity of yeast supernatants

To evaluate the killing activity of yeast against *S. aureus* D328, co-culture assays were conducted. Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD_{600} of 0.1 in 20 mL of YPD, in a 100-mL Erlenmeyer flask and grown at 30 °C and 160 rpm for 96 h. Yeast cells were then centrifuged at 5000 × g for 10 min and supernatants were recovered. This supernatant was then filtered through a 0.22-µm sterile polyethersulfone membrane filter to remove any residual yeast cells. Filtered supernatants were concentrated tenfold using Amicon Ultra-15 Centrifugal Filter Units with a 10-kDa molecular weight cut-off (Millipore Sigma, Burlington, MA, USA) by centrifugation at 5000 × g until the desired volume was reached. Concentrated supernatants were collected, filtered again to ensure sterility and stored at 4 °C if used on the same day, or at – 20 °C until further use.

When cell extracts were used, fresh yeast cells (grown as described above) were centrifuged at 5000 g for 10 min and washed with Tris buffer (50 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of ~ 10^9 CFU/mL. Cell suspensions were subjected to sonication using a Cole-Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA). Sonication was carried out in three cycles of 5 min each, with 10 intervals of 30 s ON and 30 s OFF at 40 % amplitude. The sonicated extract was then centrifuged at 10,000 × g for 10 min to remove cell debris, and the supernatant was filtered through a 0.22-µm sterile filter (Millipore-Sigma, Burlington, MA, USA) to ensure sterility. The filtered sonicated extract was stored at 4 °C if used on the same day, or at – 20 °C until further use.

S. aureus cells were grown overnight, centrifuged at 9000 × g for 10 min, washed, and resuspended and diluted in the same Tris buffer, to a concentration of ~ 10^4 CFU/mL. Yeast supernatants or extracts and bacterial cells were mixed (900 µL with 100 µL, respectively) in a 24-well plate and incubated at 150 rpm and 37 °C. Co-cultures were sampled at 3 h, 6 h, and 24 h. Viable *S. aureus* cells were quantified by plating serial dilutions onto Mannital Salt Selective Agar (Sigma Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated.

4.2.8. Protein purification using AKTA Start

Recombinant yeast cells were pre-inoculated in YPD broth overnight. Cells were then diluted to OD 0.1 in 200 mL of YPD, in a 500-mL Erlenmeyer flask and grown at 30 °C and 160 rpm for 96 h. Yeast cells were then centrifuged at 5000 × g for 10 min and supernatants were recovered. Fresh yeast cells grown as described above were centrifuged at 5000 g for 10 min and washed with Tris buffer (50 mM Tris, 200 mM NaCl, pH 8.0) to a final concentration of ~ 10^9 CFU/mL. Cell suspensions were subjected to sonication using a Cole-Parmer Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL, USA). Sonication was carried out in three cycles of 5 min each, with 10 intervals of 30 s ON and 30 s OFF at 40% amplitude. The sonicated extract was then centrifuged at 10,000 × g for 10 min to remove cell debris, and the supernatant was filtered through a 0.22-µm sterile filter (Millipore-Sigma, Burlington, MA, USA) to avoid clogging of the system. Both cell extracts and supernatants were pooled and the mixture containing either PlySA11 or CD27L(1-179), was purified using the Akta Start Purification System (Cytiva), using the Talon Crude Purification columns (Cytiva). Purification fractions were assessed for quality and purity by SDS-PAGE (4-12% gradient gel).

Turbidity reduction assays were used to assess the lytic activity of soluble protein eluted from the purification. An overnight culture of *C. difficile* was grown in liquid media to an OD600 of ~ 1.0. The cells were inactivated and then pelleted by centrifugation at 3000 x g for 5 min. The cell pellet was washed three times in PBS before being resuspended to an OD600 of 0.8. A 96-well plate was prepared to 200 µL final volume of cell suspension with the addition of purified protein or a negative control of buffer. Changes in optical density were recorded every 5 min over the course of 24 h at 37 °C using a microplate reader (Thermo Fischer, Waltham, MA, USA).

4.2.9. Evaluation of anti-Staphylococcal activity in a gut-fermentation model

Fecal samples were donated by two men and one woman between the ages of 32 and 50 years and were recruited through a trial authorized by the Regional Ethics Committee for Clinical Research (Galician Health Service, SERGAS, n° 2018/270). Based on the eligibility criteria of that trial, individuals without gastrointestinal disorders, without chronic pharmacological treatment and who had not consumed antibiotics or pre/pro/postbiotic supplements within 6 months prior to sample collection were included. The volunteers agreed to participate in the study by signing an informed consent form that detailed the utilization of their samples. They were provided with sterile containers to collect stool samples at home. Once collected at home, the sample was delivered to the laboratory within 2 h, for processing. In the laboratory, the stool samples were diluted 1:10 with phosphate-buffered saline and used immediately for the assays.

In vitro simulation of human intestinal digestion was performed based on a published study (Lopez-Santamarina et al., 2022) to evaluate the potential use of *S. boulardii* wild-type or engineered, as probiotic and its impact in the microbiota, in presence of *S. aureus*. A negative control, with pathogen and without yeast was carried out simultaneously. Conditions resembling those of the human colon, including anaerobic atmosphere, and temperature of 37 °C were replicated. The sterilized medium (composition indicated below) was mixed with 1 % (v/v) of the previously diluted feces and a final concentration of ~$5x10^5$ CFU/mL of *S. aureus* and ~$5x10^6$ *S. boulardii* were inoculated into the vessels. The assays were performed for 48 h; 2 mL samples were taken for analysis at 0, 6, 24, 30 and 48 h of fermentation. Total volume was 300 mL.

Viable *S. aureus* was quantified by enumeration, plating serial dilutions onto Baird-Parker Agar (Sigma Aldrich, Burlington, MA, USA), followed by incubation at 37 °C for 24 h or 48 h, and colony-forming units (CFU/mL) were calculated. Medium was prepared according to previous studies [19] with slight modifications

on its composition: arabinogalactan (1 g/L), pectin from apple (2 g/L), inulin (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121 °C for 15 min.

4.2.10. Statistics

All data analysis was performed in GraphPad (version 9.0.0). Unpaired t-test were used to evaluate statistical significance. The upper threshold for statistical significance for all experiments was set at $p < 0.05$.

4.3. Results

4.3.1. Integration of the endolysin expression cassette in *S. cerevisiae* and *S. boulardii*

In previous chapters, we demonstrated the feasibility of engineering *S. boulardii* to secrete an anti-*Listeria* endolysin using CRISPR-Cas9 and EasyClone-MarkerFree integrative tools. Following the same approach, we constructed new yeast strains designed to express and secrete two additional phage endolysins: CD27L (1–179), targeting *Clostridioides difficile*, and LysSA11, targeting *Staphylococcus aureus*.

Each secretion cassette was assembled to include a strong constitutive TEF1 promoter and the PGK1 terminator (Figure 4.2). The coding sequence of CD27L (residues 1–179) was fused to the *Aspergillus niger* LacA secretion signal at the N-terminus and tagged with a 10x His-tag at the C-terminus to facilitate downstream purification. The LysSA11 cassette included the *S. cerevisiae* alpha-mating factor secretion signal and a similar C-terminal His-tag. All constructs were integrated into chromosomes X, XI, or XII of *S. cerevisiae* or *S. boulardii* using CRISPR-Cas9-mediated homologous recombination. Successful genomic integration at different target loci was confirmed by colony PCR using primers flanking the corresponding insertion sites (Figure 4.3), two copies of the LysSA11 or CD27L(1-179) cassette were inserted in *S. cerevisiae* or in *S. boulardii* and three copies of the CD27L(1-179) in *S. boulardii* (Figure 4.3C).

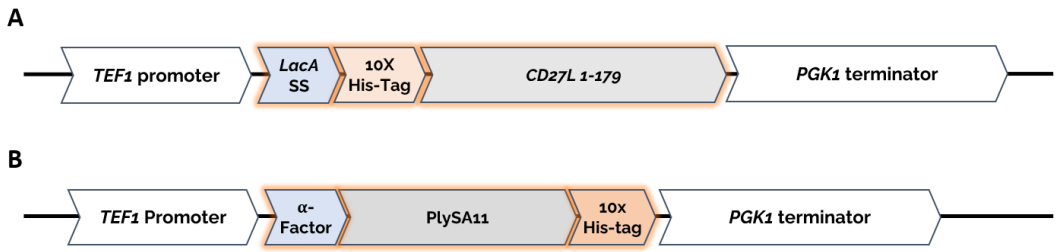


Figure 4.2. Endolysin secretion cassette integrated into yeast, containing the TEF1 promoter and PGK1 terminator. The coding sequences are highlighted in orange. A) CD27L (1-179) endolysin preceeded by the LacA secretion signal (SS) and 10 Histidine residues for purification. B) PlySA11 endolysin, preceeded by the alpha-mating factor and followed by 10 Histidine residues for purification.

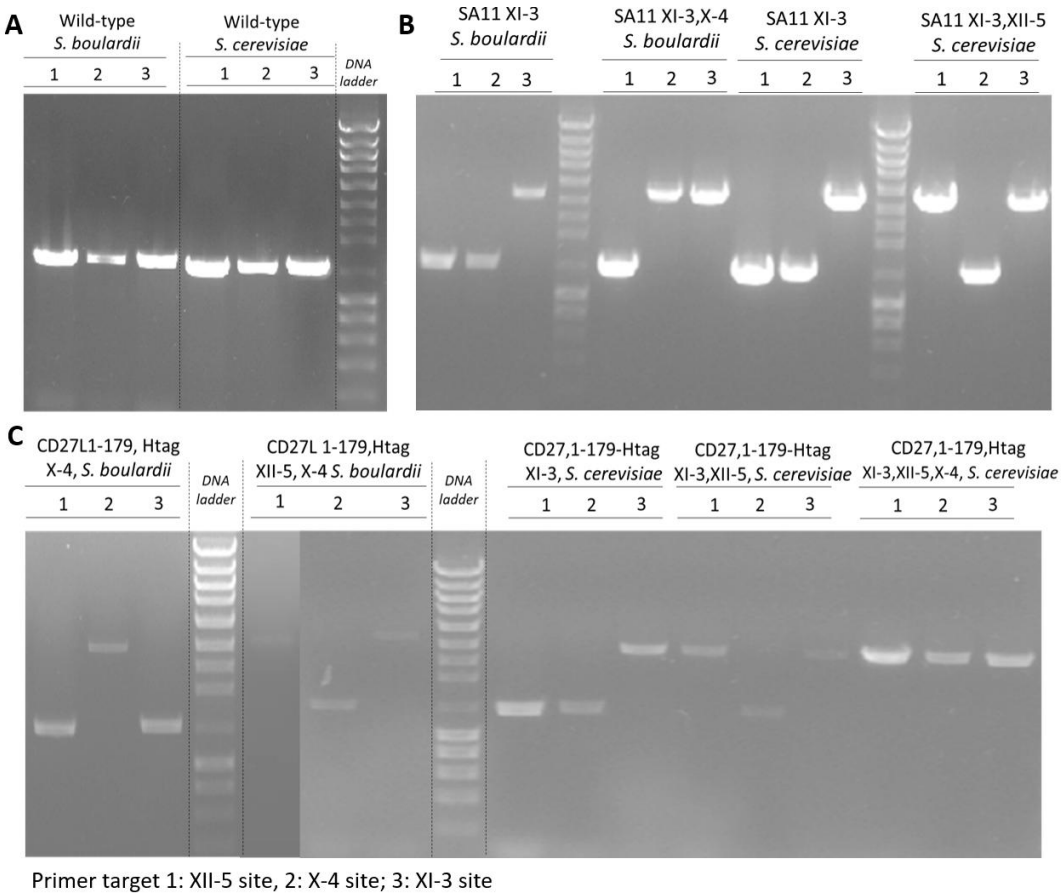


Figure 4.3. Colony PCR of *S. cerevisiae* or *S. boulardii* single, double and triple insertion of the different secretion cassettes. The picture shows the gel electrophoresis 1% agarose of the PCR products, corresponding to the numbers indicated in the figure legend: Chromosome XII site 5 (primers 899 and 900); 2: Chromosome X site 4 (primers 905 and 906) 3: Chromosome XI site 3 (primers 911 and 912), LD: DNA ladder. Sizes expected for unmodified yeast, 1 (XII-5): 1365 bp, 2 (X-4): 1394 bp, 3 (XI-3) 1450 bp. For yeast secreting the endolysin, 1 (XII-5): 3799 bp; 2 (X-4): 3828bp; 3 (XI-3): 3884 bp. A) Wild-type yeast. B) Insertion of LysSA11-containing cassette. C) Insertion of CD27L,(1-179)-containing cassette.

4.3.2. Enzymatic activity of *S. boulardii* engineered strains

To confirm the enzymatic activity of *S. cerevisiae* and *S. boulardii* expressing the endolysins CD27L(1-179) and LysSA11, we first tested their ability to degrade the peptidoglycan of heat-killed *S. aureus* and *C. difficile*, respectively. Recombinant yeast strains were spotted onto opaque YPD-agar plates containing heat-killed suspensions of each target bacterium. Plates were monitored over time for the appearance of lytic halos.

For the yeast expressing LysSA11, clearing zones around yeast colonies appeared after approximately three weeks in the single and double copy strains (Figure 4.4). These clearing zones did not appear in the wild-type strains. The slow peptidoglycan degradation could reflect an indication of the low levels of endolysin expression or a low enzyme activity under the assay conditions. On the contrary, for CD27L(1-179)-expressing strains, we were unable to visualize any clearing on *C. difficile* lawns. One reason might be that the preparation of lawns with *C. difficile* was technically challenging due to uneven distribution and cell aggregation on the agar surface, which prevented consistent formation of an opaque background. No lysis zones were observed under these conditions, possibly indicating a lack of enzymatic activity.

Since we previously found cell extracts to contain more active protein than supernatants, as reflected by their activity, we tested cell extracts of the modified yeast against heat-killed *C. difficile.* Again, no lysis zones were identified indicating either a lack of activity, or a lack of validity of the method for this bacterium (data not shown).

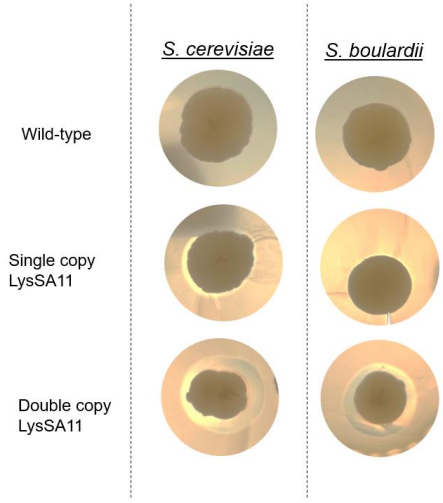


Figure 4.4. Enzymatic activity of *S. cerevisiae* or *S. boulardii* secreting endolysin PlySA11, one copy, two copies or wild type, as indicated in the legend, over a heat-killed layer of *S. aureus*.

4.3.3. Growth kinetics of *S. boulardii* engineered strains.

To evaluate whether genomic modifications affected yeast growth, we monitored the OD_{600} of wild-type and engineered *S. cerevisiae* and *S. boulardii* strains over 24 h at 37 °C (Figure 4.5). *S. boulardii* strains showed a slightly slower growth than *S. cerevisiae* under these conditions, which can be related to strain-specific temperature tolerance or cell aggregation, common of probiotic *S. boulardii*. Yeast strains expressing the truncated CD27L(1-179) enzyme (CD27LΔ) exhibited growth kinetics similar to their respective wild-type counterparts, indicating that this modification did not significantly impact cell fitness (Fig. 4A). However, expression of the LysSA11 endolysin had a strong impact on yeast growth. Both *S. cerevisiae* and *S. boulardii* strains secreting LysSA11 showed a slower growth rate compared to their wild-type controls (Fig. 4B). This suggests a possible metabolic burden or even cytotoxicity associated with LysSA11 expression cassette. These results are in agreement with the delayed appearance of enzymatic activity halos in the *S. aureus* lawn assay, indicating that the secretion of LysSA11 affects yeast growth.

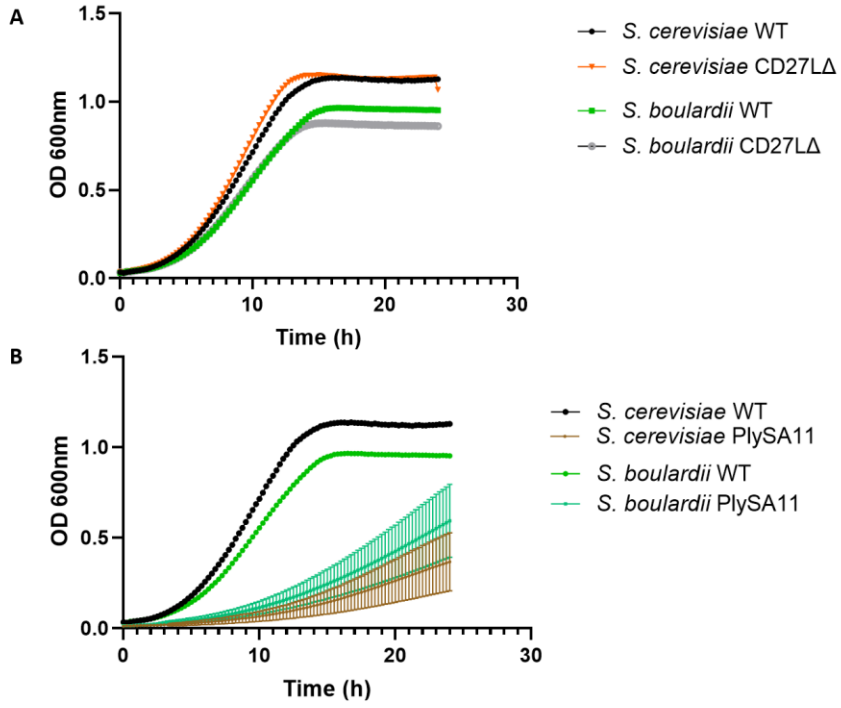


Figure 4.5. Growth kinetics of wild-type and endolysin-expressing yeast strains. A) OD_{600} of *S. cerevisiae* and *S. boulardii* strains expressing CD27LΔ, measured over 24 h at 37 °C. B) OD_{600} of *S. cerevisiae* and *S. boulardii* strains expressing LysSA11, measured over 24 h at 37 °C.

4.3.4. Evaluation of anti-*C. difficile* activity of yeast engineered strains

To further understand if CD27L(1-179) was being expressed, the protein was purified from the supernatant of engineered *S. boulardii* or *S. cerevisiae*, using immobilized metal affinity chromatography (IMAC) via an ÄKTA system. For *S. cerevisiae*, the SDS-PAGE analysis of the purified fractions revealed a weak and diffuse band close to the expected size range (Figure 4.6A), suggesting low or no expression of the target protein. To evaluate if the band corresponded to the purified endolysin, we concentrated the fractions where the band was visible and performed turbidity reduction assays against *C. difficile* APC43. However, there was no observable difference in OD reduction when comparing the purified protein fractions to the buffer control (Figure 4.6B), indicating a lack of lytic activity under the tested conditions. No lysis was observed in spot assays in *S. cerevisiae* not *S. boulardii* strains (data not shown). We attributed this outcome to insufficient production or misfolding of the CD27L(1-179) endolysin in the yeast expression system. Due to time constraints and the lack of observable activity, we did not pursue further optimization steps for the expression of this endolysin.

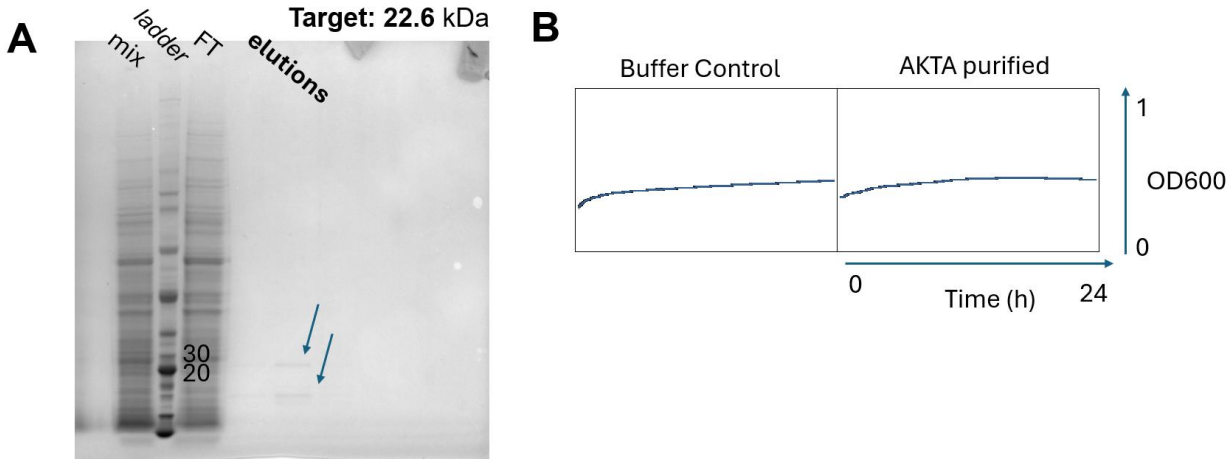


Figure 4.6. A) SDS-page PAGE analysis of the expression and purification of fractions in *S. cerevisiae*. B) OD600 reduction assay over 24 h in anaerobic conditions (37 °C) target protein CD27L(1-179) (22.6 KDa estimated size).

4.3.5. Activity of anti-staphylococcal yeast strains

Despite not being able to recover PlySA11 by IMAC, since the engineered yeast cells previously showed enzymatic activity against *S. aureus* heat-killed cells, we aimed to assess if the enzymatic activity could correspond to antimicrobial activity against live *S. aureus*. We tested both concentrated (10×) culture supernatants and concentrated yeast cell extracts from *S. cerevisiae* and *S. boulardii* strains after 96 h of growth. These were mixed

with early exponential-phase cultures of *S. aureus* and incubated under standard conditions to check for killing. *S. boulardii* supernatants, regardless of whether they originated from wild-type or PlySA11-expressing strains, exhibited a mild bacteriostatic effect, slowing bacterial growth relative to *S. cerevisiae*. In fact, *S. cerevisiae* supernatants seem to promote *S. aureus* growth, possibly serving as a nutrient source. No reduction in *S. aureus* growth was observed in strains expressing the PlySA11 endolysin, indicating that the enzyme, under these conditions was either inactive or not present at sufficient levels to impact the target cells (Figure 4.7).

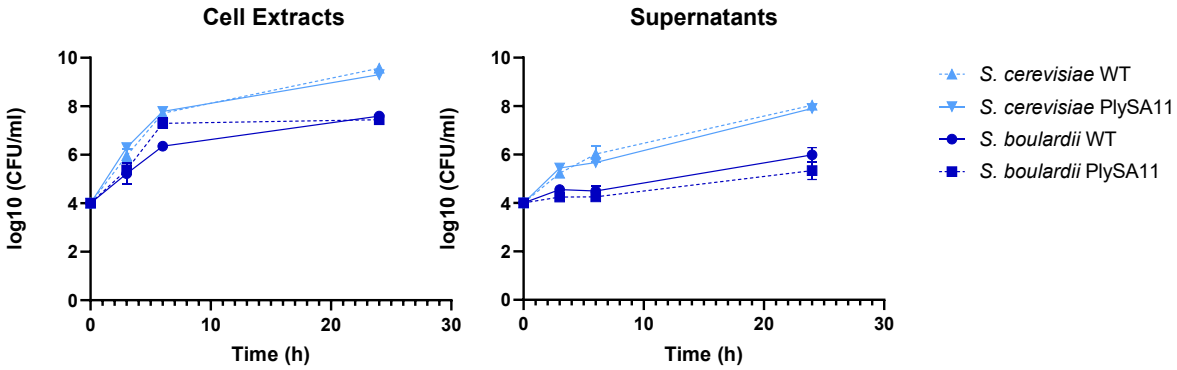


Figure 4.7. S. aureus D328 colony forming units after treatment with 10x concentrated supernatants or cell extracts of *S. boulardii* or *S. cerevisiae*, wild-type (WT) or expressing PlySA11, as indicated in the legend, over a 24-h period.

4.3.6. Activity of anti-staphylococcal *S. boulardii* engineered strains in a simulated GI fermentation

To further investigate the bacteriostatic effect of *S. boulardii* supernatants, in both the wild-type and engineered strains in a more complex environment, we conducted *in vitro* simulated gastrointestinal fermentation assays in fermenters, in the presence of *S. aureus*. No differences in viable *S. aureus* were observed between the *S. aureus*-only control, the *S. boulardii* wild-type, or the *S. boulardii* secreting PlySA11 strain, indicating that there was no antimicrobial effect under the tested conditions.

In some of the samples collected at different time points, plating of several samples yielded no visible *S. aureus* colonies (Figure 4.8., red circles). This may be due to the formation of viable but non-culturable (VBNC) *S. aureus* cells under the stress of the fermentation conditions, a phenomenon that has been reported in similar *in vitro* systems with varying concentration of acids and nutrients [20]. However, this issue was not further investigated. Overall, these results indicate that *S. aureus* remained viable and proliferated during the assay, and that neither the probiotic yeast nor the secreted endolysin had a detectable inhibitory effect under these conditions. The fact that bacterial numbers increased during the fermentation also

indicates that *S. aureus* proliferation is difficult to control, especially considering the previously demonstrated low activity and secretion efficiency of the LysSA11 construct in yeast.

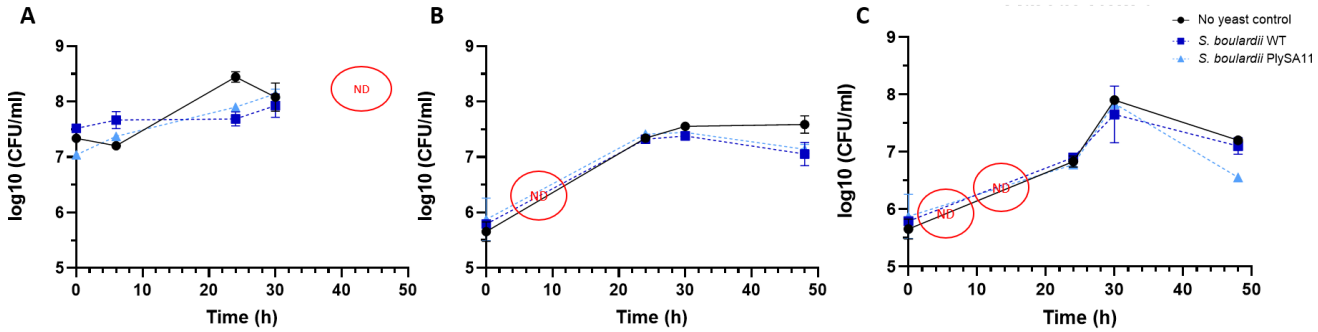


Figure 4.8. *S. aureus* D328 colony forming units after treatment with a no-yeast control, *S. boulardii* wild-type (WT) or *S. boulardii* expressing PlySA11, as indicated in the legend, over a 48-h period. ND: refers to not detected and is indicated at the time points where no cells were detected in the assay.

4.4. Discussion

The aim of this study was to engineer S. *cerevisiae* and S. *boulardii* strains to express and secrete two phage-derived endolysins, CD27L (targeting *C. difficile*) and LysSA11 (anti- *S. aureus*), to enhance the therapeutic potential of probiotic yeast against those pathogens, respectively. Previously, endolysin Ply511 against *Listeria monocytogenes* was successfully expressed and secreted in both yeast species, therefore we constructed new secretion cassettes and integrated them in two copies in each of the yeasts´ genome using the validated CRISPR-Cas9-based approach.

The lack of observable antimicrobial activity in all yeast strains tested suggests possible issues with protein expression, folding, or secretion. Unlike anti-*Listeria* Ply511, which we previously expressed successfully in both *S. cerevisiae* [21] and *S. boulardii* (previous chapter), PlySA11 or CD27L endolysins may be more difficult to express in yeast systems. Differences in protein size, folding requirements, glycosylation patterns, or stability could all influence the outcome.

Intriguingly, PlySA11 was previously expressed in yeast by Chun *et al.*, (2020) [11] using surface display, suggesting that *S. cerevisiae* is in fact capable of expressing the active protein, something that suggests that not the host, but the expression cassette, can be another factor influencing the outcome we report. Since we could previously secrete an active endolysin (Ply511) using both *S. cerevisiae* [21] and *S. boulardii* (Chapter III), we aimed to secrete PlySA11 using a similar approach. For that, we used the alpha-mating factor secretion signal, while our previous work with endolysin Ply511 relied on *Sed1*, a secretion signal that

has shown superior performance in yeast for certain heterologous proteins [22],[23]. Chun *et al.*, (2020) [11] used Aga2p cell surface display signal to direct the protein to the surface, however, it is unknown whether the same signal would allow the secretion of a functional endolysin into the extracellular media. A strategy developed by Cruz-Teran et al., (2017) was to use this signal to both secrete and display protein using "self-cleaving" 2A peptides [24]. The choice of secretion signal has been shown to be of utmost importance in protein secretion and some of the combinations of secretion signal and protein might not lead to secretion, requiring a case-by-case optimization[25]. Furthermore, the promoter–terminator pair used here (TEF1 and PGK1) differs from the Sed1 promoter and Sag1 terminator used previously, or the GAL1 promoter used by *Chun et al.,* (2020). The combination of TEF1 promoter and PGK1 terminator demonstrated a previous high-level expression with different molecules [26],[27]. Promoter strength is highly dependent on the carbon source[26],[28], and Sed1 promoter might be more appropriate than TEF1 for longer cultivation times and a slower but continuous expression during stationary growth phases of yeast. Again, a fine-tune optimization of the promoter for the expression of each single protein should be taken into account.

Another important difference between the current and previous designs is the number of integrated copies. In our earlier work[21], we achieved the triple integration of the Ply511 cassette in both yeast, whereas in this study, only two chromosomal copies were introduced. Further optimization of the chassis strain, such as improving stress tolerance, anaerobic growth, reducing metabolic burden, or introducing supportive folding and secretion machinery, may enhance the expression and activity of more challenging heterologous proteins in future studies. These design differences could have had a cumulative effect on expression levels and affect yeast metabolism, leading to a low activity, not sufficient to reduce the pathogen.

Regarding the anti-*S. aureus* activity of yeast strains, *S. boulardii* supernatants showed a slight bacteriostatic effect against *S. aureus in vitro*, which proved to be independent of endolysin expression, something that has been reported previously, affecting also other clinical pathogens [29]. This effect was not replicated in our study under simulated colonic fermentation conditions, likely due to the higher initial bacterial load in those assays, and the lower supernatant concentration, which was diluted in the fermenter.

No activity was detected for CD27L(1-179), even in heat-killed lawn assays, likely due to both technical difficulties in establishing consistent *C. difficile* lawns and the lack of a reliable positive control, together with the fact that the protein expression could not be confirmed. Since we confirmed that yeast metabolism was not affected, the design of the expression cassettes with LacA secretion signal or the nature of the protein,

as explained earlier, might be responsible for the lack of activity. Resource and time constraints limited the optimization of this analysis.

4.5. Conclusion

In conclusion, using yeast as a delivery platform for endolysins remains a promising but challenging strategy. The approach we used, to express these two endolysin involved multiple variables, such as expression cassette architecture, different endolysins and target bacteria. Future efforts may benefit from a modular and systematic approach, such as the described a GoldenGate [30] or more specifically VersaTile strategy [31] where combinatorial libraries containing different secretion signals, promoters, endolysin coding sequences and terminators, can be tested and optimized individually or in combination, while being assembled into a final expression system. These approaches could be optimized to include selection filters such as conditions resembling the GIT (low pH or the presence of different proteolytic enzymes), leading to a robust platform for endolysin delivery to the gut.

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Chapter V.

General conclusions and future perspectives.

This chapter offers the major conclusions from this doctoral project and highlights the next steps of this work, emphasising its contribution to the development of alternatives to antibiotics in the context of gastro-intestinal infections, using phage-based tools and genetically engineered probiotics.

The antibiotic resistance crisis is speeding up the need for alternatives to conventional antibiotics, particularly in bacterial infections affecting the gastrointestinal tract, where relapse and recurrence are frequently linked to the use of antibiotics and its consequential microbiota disruption. Throughout this thesis we explored the engineering of probiotic yeast to express phage endolysins, to be used as precision antimicrobials —*Saccharomyces cerevisiae* and *Saccharomyces boulardii*—as chassis for their delivery to the gut. We used a CRISPR/Cas9–based approach resulting in a marker-free, chromosomal integration of endolysin expression cassettes, evaluated secretion and activity in gastrointestinal settings, and aimed to extend the platform beyond *Listeria monocytogenes* to *Staphylococcus aureus* and *Clostridioides difficile*. Taken together, our results provide a proof-of-concept for probiotic yeast-mediated endolysin delivery, while also clarifying the major technical bottlenecks and the most promising routes for improvement that the development of such platform would face.

In **Chapter I** we introduced the need for microbiome-sparing antimicrobials in the context of the antimicrobial resistance crisis, outlined the therapeutic rationale for the use of phages and their endolysins, and the advantages of yeast as a production and/or delivery platform. We highlighted the limitations of a yeast expression system for production of whole phage particles and the advances in probiotic engineering. These advances have made it possible to produce molecules of interest, in our case, phage endolysins, aiming to target intestinal pathogens while sparing the commensal gut microbiome.

In **Chapter II**, the feasibility of a yeast-based system for expression of Ply511 was demonstrated. To attain this, *Saccharomyces cerevisiae* was successfully engineered, using a CRISPR-Cas9 system, obtaining a strain featuring a triple integration of the expression cassette featuring either Sed1-driven secretion or surface-display of the endolysin. We assessed this strain for anti-*Listeria* activity using whole cells, supernatants and cell extracts. We demonstrated that engineered *S. cerevisiae* was able to supress *L. monocytogenes* when secreting Ply511, and that the Sed1-driven surface display system was not effective whatsoever. CRISPR-Cas9 enables a stable, marker-free integration of endolysin constructs into the yeast genome, overcoming limitations of plasmid-based expression. Our data establish secretion (rather than display) as the preferred mode for yeast-based endolysin delivery and validated a CRISPR-Cas9-based integration as a robust alternative to plasmids, for example, *S. cerevisiae* has previously been used to produce or display endolysins [11],[32],[33], showing that *S. cerevisiae* can be used for endolysin production, however, they relied on the use of plasmids (and their selective markers) or induction strategies, which

would make application and commercialization challenging. In contrast, our approach assures a marker-free, stable and scarless chromosomal integration [13].

In **Chapter III** we translated the findings on the design of the secretion of Ply511 from *S. cerevisiae* to the probiotic *S. boulardii*. Using the same multi-locus integration strategy, we created strains secreting Ply511 and assessed growth and activity under conditions relevant to the gut. We show that the endolysin Ply511 can be expressed in *S. boulardii*, showing for the first time the expression of an endolysin in this host. In simulated gastrointestinal digestions, using an INFOGEST gastric–intestinal digestion we found that both *S. cerevisiae* and *S. boulardii* can reduce *L. monocytogenes*, reinforcing the feasibility of yeast delivery of endolysins throughout the gastrointestinal conditions. We then evaluated activity in a simplified intestinal community (SIHUMI) including *L. monocytogenes*, showing that yeast extracts containing Ply511 have selective activity against the pathogen, indicating no detrimental impact of yeast extracts on the non-target bacteria, consistent with endolysin specificity. However, when we tested the yeast in faecal fermentations resembling the small intestine*, L. monocytogenes* levels did not decrease, highlighting the impact of matrix complexity and community interactions on endolysin activity and bacterial killing.

In **Chapter IV** we aimed to expand our yeast expression system towards two other pathogens, *S. aureus* and *C. difficile* expressing endolysins PlySA11 or CD27L(1-179), respectively, with new constructions featuring TEF1-driven expression cassettes, and two different secretions signals. Both engineered yeast failed to show activity against each pathogen, proving that secretion efficiency and enzyme stability are highly endolysin and construct dependent.

Across the conclusions of this work, there are highlights which we would like to point out as contributions to the field.

First, we proved that secretion outperforms surface display for endolysin Ply511. Despite successful display cassette integration and epitope detection, anchoring Ply511 to the yeast surface did not reduce *L. monocytogenes*, whereas secretion consistently yielded activity—including in a food matrix and through simulated digestion—making secretion the default mode for endolysin delivery in yeast. Interestingly, PlySA11 did achieve a bactericidal effect against *S. aureus* in a surface-display system, albeit with high yeast concentrations (OD_{600} 50). Authors did not test secretion [11] .

Second, we showed that host background and growth conditions have a relevant impact on expression and endolysin activity, as previously shown with other proteins [26],[28]. *S. boulardii* was able to secrete Ply511 but generally exhibited lower activity than *S. cerevisiae* across assays, in line with anaerobic growth differences and likely reflecting the fact that this expression cassette was optimized for *S. cerevisiae* (promoter/terminator and secretion signal choices) and would require further optimization to overcome strain-specific differences. We recognise that the optimization of protein expression and evaluation of protein activity early in the development of the work is important. For example, evaluating expression under anaerobic conditions, or protein activity in gut-like conditions.

Third, we further prove that endolysin Ply511 was specific, as shown previously by [34] and its activity was maintained in more complex settings: in SIHUMI-L, yeast extracts reduced the target pathogen without suppressing the non-target community members, aligning with the expected narrow spectrum of endolysins. Testing for this desirable property is of utmost important for microbiome-sparing interventions.

Fourth, in the same regard, we found differences between different assays in the anti-*Listeria* activity, being non-effective in assays with faecal samples, where a more varied microbial community is present. Metagenomic sequencing or further studies should be done to confirm whether the same would happen *in vivo* and would further contribute to the claim of endolysin specificity, as previously studied for CD27L_EAD [7].

Fifth, we would like to highlight the difference in expression and activity seen between endolysins. The contrasting outcomes with LysSA11 and CD27L(1-179) indicate that secretion signals, genomic cassettes, protein differences and host, can influence the expression and protein activity, and there is still room for improvement in this regard.

Our work also presents several limitations that would set the base of the next steps of the work:

First, protein secretion yields in *S. boulardii* were lower than in *S. cerevisiae*, affecting its activity in complex matrices; we did not directly quantify secreted protein concentrations, which hampers precise correlations between hosts and effect. Our proposal for future efforts would be to reoptimize using promoters and terminators from *S. boulardii*, as done in [29] , screening secretion signals beyond Sed1/α-factor/LacA, using a similar approach to [25] and using *S. boulardii* strains engineered for secretion, such as protease-deficient strains [35]. We believe there is also room for optimization in the endolysin cassette, were truncation and

domain swaps have enhanced Ply511 in other contexts[36] and applying rational engineering to increase lytic activity in certain environments such as stability at gut pH/ionic strength could also improve the overall outcome. A strategy such as Golden-Gate-like [30] or VersaTile [37] would enable rapid assembly of promoters, secretion signals, coding variants of endolysins and terminators into random libraries, ideally screened under selection filters mimicking gut constraints (low pH, bile, proteases). Then, once the best architecture is defined, this could be used as a platform where not only Gram-positive, but also Gram-positive targets can be envisioned; such as *Helicobacter pylori*, or enterotoxigenic *E. coli*, which are also prevalent and associated with diseases in the gastrointestinal tract. Second, a direct comparison between the free endolysin, different delivery systems such as the one described in this thesis, using yeast probiotics or encapsulation, should also be performed in the future to evaluate whether the yeast is synergizing with the endolysin effect.

Third, most of the evidence of anti-*Listeria* activity comes from *in vitro* experiments. Thus, *in vivo* performance should be a future priority. The small-intestine faecal fermentation model showed no *L. monocytogenes* reduction despite positive results in simpler systems, highlighting the need to better understand matrix and community effects.

Finally, metagenomic sequencing was not performed in the *ex vivo* assays, this is an important future direction to determine the broader impact of this delivery system on the gut microbiome.

There are several applications which we could envision for an engineered probiotic capable of producing endolysins. A first application can be envisioned in the food sector, where engineered yeasts could be used as natural biopreservatives in ready-to-eat foods and dairy products, such as fermented cheese, where yeast is already used[38]. Another application we envision is in the probiotic field, where *S. boulardii* strains engineered for endolysin secretion could be administered as preventive supplements, particularly in high-risk populations such as the elderly, immunocompromised individuals, or pregnant women, to reduce the risk of intestinal colonization and infection, particularly against *L. monocytogenes*. In parallel, the activity of yeast supernatants or yeast extracts opens the possibility for postbiotic formulations, where the antimicrobial effect is delivered without the use of a live organism, specially accounting for its genetically modified nature. This could potentially simplify some of the regulatory and safety challenges associated with the commercialization of such product. Finally, given the high specificity of endolysins, probiotic or postbiotic applications could extend beyond pathogen control to precision microbiome modulation, allowing

selective removal of harmful bacteria while preserving commensal diversity. For such applications, future work should also address the safety and regulatory aspects of applying engineered probiotic yeasts to humans. For this, incorporating biocontainment strategies such as auxotrophic mutants or kill-switches such as the ones described for *S. boulardii* [39],[40], are of utmost importance.

This work demonstrates that probiotic yeast can be genetically engineered to deliver phage endolysins. While secretion efficiency remains a critical hurdle, the results provide a foundation for probiotic-based precision antimicrobials and their potential delivery into the gut. This combined approach—linking the already proven health-promoting effects of probiotics with the marker-free engineering using CRISPR/Cas9 and the specificity of phage endolysins— is an approach that has the potential to contribute target gut pathogens while sparing commensal microbiota in intestinal infections.

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Table A1.1. Plasmid expression systems reported in *S. boulardii.* (a)- Commercially available product containing the CNCM I-745 strain; (b)- GDP and TDH3 are different nomenclatures for the same promotor; (c)- Not disclosed (n/d): Information was not available; (d) Promotors- TDH3, CCW12, HHF1, HHF2, TEF1, TEF2, HTB2, RPL18B, ALD6, PAB1, RET2, pRNR1, pREV1, SAC6, RNR2, POP6, RAD27, PSP2; Terminators- ENO1, ENO2, SSA1, PGK1, ADH1, and TDH1; Selective markers- URA3, HIS3, TRP1 (Auxotrophic) and KanMX, ZeoR, NatR, HygR (Dominant); (e) Promotors- TDH3, CCW12, HHF2, TEF1, TEF2, HHF1, HTB2, PAB1, RNR2; Terminators- SSA1; ADH1; TDH1; ENO; (f) The strain used was isolated from a commercially available product from Jarrow Formulas and referred as CLPY01 in the study; (h) The Medium chain fatty acids pathway was constituted by the following genes: mutant gene of Homo sapiens fatty acid synthase with its native thioesterase domain replaced by the Rattus norvegicus thioesterase II gene (mhFAS-rTEII) and H. sapiens phosphopantetheinyl transferase gene (hSFP). References are listed in Chapter I.

Study	Strain(s)	Origin(s) of replication	Promotor(s)	Terminator(s)	Marker(s)	Protein(s) expressed
[258]	Perenterol [a]	2μ	GPD [b]	ADH I	KanMX (G418)	HCMV IL-10; EBV IL-10
[259]	Perenterol [a]	2μ	GPD [b]	ADH I	KanMX (G418)	IL-10
[208]	*Iyo* (DiarSafe)	2μ	Native Promotor	Native Terminator	URA3	Ura3p
[205]	CNCM I-745	2μ	TEF1; PGK1	n/d [c]	KanMX (G418)	EGFP; Eimeria *tenella* Microneme-2 proteins
[204]	ATCC MYA-797	2μ	TEF1	n/d [c]	URA3	GFP
[198]	ATCC MYA-796	ARS1	n/d [c]	n/d [c]	Hygromicin B	Hygromycin B Phosphotransferase
[207]	ATCC MYA-796	2μ	TDH3 [b]	CYC1	KanMX (G418)	β-galactosidase (lacZ)
[207]	ATCC MYA-796Δura3	2μ	ADH1	ADH1	URA3	mRuby
[260]	CNCM I-745	2μ	TEF1	TPI1	BlastS	Leucocin C
[199]	ATCC MYA-796	2μ	TEF1	n/d [c]	Hygromicin B	mCherry
[185]	ATCC MYA-796	2μ	TEF1	CYC1	URA3	Heavy chain antibodies fusion
[196]	ATCC MYA-796 Δura3Δtrp1Δhis3	2μ; CEN6/ARS4	18 promotors [d]	5 terminators [d]	7 markers [d]	mRuby
[196]	ATCC MYA-796Δura3	CEN/ARS	9 promotors [e]	4 terminators [e]	URA3	β-carotene pathway; Violacein pathway
[261]	ATCC MYA-796 Δura3Δtrp1Δhis3	2μ	GPD [b]	CYC1	URA3; TRP1; HIS3	Endo-type β-agarase

[262]	ATCC MYA-796 Δura3Δleu2Δhis3	2μ	GPD [b]	CYC1	URA3;LEU2;HIS3	l-fucose metabolic pathway
[263]	CLPY01 [f]	2μ	GAL1; GAL10	CYC1; ADH1	URA3	Medium chain fatty acids pathway [g]
[206]	CNCM I-745 Δura3	2μ	TEF1	CYC1	URA3	EGFP; Lipase inhibitor peptide 1 and 2
[245]	ATCC MYA-796Δura3	2μ; CEN6/ARS4	TDH3 [b]	TDH1	URA3	*C. difficile* Toxin A Neutralising Peptide (NPA)
[197]	ATCC MYA-796Δura3	2μ	TEF1	n/d [c]	URA3	Erythromycin destructase (ereA)
[264]	ATCC MYA-797	2μ	TEF1	TPI1	BlastS	haPD-1; rPD-L1nb; Gaussia luciferase
[81]	ATCC MYA-797Δura3	2μ	TDH3 [b]	CYC1	URA3	yeGFP; Aspergillus aculeatus β-glucosidase

Table A1.2. YIp, Cre-loxP and Ty elements integration/gene disruption strategies used in S. boulardii. Chr stands for chromosome. (a)- *CNCM I-745* and *BIOCODEX* are different nomenclatures for the same strain; (b)- Not disclosed (n/d): Information was not available; (c) The strain used was isolated from a commercially available product from Jarrow Formulas and referred as CLPY01 in the study; (d) The Medium chain fatty acids pathway was constituted by the following genes: mutant gene of Homo sapiens fatty acid synthase with its native thioesterase domain replaced by the Rattus norvegicus thioesterase II gene (mhFAS-rTEII) and H. sapiens phosphopantetheinyl transferase gene (hSFP). References are listed in Chapter I.

Study	Method	Strain	Chr	Target locus/ Ty element	Promotor	Terminator	Protein(s) expressed/Gene disrupted
[207]	YIp	ATCC MYA-796 Δura3	V	YEL021W	TDH3; PGK1	TDH3; PGK1	Xylose metabolic pathway
[211]	YIp	CNCM I-745 Δura3 [a]	n/d [b]	n/d [b]	PGK1 GAL-1; TEF1	CYC1	Chicken ovalbumin - *C. perfringens* enterotoxin
[199]	YIp	ATCCMYA-796	XII	XII-1; XII-2 XII-4	PGK1; TEF1	n/d [b]	mCherry; EGFP
[265]	YIp	ATCC MYA-796	X	X-2	TEF1	CYC1	GFP
[212]	YIp	ATCC MYA-796 Δura3	n/d [b]	n/d [b]	TDH3; TPI1	MATa	mSA-AGA1-AGA2
[266]	YIp	ATCC MYA-796	X	X-2	TEF1	CYC1	GFP
[263]	YIp	CLPY01 Δura3 [c]	V	YEL021W	TDH3; TEF1	CYC1; ADH1	Medium chain fatty acids pathway [d]

[218]	Cre-loxP	CNCM I-745 [a]	V	YEL021W	-	-	URA3
[185]	Cre-loxP	CNCM I-745 [a]	V	YEL021W	-	-	URA3
[219]	Cre-loxP	CNCM I-745 [a]	XV	YOR128C	-	-	ADE2
[199]	Ty elements	ATCC MYA-796	-	Ty1.1; Ty1.2; Ty2; Ty3	TEF1	n/d [b]	IL-10; TNFR1-ECD; AP; ANP
[267],[268]	Ty elements	ATCC MYA-796Δoaz1	-	Ty2	TDH3	CYC1	Overexpression of Spermidine pathway related genes
[269]	PiggyBac Transposon	BIOCODEX Δura3Δtrp1 [a]	-	-	TEF2	n/d [b]	EGFP; l-Ergothioneine pathway

Table A1.3. CRISPR-Cas systems used in *S. boulardii*. Chr stands for chromosome. (a) Strain description- *S. boulardii ATCC-MYA796Δura3Δhis3* with *PGM2* mutation corrected; (b)- Enterol- Commercially available probiotic product which contains the *CNCM I-745* strain; *Sb.P*- Natural isolate that accumulates elevated levels of acetic acid in YPD 2%, sensitive to low pH. [270]; (c)- Not disclosed (n/d): Information was not available; (d)- Promotors: *ALD6*; *CYC1*; *CYC7*; *DAN1*; *HSP26*; *HXT7*; *JEN1*; *SSA1*; *SUC2*; *TDH3*; *TEF1* and *TPI1*; (e)- Modified version of the *DIT1* terminator: *DIT1*$^{[32\text{-}34delTGT+74\text{-}76delCAT]}$; (f)- Genes KO: *ROX1*; *PAH1*; *DER1*; *HRD1*; *GOS1*; *OCH1*; *MNN9*; *VPS5*; *VPS17*; *TDA3*; *YPS1*; *PRB1*; *PEP4* and *APE1*; (g)- Genes KO: *URA3*; *HIS3*; *TRP1*; *SNO1*; *SNZ1*; *THI2*; *THI6* (auxotrophic) and *REI1*; *BTS1* (temperature resistance); (h)- Cell penetrating peptides produced: RRL helix; Shuffle; Penetramax and PN159. References are listed in Chapter I.

Study	Strain	Chr	Locus	Promotor	Terminator	Protein (s) expressed/ targeted gene (s)
[207]	ATCC MYA-796	-	-	-	-	URA3; HIS3; TRP1; LEU2 (Gene disruption)
[207]	ATCC MYA-796	XVI	CS8	TDH3	CYC1	Human lysozyme (Protein expression)
[246]	ATCC MYA-796	-	-	-	-	PGM2 (Mutation correction)
[196]	ATCC MYA-796 Δura3	V; XI; XVI; VIII; XV	INT1; INT2; INT3; INT4; INT5	TDH3	TDH1	mTurqouise2; mRuby2 (Protein expression)
[261]	ATCC MYA-796	XV	CS5	TDH3	CYC1	BpGH16A (Protein expression)
[261]	ATCC MYA-796 Δura3Δtrp1	-	-	-	-	HIS3 (Gene disruption)
[265]	ATCC MYA-796	-	-	-	-	URA3 (Gene disruption)
[245]	ATCC MYA-796 Δura3	V	INT1	TDH3	TDH1	NPA (Protein expression)

[245]	ATCC MYA-796 Δura3	-	-	-	-	KO of 14 genes involved in protein secretion pathway [f]
[242]	ATCC MYA-796	VII; XII; XVI	CS6; CS7 CS8	TDH3	CYC1	Human lysozyme (Protein expression)
[271]	ATCC MYA-796 Δura3	X; XI XII	X-2; X-4 XI-3; XII-5	TDH3	DIT1** [e]	GLP-1; Exendin-4 (Protein expression)
[244]	ATCCMYA-796 Δura3	-	-	-	-	KO of 7 auxotrophic genes and 2 genes involved in temperature resistance [g]
[241]	ATCC MYA-796	VII	CS6	PGK	CYC1	Firefly luciferase (Protein expression)
[240]	ATCC MYA-796 Δura3	XI; XII	XI-3; XII-5	12 promotors [c]	ADH1	yEGFP; mKate2 (Protein expression)
[247]	ATCC MYA-796	-	-	-	-	URA3; HIS3 (Gene disruption)
[247]	ATCC MYA-796 Δura3Δhis3	-	-	-	-	PGM2 (Mutation correction)
[247]	SbGal- [a]	XV	INT5	GAL1; TET	n/d [c]	NanoLuc /Protein expression/
[219]	CNCM I-745	n/d [c]	n/d [c]	TET4; TDH3 MYO2	ADH1	yEGFP; rtTA (Protein expression)
[272]	Enterol; Sb.P [b]	II; IV; XII; XVI	II-1; IV-1 XII-1; XVI-1	TDH3; TEF1; RPL18B; SAC6	ENO2; CYC; ADH1	Abscisic acid pathway related genes
[273]	ATCC MYA-796	VII	int#1	CCW12	CYC1	EGFP; Endo-inulinase (Protein Expression)
[267],[268]	ATCC MYA-796	-	-	-	-	OAZ1 (Gene disruption)
[274]	ATCC MYA-796	XI; XII	XI-3; XII-5	TDH3	DIT1** [e]	Production of 4 cell penetrating peptides [h]
[81]	ATCC MYA-797	-	-	-	-	URA3; CCW12; CCW14; FYV5 (Gene disruption)
[81]	ATCC MYA-797 Δura3Δccw12Δccw14Δfyv5	XV	int#11	TDH3	CYC1	yeGFP; Aspergillus aculeatus β-glucosidase (Protein expression)

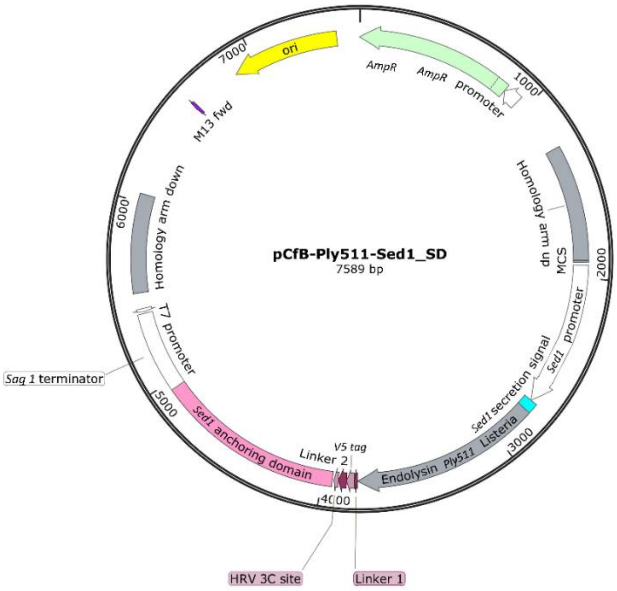


Figure A2.1. pCfB-Ply511-Sed1_SD genome map annotated. Homology regions used in this study belong to X-4, XI-3 and XII-5, sites in pCfB3035-Ply511-Sed1_SD, pCfB2904-Ply511-Sed1_SD, pCfB2909-Ply511-Sed1_SD respectively.

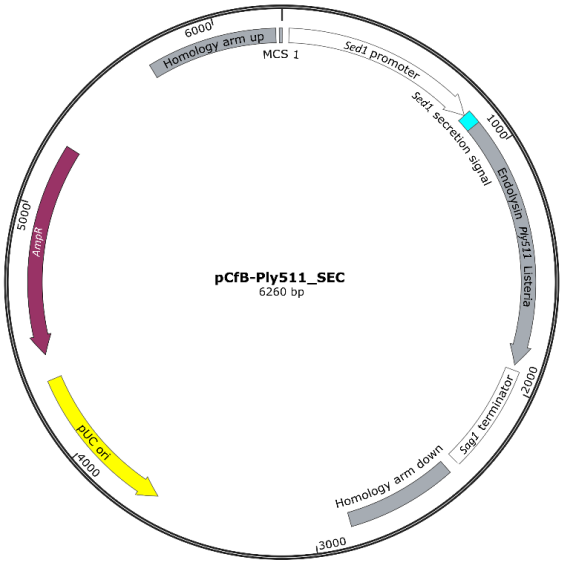


Figure A2.2. pCfB-Ply511_SEC genome map annotated. Homology regions used in this study belong to X-4, XI-3 and XII-5, sites in pCfB3035-Ply511_SEC, pCfB2904-Ply511_SEC, pCfB2909-Ply511-SED1_SEC, respectively.

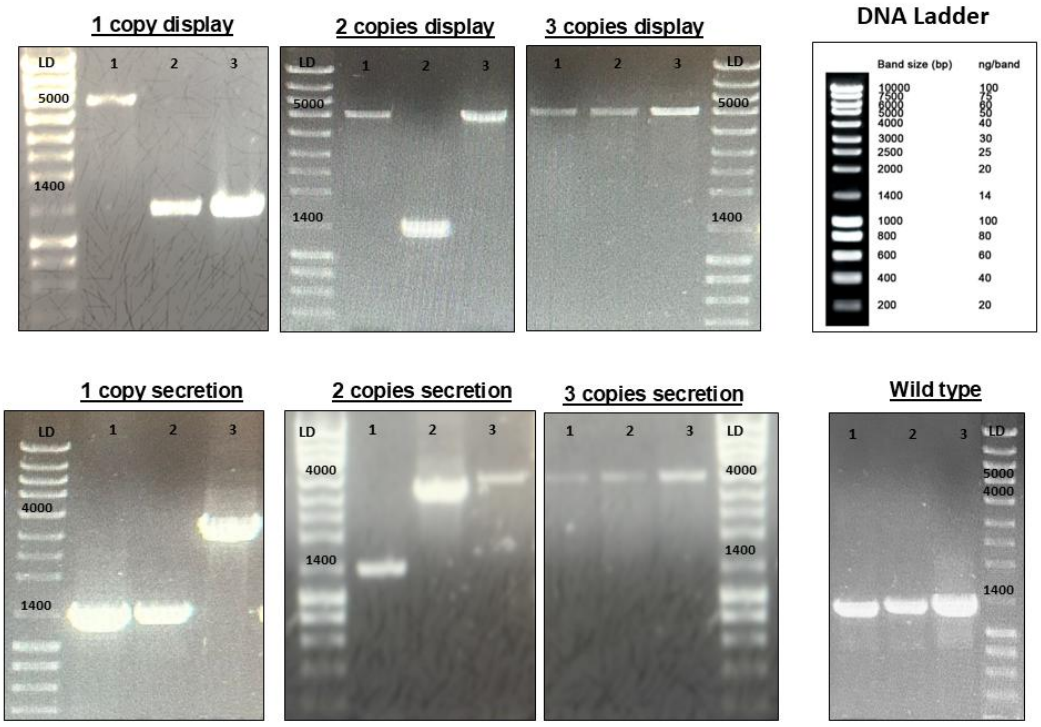


Figure A2.3. Colony PCR of *S. cerevisiae* single, double and triple insertion of the Ply511 display (upper panel) or secretion cassette (lower panel). The picture shows the gel electrophoresis 1% agarose of the PCR products, corresponding to the numbers indicated in the figure legend 1: Chromosome XII site 5 (primers 899 and 900); 2: Chromosome X site 4 (primers 905 and 906) 3: Chromosome XI site 3 (primers 911 and 912), LD: DNA ladder. Sizes expected for unmodified yeast, 1 (XII-5): 1365 bp, 2 (X-4): 1394 bp, 3 (XI-3) 1450 bp. For yeast display, 1 (XII-5): 5128 pb; 2 (X-4): 5157 bp; 3 (XI-3): 5213 bp. For yeast secreting the endolysin, 1 (XII-5): 3799 bp; 2 (X-4): 3828bp; 3 (XI-3): 3884 bp.

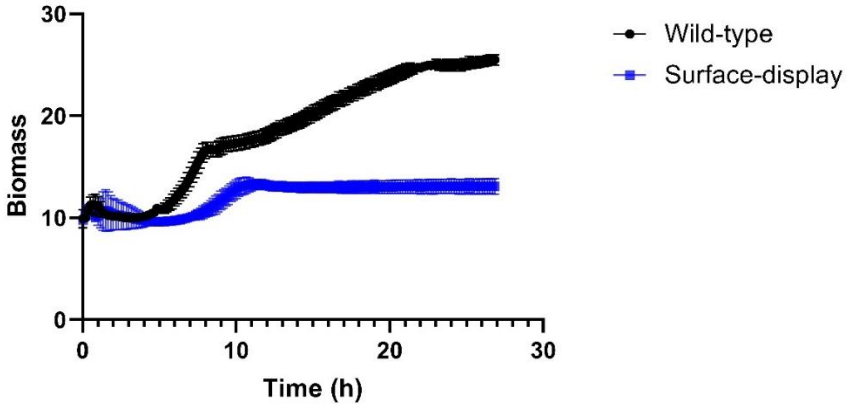


Figure A2.4. Biomass of different yeast over time, as indicated in the legend

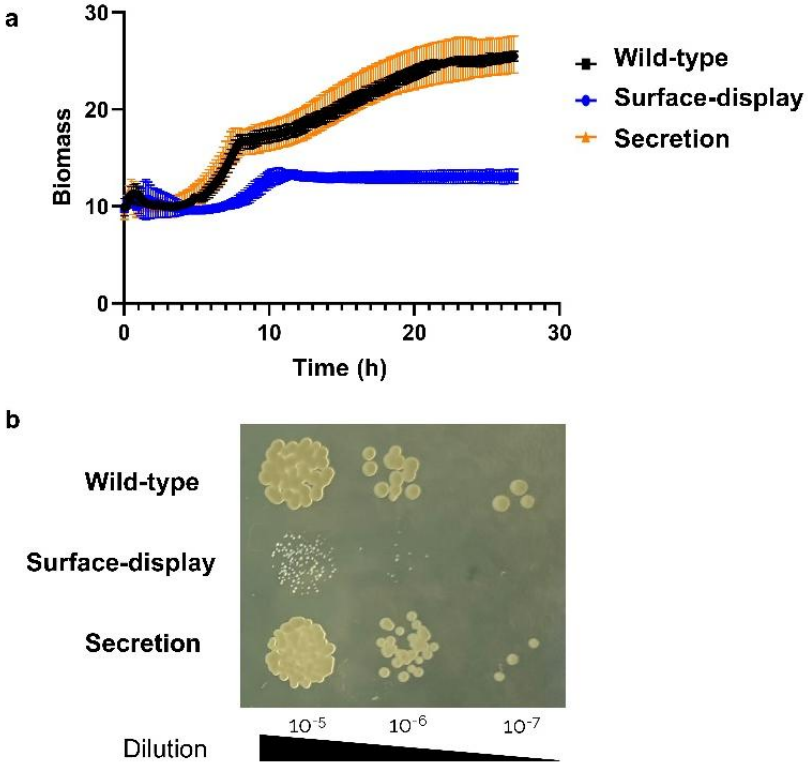


Figure A2.5. a) Biomass over time of different yeast as indicated in the legend. b) Growth of colonies in agar plates after 24 h incubation. (WT: Wild-type yeast, SD: Ply511 Surface-Display yeast; SEC: Ply511 secretion yeast).

Table A2.1. Primers used in this study for plasmid assembly and colony PCR.

Primer name	Primer sequence (5'->3')	Aim
p59_FW	actactttggcccaaGTTAAATACACTGTCGAGAAC	Amplification of the endolysin cassette Ply511-GS-based linker (GSSGGS)-V5tag-GS-based linker(G4S)3-HRV3C cut site
p54_RV	taatttactcgagccTGGACCTTGGAATAAGACTT	
p49_FW	GGCTCGAGTAAATTATCAAC	Amplification of the plasmid backbone for surface display from pI2-EG-kanMX (*Sed1* promoter, Sed1SS, Sed1 anchor, *Sag1* terminator)
p56_RV	TTGGGCCAAAGTAGTC	
pJ_05_FW	CCAATTCGCCCTATAGTGAG	

pCfB_Iin_RV	ATCGCACGCATTCCATG	Amplification of the insertion site cassette with homology arms for site XI-3, X-4 or XII-5
p_23_9_FW	tggaatgcgtgcgatttggatatagAAAATTAACGTAAGGCAGTATC	Amplification of surface display of Ply511 cassette
p_23_10_RV	tatagggcgaattggTTTGATTATGTTCTTTCTATTTGAATGAGATATGAGAGAG	
p460_FW	gctgttatcaaaaag**TAA**TGTACAGTTAGTACATTGAGTC	Amplification of the endolysin secretion without the Sed1 anchor, adding a stop codon (in bold) to close the plasmid.
p46_RV	**TTA**CTTTTTGATAACAGCACCCG	
p62_ply511_FW	ATTAAGTAAAATCAAGGG	Colony PCR to confirm the insertion of Ply511-GS-based linker (GSSGGS)-V5tag-GS-based linker(G4S)3-HRV3C cut site into the surface display cassette
pSAG_RV	GAAGAAAAAAAGAGCCAGGATG	
p47_FW	TAAATATACGAAATTATTGTTC	Colony PCR to confirm the insertion of Ply511-surface display cassette into the homology arms for sites XII-5, X-4 and XI-3
p770_RV	TTTGCTGGCCTTTTGCTC	

Table A2.2. Serovars of *Listeria monocytogenes* used for enzymatic activity confirmation.

Serovar	Strain name	Clearance around yeast
1/2c	CECT 911	Yes
3b	CECT 937	Yes
3c	CECT 938	Yes
4b	Scott A	Yes
4c	CECT 939	Yes

Table A2.3. Values of Log_{10} (CFU/mL) of *L. monocytogenes* Serovar 4c after 3, 6 and 24 h of contact with different yeast concentrations as indicated in the legend. Results are the mean and standard deviation of three replicates.

Yeast $5x10^7$	Wild-type	Surface display
3 h	3.12 ± 0.45	3.13 ± 0.11
6 h	4.25 ± 0.10	4.09 ± 0.12
24 h	7.57 ± 0.19	7.58 ± 0.14
Yeast 10^9	**Wild-type**	**Surface display**
3 h	3.26 ± 0.02	3.33 ± 0.01
6 h	4.55 ± 0.07	4.29 ± 0.02
24 h	8.6 ± 0.29	8.25 ± 0.13

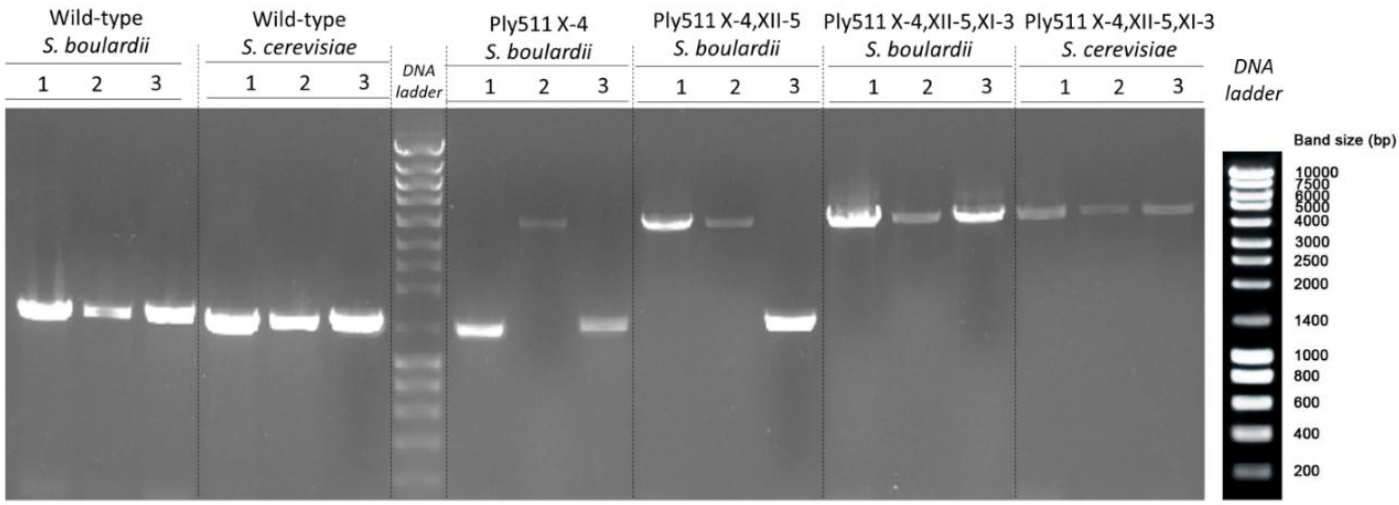


Figure A3.1. Colony PCR of *S. boulardii* single, double and triple insertion of the Ply511 secretion cassette. The picture shows the gel electrophoresis 1% agarose of the PCR products, corresponding to the numbers indicated in the figure legend 1: Chromosome XII site 5 (primers 899 and 900); 2: Chromosome X site 4 (primers 905 and 906) 3: Chromosome XI site 3 (primers 911 and 912), LD: DNA ladder. Sizes expected for unmodified yeast, 1 (XII-5): 1365 bp, 2 (X-4): 1394 bp, 3 (XI-3) 1450 bp. For yeast secreting the endolysin, 1 (XII-5): 3799 bp; 2 (X-4): 3828bp; 3 (XI-3): 3884 bp.

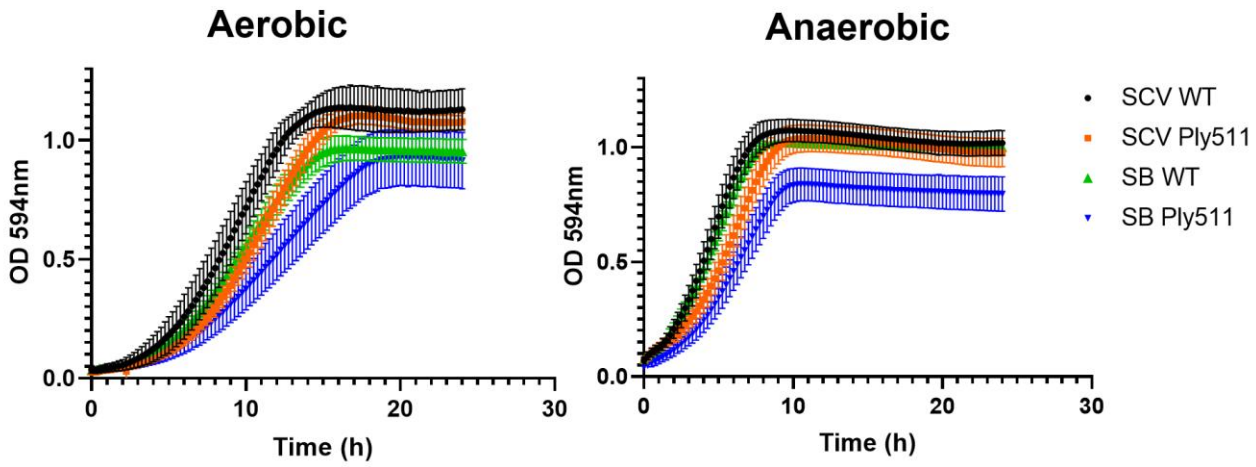


Figure A3.2. OD600 measurements over 24h, of different yeast over time, as indicated in the legend (SCV refers to S. cerevisiae, SB refers to S. boulardii and WT refers to Wild-Type).

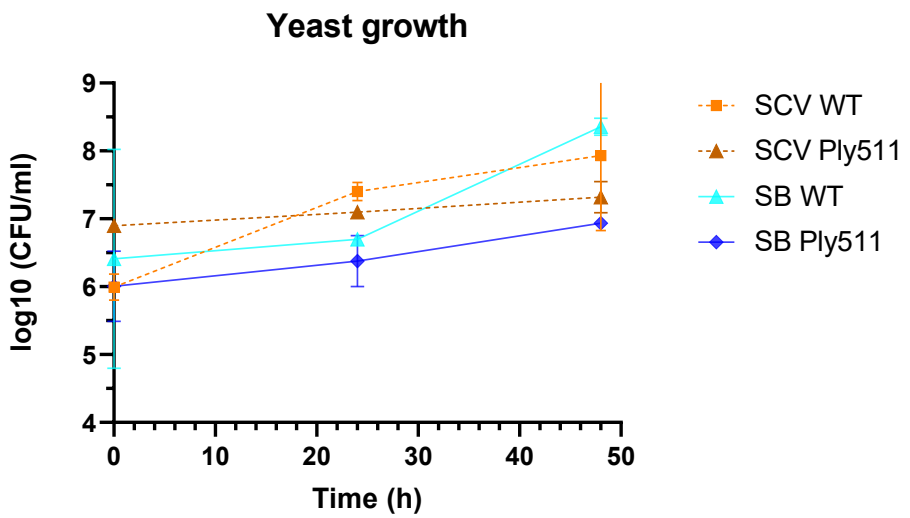


Figure A3.3. Log10 (CFU/mL) of S. boulardii (SB) or S. cerevisiae (SCV), wild-type (WT) or secreting Ply511 (Ply511), as indicated in the legend, after 0h, 24 h or 48 h of simulated intestinal fermentation. Figure shows the log10 (CFU/mL) mean of three replicates, standard deviation is represented as error bars.

Table A3.1. Analysis of the compatibility of the homology arms (upstream and downstream) for each site available in the EasyClone-MarkerFree vector set. Both homology arms from each integration plasmid of the set were compared with *S. boulardii* genome (GenBank accession number: GCA_001298375.2) to evaluate if they were compatible with genetic engineering in the probiotic yeast.

	Upstream homology arm			Downstream homology arm			
Target site	Aligned/total arm length	Mismatches/gaps or insertions	Identity	Aligned/total arm length	Mismatches/gaps or insertions	Identity	Compatible with SB?
X-2	530/533	2/1	99.44 %	488/491	2/1	99.40 %	Yes
X-3	563/572	1/1	98.43 %	539/541	2/0	99.63 %	Yes
X-4	521/522	1/0	99.80%	446/446	0/0	100%	Yes
XI-1	443/473	13/4	93.66 %	555/585	16/3	95.87%	No
XI-2	565/569	4/0	99.30%	544/569	19/5	95.61%	Yes
XI-3	564/575	11/0	98.10%	530/536	5/1	98.88%	Yes
XI-5	662/663	1/0	99.85%	455/457	2/0	99.56%	Yes
XII-1	583/590	7/0	99.81%	508/514	6/0	98.83%	Yes
XII-2	522/536	14/1	97.38%	545/550	5/0	99.09%	Yes
XII-4	484/494	10/0	97.98%	484/502	14/1	96.41%	Yes
XII-5	449/454	5/1	98.90%	666/677	9/1	98.38%	Yes

Table A3.2. Guide RNA analysis. Protospacer identity and PAM analysis for each site available in the EasyClone-MarkerFree vector set. The protospacer sequences were aligned with *S. boulardii* genome (GenBank accession number: GCA_001298375.2) using BLAST and the PAM sequences were identified in SnapGene after locating the protospacer. *Due to the mismatches the CAS9 efficiency would likely be too low therefore, these sites were deemed not compatible.

Target site	PAM distal region								PAM proximal or seed region												PAM			Protospacer Identity	Compatible with SB?
	+20	+19	+18	+17	+16	+15	+14	+13	+12	+11	+10	+9	+8	+7	+6	+5	+4	+3	+2	+1	N	G	G		
X-2	C	T	C	T	C	G	A	A	G	T	G	G	T	C	A	C	G	T	G	C	G	G	G	100 %	**Yes**
X-3	C	T	A	A	T	G	T	G	T	C	C	G	C	G	T	T	T	C	T	A	A	G	G	100 %	**Yes**
X-4	C	G	C	C	A	T	T	C	A	A	G	A	G	C	A	G	C	A	A	C	A	G	G	100%	**Yes**
XI-1	G	C	A	A	T	G	C	G	A	T	G	T	T	A	G	T	T	T	A	G	C	G	G	80%	**No***
XI-2	G	T	T	G	A	C	C	A	G	T	T	G	A	T	C	A	G	T	T	G	A	G	G	80%	**No***
XI-3	A	T	A	T	G	T	C	T	C	T	A	A	T	T	T	T	G	G	A	A	G	G	G	100 %	**Yes**
XI-5	T	G	A	G	A	A	T	A	C	T	G	T	T	G	T	A	A	A	A	C	A	G	G	95%	**No***
XII-1	G	G	T	A	T	G	T	G	C	A	G	T	T	G	A	T	T	C	A	C	G	G	G	100%	**Yes**
XII-2	T	G	A	A	A	C	T	C	T	A	A	T	C	C	T	A	C	T	A	T	C	G	G	100%	**Yes**
XII-4	G	C	T	T	C	A	A	G	A	A	T	T	G	A	G	T	A	A	A	C	G	G	G	100%	**Yes**
XII-5	T	T	G	T	C	A	C	A	G	T	G	T	C	A	C	A	T	C	A	G	C	G	G	100%	**Yes**