Engineering antimicrobial strains of *Saccharomyces cerevisiae* as industrial platform for non-sterile bioprocesses

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I declare that "**Engineering antimicrobial strains of *Saccharomyces cerevisiae* as industrial platform for non-sterile bioprocesses**" is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used have been indicated and acknowledge by complete reference.

Signature: 

Date: December 2023

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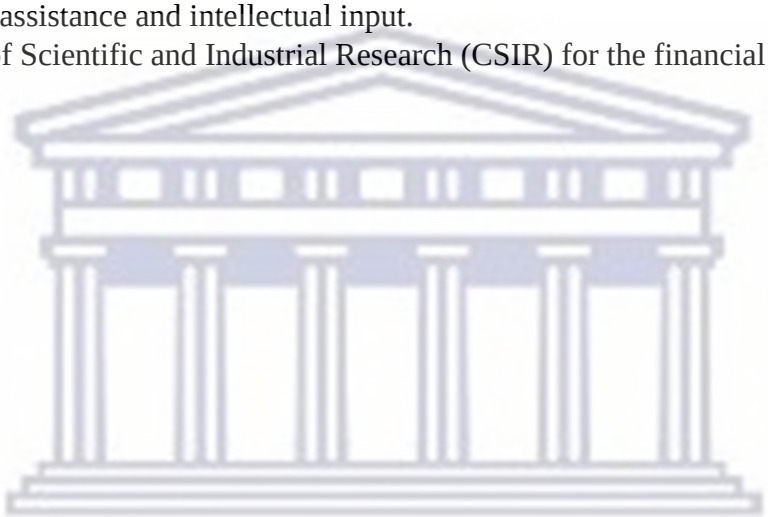

LIST OF ABBREVIATIONS

AI - Artificial Intelligence

AMP - Antimicrobial Peptide

AMR - Antimicrobial Resistance

ATCC - American Type Culture Collection

BHI - Brain Heart Infusion

CFS - Cell Free Supernatant

CFU/ml - Colony forming units per millilitre

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

DNA - Deoxyribonucleic acid

DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen

GHG - Greenhouse Gas

HPLC - High Performance Liquid Chromatography analysis

IMBM - Institute for Microbial Biotechnology and Metagenomics

LAB - Lactic Acid Bacteria

Man-PTS - Mannose phosphotransferase system

MFα1 - Alpha mating factor

MOA - Mechanism of Action

MRS - Man De Rogosa medium

PCR - polymerase chain reaction

PTM - Post-Translational Modification

RNA - Ribonucleic Acid

rpm - Rotations per minute

SU - Stellenbosh University

Tricine-SDS-PAGE - Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

USA - United States of America

UWC - University of the Western Cape

YPD - Yeast peptone dextrose

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ABSTRACT

Microbial contamination is a major challenge in fuel ethanol facilities, especially during the fermentation stage. Bacterial contaminants compete with yeast for fermentable sugars and nutrients, which obstructs starch to ethanol conversion. This leads to a reduction in ethanol yield. At large production scales, prior sterilization of the feedstocks is economically unfeasible. Therefore, antibiotic supplementation is common practice to limit the growth of contaminating bacteria. In addition to the high cost of the antibiotics, concerns and evidence are mounting that antibiotic use in non-clinical settings is driving the emergence of drug resistant microbes. This study focused on heterologous expression of antimicrobial peptides (AMP) as an alternative strategy for combating microbial contamination. This work aimed to engineer an industrial *Saccharomyces cerevisiae* strain that produces AMPs active against lactic acid bacteria and Enterobacteriaceae which are some of the main bacterial contaminants found in industrial biofuel fermenters. Seven candidate AMPs were selected from curated online databases, and their amino acid sequence was analyzed using bioinformatic tools. AlphaFold, Clustal Omega, and SNAP2 were used to predict AMP tertiary structures, construct a phylogenetic tree, and analyse mutation effects, respectively. The amino acid sequences of the selected AMPs were used to produce codon optimized genes for expression in *S. cerevisiae* on expression plasmids. These plasmids were successfully cloned into *S. cerevisiae* Y294. The soft agar-overlay and well diffusion method was used to determine antimicrobial activity, however no antimicrobial activity was found for any of the genes. The inducible *GAL1*$_{P}$-*CYC1*$_{T}$ was replaced with the constitutive *ENO1*$_{P/T}$ and the newly constructed plasmids were cloned into *S. cerevisiae* Y294. These transformants were subjected to antimicrobial activity testing against a range of microorganisms. Transformants expressing the AMPs Garvieacin Q, Carnobacteriocin BM1 and Piscicolin 126 respectively, showed antimicrobial activity against *Listeria spp.* and *Enterococcus spp.* An attempt was made to create antimicrobial industrial *S. cerevisiae* strains. The Carnobacteriocin BM1 gene was integrated into the genome of the industrial strain *S. cerevisiae* Ethanol Red, targeting either δ-sequences or the intergenic regions of chromosome 11. Even though the presence of the gene was confirmed, no antimicrobial activity was displayed by these strains or an alternative *S. cerevisiae* YI13 strain. These strains were created using the CRISPR-Cas9 method for gene integration. Traditional plasmid-based methods for genome integration were then used, which required the subcloning of our AMP genes. The Garvieacin Q and

Carnobacteriocin BM1 encoding genes, respectively were integrated into both *S. cerevisiae* Ethanol Red and *S. cerevisiae* Y294. None of the mentioned clones created showed antimicrobial activity, even though the presence of the genes were confirmed in each case. a *S. cerevisiae* Ethanol Red strain with the AMP Enterocin A gene was obtained from a collaborator. When screened for antimicrobial activity, this strain was active against *Listeria monocytogenes* EDG-e. This strain was co-cultured with *L. monocytogenes* EDG-e and the growth of both the yeast and bacteria was measured using the plate count method. The Enterocin A producing yeast successfully suppressed the growth of the bacterial contaminant *L. monocytogenes* EDG-e. In anaerobic fermentation co-culture conditions the Enterocin A expressing *S. cerevisiae* Ethanol Red strain produced a 4% higher ethanol yield compared to the wild type. This study showed that an engineered antimicrobial industrial *S. cerevisiae* strain grown under fermentation conditions with a bacterial contaminant, produces a higher ethanol yield compared to the wild type.

CHAPTER 1: LITERATURE REVIEW

1.1. Bioenergy

The world's increasing energy demand and the inevitable exhaustion of conventional fossil-based energy sources have driven a global interest in exploring alternative energy solutions (Aristidou and Penttilä, 2000). Among these alternatives, biofuels have emerged as a promising option. Biofuels, encompassing liquid or gaseous fuels such as bioethanol, biobutanol, biomethane, biohydrogen, and biodiesel, are derived from organic materials or biomass (Patinvoh and Taherzadeh, 2019). Biomass is classified as non-lignocellulosic or lignocellulosic in nature and exists in various forms such as woody, herbaceous, aquatic debris, farming manure and byproducts, and other forms (Osman *et al.*, 2019; Kaloudas, Pavlova and Penchovsky, 2021). Biomass has been identified as the world's fourth largest available energy resource (Haykiri-Acma and Yaman, 2010). This abundance of biomass offers the potential for its conversion into biofuels within a relatively short timeframe, unlike fossil fuels, which are limited in supply.

Researchers are working on various processes and methods to increase the production of biofuels or alternate fuels (Singhal *et al.*, 2023). Fossil fuels are categorized as non-renewable resources due to their extensive carbon cycle, which involves millions of years of organic material decomposition under high pressure and heat. In contrast, biofuels have a shorter carbon cycle, making them readily renewable and offering a sustainable energy alternative. Figure 1.1 illustrates the contrasting carbon cycles of fossil fuels and biofuels, highlighting the finite nature of fossil fuel reserves and the renewable nature of biofuel sources.

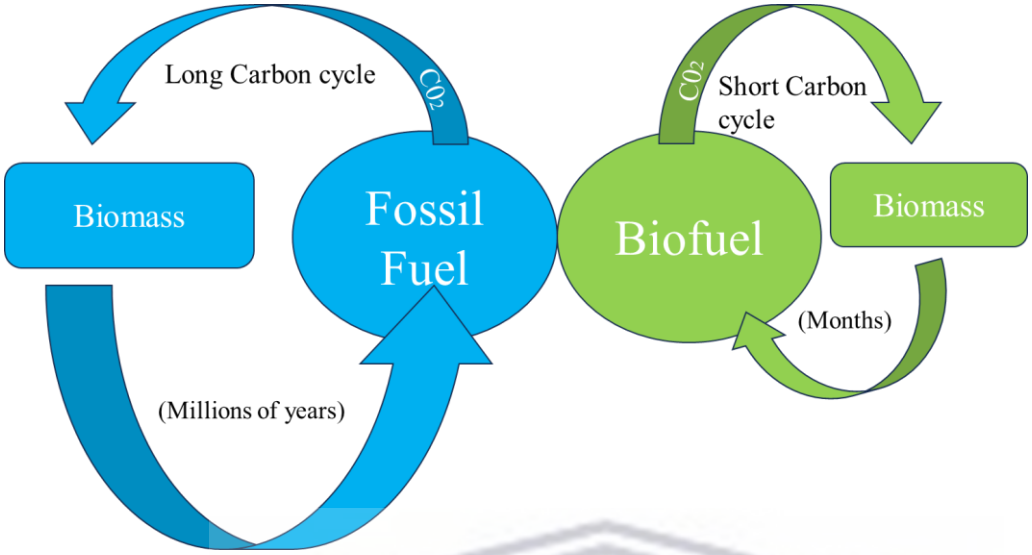

Figure 1.1: Fossil fuel and Biofuel carbon cycle.

The transportation sector heavily relies on fossil fuels, with about 30% of the world's total energy consumption being attributed to the shipping industry, which relies on 96% fossil fuels (Atabani *et al.*, 2012; Sani, Daud and Abdul Aziz, 2014; Singh *et al.*, 2014). This high fossil fuel usage has raised environmental concerns because the combustion of fossil fuel causes the formation of exhaust gases, which is the major cause of the greenhouse effect (Zhao *et al.*, 2009), leading to global warming. As the global temperature increases it leads to a rise in sea level, depletion of glaciers, loss of biodiversity, and a change in climate (Gullison *et al.*, 2007; Nigam and Singh, 2011). Greenhouse gas (GHG) emissions from the transportation sector have been rising at a faster pace compared to any other sector (Sims *et al.*, 2015). To combat these challenges, a transition from petroleum-based fuels to biofuels presents a viable solution as it would lead to lower emissions of GHG, which can reduce global warming (Malode *et al.*, 2021).

In the last decade, policies on climate change mitigation strategies, with the goal to reduce GHG emissions from the transport sector, have further increased the interest in biofuels (Jeswani, Chilvers and Azapagic, 2020). Despite the 67% increase in global bioethanol and threefold increase in biodiesel production over the decade of 2008 - 2018, biofuels only account for about 3.4% of total transportation fuels worldwide (IEA, 2019). Thus, further research in biofuels is essential for optimizing production processes, advancing sustainable energy solutions, and mitigating climate change through reduced GHG emissions.

First generation ethanol production has been commercialized at a large scale in many countries, including the United States of America (USA), Brazil and China (Xu *et al.*, 2018; Yu *et al.*, 2019). The USA is the world's largest producer of ethanol, having produced over 50 billion litres in 2021, and together with Brazil they produce 82% of the world's ethanol (U.S. Department of Energy, 2023). Presently, ethanol is produced from approximately 60% corn, 25% from sugar cane, 3% from wheat, 2% from molasses, and the rest from other grains, cassava, and sugar beets. (Hoang and Nghiem, 2021).

1.2. Industrial biofuel fermentation

The biomass to fuel conversion can occur using various technologies such as gasification, combustion, pyrolysis, enzymatic hydrolysis, and fermentation (Peng *et al.*, 2020; Abou Rjeily *et al.*, 2021). Fermentation employs microorganisms, including yeast, for enzymatic conversion of complex sugar substrates present in biomass into a usable byproduct. Various useful organic products such as alcohols, gases and acids are produced by microorganisms during fermentation (Wilkins and Atiyeh, 2012). Alcohol extracted from these microbial fermentations can be used as a biofuel. Microbial contamination during the fermentation process can lead to suboptimal growth conditions, resulting in lower product yield. To ensure optimal performance of the fermentation process and maximize alcohol yield, it is important to choose the appropriate bioreactor. The choice of appropriate microbial bioreactor depends on (i) the genetic stability of the fermenting microorganism, (ii) type of substrate, (iii) productivity of the fermentation process, (iv) flexibility in operation, (v) optimal growth conditions, (vi) risk of bacterial contamination, and (vii) the economy of the process (Patinvoh and Taherzadeh, 2019). There are three main types of fermentation systems: batch, fed-batch, and continuous. The batch fermentation is a closed system, where the substrate and producing microorganism are added to the bioreactor at the beginning and not removed until the fermentation is complete (Burke *et al.*, 2013). A batch bioreactor is advantageous because there is low risk of contamination (Zabed *et al.*, 2017). In a fed-batch bioreactor, a very small amount of feedstock is added at the start of the process and thereafter there is continuous feeding without removal of any fermentation broth (Zabed *et al.*, 2017). In a continuous bioreactor, the substrate is fed continuously into the reactor, and an equal amount is removed to attain a constant working volume (Patinvoh and Taherzadeh, 2019). Nevertheless, the choice of bioreactor becomes inconsequential if the fermenting microorganism's capability to efficiently convert the substrate into the desired product is compromised. There are three

types of biomass to biofuel fermentation systems, each using a different substrate. First- and second-generation biofuels employ edible and non-edible organic materials as substrates, respectively, whereas third-generation biofuels rely on fast-growing algae as their organic substrate. Figure 1.2 illustrates the important steps for converting second-generation lignocellulosic feedstocks to biofuel, highlighting the critical importance of the fermentation process, as it directly drives the production of the final biofuel product.

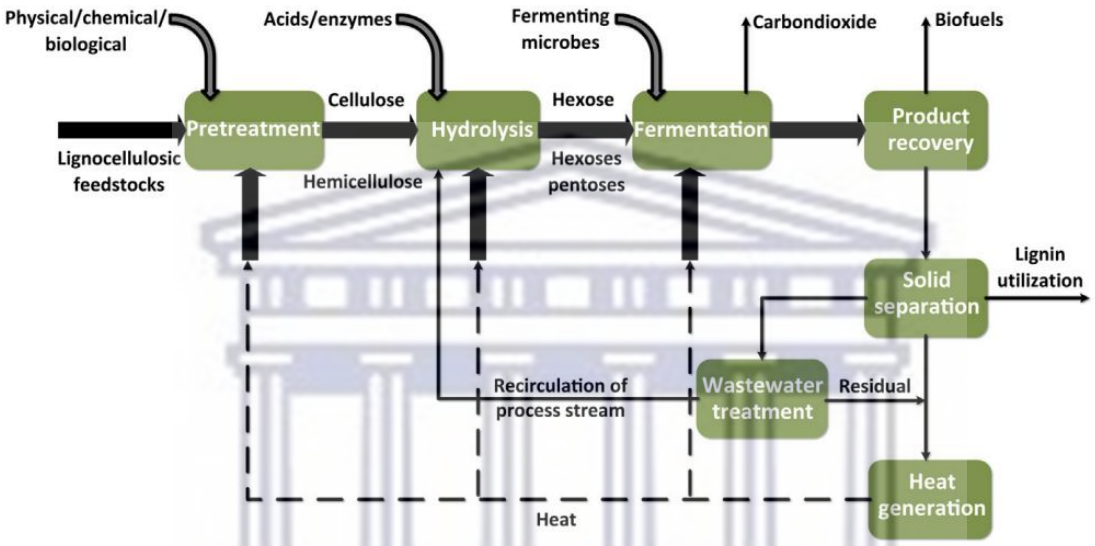

Figure 1.2: Biochemical conversions of second-generation feedstocks to biofuels (Patinvoh and Taherzadeh, 2019).

1.3. Microbial contamination in fermentation

Contamination is the unwanted and unexpected presence of any organic or inorganic substance, including bacteria, during the fermentation process. Microbial contamination presents an unavoidable challenge in the biofuel industry, particularly during the propagation and fermentation processing stages (Bayrock and Ingledew, 2004; Skinner and Leathers, 2004; Olmstead, 2009; Beckner, Ivey and Phister, 2011; Carlos, Olitta and Nitsche, 2011).

The yeast *S. cerevisiae* is the primary microorganism used for ethanol fermentation (Patinvoh and Taherzadeh, 2019). Bacterial contaminants compete with yeast for fermentable sugars and nutrients, which obstructs starch to ethanol conversion by yeast (Skinner and Leathers, 2004; Beckner, Ivey and Phister, 2011). In addition, many bacteria produce inhibitory metabolites, such as lactic and acetic acids, which have a negative impact on yeast health (Skinner and Leathers, 2004; Beckner, Ivey and Phister, 2011), resulting in prolonged lag

times, decreased growth rates and reduced microbial yields (Makanjuola, Tymon and Springham, 1992; Narendranath, Thomas and Ingledew, 2001; Thomas, Hynes and Ingledew, 2001, 2002; Abbott and Ingledew, 2004; Graves *et al.*, 2006). One of the primary contaminants in ethanol fermentation, namely *L. fermentum*, has been shown to reduce ethanol production in *S. cerevisiae* fermentation cultures by 27% (Bischoff *et al.*, 2009)

The non-aseptic conditions of industrial ethanol production favour microbial growth (Essia Ngang *et al.*, 1990), which is why fuel ethanol fermentations are designed to be carried out in the presence of chronic microbial contamination (Bayrock and Ingledew, 2004). It is difficult to eliminate bacterial contamination due to their ability to accumulate and colonize different parts of the production system, such as piping, heat exchangers, valves, and crevasses (Olmstead, 2009). However, there are attempts to reduce microbial contamination by using antimicrobial treatments. Bacterial contamination can be controlled using antibiotics, acid treatment, ammonia (Broda and Grajek, 2009) and urea hydrogen peroxide (Muthaiyan and Ricke, 2010). Some of these control methods however can be harmful towards *Saccharomyces* strains, create waste disposal problems, and may be required in large quantities, rendering them less viable as long-term solutions for bioethanol production facilities (Beckner, Ivey and Phister, 2011).

The main contaminants of wine and fuel-ethanol production processes are Gram-positive lactic acid bacteria (LAB) and wild yeasts (De Souza Liberal *et al.*, 2007; De Barros Pita *et al.*, 2011). LAB can tolerate high concentrations of alcohol and low pH conditions (Bischoff, Skinner-Nemec and Leathers, 2007). During fermentation, the alcohol concentration increases while the pH decreases, which means LAB are naturally better adapted to the harsh conditions occurring during the fermentation process, compared to other microbial contaminants. Contamination by Gram-negative organisms can still occur in brewing; however, it is generally limited to the *Aerobacter spp., Acetobacter spp., Acetomonas spp., Obesumbacterium spp.,* and *Zymomonas spp.* (Kleyn and Hough, 1971). Wild yeasts are also a persistent problem in all types of fermentations, including biofuel production (Muthaiyan, Limayem and Ricke, 2011). These wild yeasts may include species such as *Dekkera bruxellensis*, *Candida tropicalis* and *Pichia galeiformis* (Basílio *et al.*, 2008).

These contaminants can negatively affect fermentation efficiency and even lead to a stuck fermentation, resulting in the shutdown of the plant for cleaning before restarting the process (Skinner and Leathers, 2004). This, in turn, leads to significant production losses during the

cleaning of the fermentation system, resulting in decreased overall yield and increased economic loss. LAB contamination can cause a loss of ethanol yield, ranging between 1% to 22% (Skinner and Leathers, 2004; Beckner, Ivey and Phister, 2011). According to Abbott and coworkers (2005) even as little as 1% ethanol loss of current production levels could be considered detrimental to the financial health of fuel alcohol plants, some of which already operate with narrow profit margins. Thus, implementing effective control measures becomes crucial to mitigate the risk of contamination, thereby ensuring optimal ethanol production. This study was mainly focused on the elimination of LAB contamination.

The most problematic LAB class in fermentation includes the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* (Bischoff *et al.*, 2009), with *Lactobacillus spp.* being the single most problematic genus of bacteria in the fermentation industry throughout its history (G-Alegría *et al.*, 2004). LAB are Gram-positive, catalase negative, microaerophilic to anaerobic, asporogenous, and low in GC content (Klein *et al.*, 1998). They are the most common bacterial contaminants in corn-based ethanol production facilities, particularly *Lactobacillus spp.* (Narendranath, Thomas and Ingledew, 2001; Skinner and Leathers, 2004; Bischoff *et al.*, 2009; Beckner, Ivey and Phister, 2011). They limit ethanol production by producing lactic and acetic acids, or by competing for nutrients (Narendranath *et al.*, 1997). This occurs when LAB ferment carbohydrates to lactic acid, reducing ethanol yield. Furthermore, as the acid levels increase, it concomitantly inhibits yeast fermentation (Makanjuola, Tymon and Springham, 1992). LAB have demanding nutritional requirements, relying on a broad spectrum of amino acids, nitrogenous bases, and vitamins for growth (Chin and Ingledew, 1994; Bayrock and Ingledew, 2004). Their rapid growth outpaces that of yeast, resulting in a depletion of available nutrients for the yeast population.

1.4. Antibiotic treatment as a counter measure to microbial contamination

Antibiotics have traditionally been used to control microbial contamination in commercial fermentations (Lu *et al.*, 2020). However, the persistent and excessive use of antibiotics has sparked growing concerns regarding its contribution to the development of antimicrobial resistance (AMR) (Bischoff, Skinner-Nemec and Leathers, 2007; Walter *et al.*, 2019). AMR is defined as the resistance of microorganisms to antimicrobial agents against which they were once sensitive (Maria-Neto *et al.*, 2015; Andersson, Hughes and Kubicek-Sutherland, 2016). AMR is a major problem, particularly when pathogens evolve resistance against the

primary antibiotics intended to combat them. More than 70% of pathogenic bacteria are resistant to at least one type of antibiotic (Watkins and Bonomo, 2016). The limited development of novel classes of antibiotics over the past four decades has led to a scenario in which some infections are no longer treatable with available antibiotics (Maria-Neto *et al.*, 2015). This has led to the current problem in which antibiotic resistant bacteria are rapidly emerging while the development of new antimicrobial agents is decelerating (Ghosh *et al.*, 2019).

The agriculture and ethanol industries have been identified as major contributors to non-medical misuse of antibiotics, leading to an increase in AMR (Olmstead, 2009; Meek, Vyas and Piddock, 2015). Thus, it is important to find alternatives to classical chemical preservatives, such as sulphur dioxide and antibiotics, which perform a similar role in industrial ethanol fermentations (Santos *et al.*, 2009; Beckner, Ivey and Phister, 2011; Mehlomakulu, Setati and Divol, 2014). Ideally, potential antimicrobials should be (i) non-toxic to yeast strains used in fermentation, (ii) should have broad spectrum, of high antimicrobial activity against most if not all contaminant bacteria, (iii) should have minimal opportunities for development of resistance, (iv) should be economical, (v) easy to administer, and (vi) environmentally friendly with no toxicity towards plants, animals, and humans (Muthaiyan, Limayem and Ricke, 2011). This study will focus on antimicrobial peptides (AMP) and their application as a strategy for combating microbial contamination.

1.5. Antimicrobial peptides as viable antimicrobial treatment during fermentation

Even though AMPs have antimicrobial properties, they are not termed antibiotics to distinguish them from therapeutic antibiotics (Verma *et al.*, 2014). Therapeutic antibiotics are derived from microorganisms or fungi, whereas AMPs are synthesized within an organism as components of its innate immune system, encoded by deoxyribonucleic acid (DNA) and produced through cellular metabolism to form small proteins or peptides with antibacterial properties. They are typically composed of 5-100 amino acids and are present in both prokaryotic and eukaryotic organisms, as multipotent components that constitute their innate immune defence systems (Zasloff, 2002; Ganz, 2003). AMPs have a distinct and efficient mechanisms of action (MOA), broad-spectrum antibacterial activity, high efficacy at low concentrations, low risk of developing resistance, biodegradability, small size, and synergistic

action with classical antibiotics (Huerta-Cantillo and Navarro-García, 2016; Da Cunha *et al.*, 2017). Certain AMPs can kill target cells in seconds after the initial contact with the cell membrane (Loeffler, Nelson and Fischetti, 2001). AMPs are produced by most life forms, leading to considerable diversity that poses challenges in their classification. As illustrated in Figure 1.3, AMPs can be classified according to their source, activity, structural characteristics, and amino acid-rich species (Huan *et al.*, 2020).

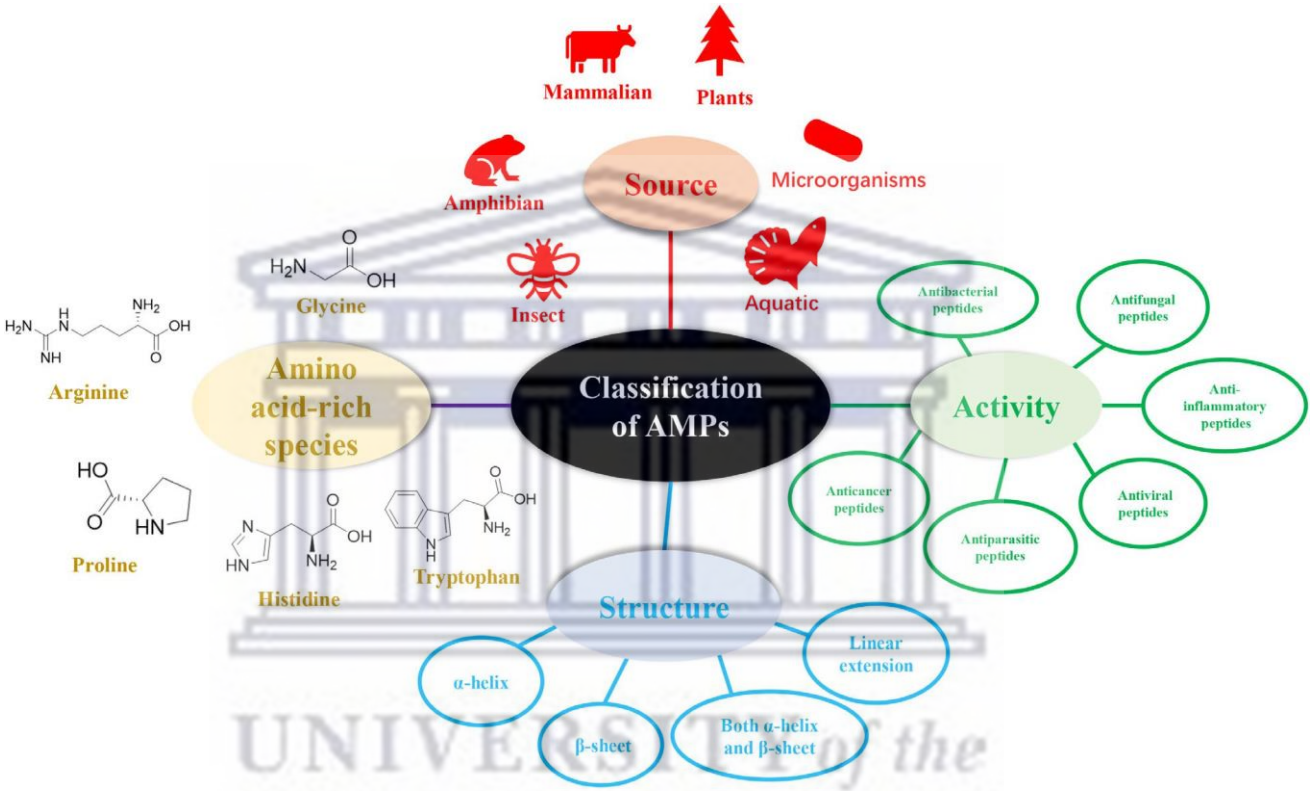

Figure 1.3: Classification of AMPs (Huan *et al.*, 2020).

Bacteriocins are AMPs synthesized in the ribosome by bacteria that inhibit or kill other related or unrelated microorganisms (Leroy and De Vuyst, 2004; Cotter, Hill and Ross, 2005). They function to establish a competitive advantage in the surrounding environment by eliminating competitors to gain more available resources (Lohans and Vederas, 2012). Unlike antibiotics, they are typically active at nanomolar concentrations (Kjos, Nes and Diep, 2011). Bacteriocins also show low toxicity and stability against proteases and temperature (García *et al.*, 2010). Bacteriocins are either processed by additional post-translational modification (PTM) enzymes or left unmodified and exported to the extracellular medium (Cotter, Hill and Ross, 2005). Bacteriocins can exhibit either a narrow target spectrum, selectively inhibiting bacteria that are taxonomically closely related, or a broad spectrum, targeting a diverse array

of bacterial species (Cotter, Hill and Ross, 2005). Additionally, there is no information suggesting toxicity of bacteriocins to humans and animals (Rea *et al.*, 2010; Belguesmia *et al.*, 2020).

Bacteria have a more difficult time developing resistance to bacteriocins due to their quick killing mechanism and the multiple sites they target (Peschel and Sahl, 2006; Fjell *et al.*, 2012). Bacteriocins kill bacteria with a MOA distinct from those used by traditional antibiotics (Lei *et al.*, 2019), which means that they could also be affective against bacteria that already acquired AMR against antibiotics. Nevertheless, it is still possible that bacteria develop resistance against bacteriocins. There are two main types of resistance mechanisms against bacteriocins: constitutive and inducible resistance. Constitutive resistance mechanisms include electrostatic shielding and changes in membrane potential, while inducible resistance mechanisms include substitution and modification of membrane molecules, activation of proteolytic enzymes, and the presence of efflux pumps (Yeaman and Yount, 2003). These resistance mechanisms essentially prevent the bacteriocin from binding to the microbial cell membrane. By interfering with the binding process, the microorganisms can evade the antimicrobial activity of the bacteriocin. Fortunately, the resistance against bacteriocins observed to date is weaker compared to that against antibiotics, and it only covers a limited number of bacteriocins (Bahar and Ren, 2013). Therefore, bacteriocins are of great interest in the mitigation of bacterial contamination.

The history of studies on AMPs dates to 1939 when Dubos (Dubos, 1939b, 1939a) discovered an antimicrobial agent, extracted from a soil *Bacillus* strain, that was able to protect mice from pneumococci infection. Today, AMPs are recognized as indispensable components of the innate immune system in various species, including humans, animals, and plants, as they serve as the first line of defence against foreign attacks (Reddy, Yedery and Aranha, 2004; Kościuczuk *et al.*, 2012a). Since the first AMP was discovered, more than 1400 AMPs have been isolated from bacteria, plants, insects, and other invertebrates, as well as vertebrates such as amphibians, birds, fishes, and mammals (Pasupuleti, Schmidtchen and Malmsten, 2012). However, only a few AMPs, such as Nisin A and Pediocin PA-1, have been commercialized so far for food preservation (Arthur, Cavera and Chikindas, 2014).

1.6. AMP advantages and limitations

AMPs offer several advantages, such as easy chemical modification and surface immobilization due to the diverse amino acid chemistry (Onaizi and Leong, 2011; Bahar and Ren, 2013). Since AMPs are peptides, it allows relatively easy gene modifications for structural changes, if required. AMPs offer the added advantage of biodegradability, ensuring they do not pose waste disposal challenges, like that of chemical antibiotics. AMPs can target a variety of microorganisms, by recognising various cell wall components such as lipopolysaccharide from Gram-negative bacteria, lipoteichoic acid from Gram-positive bacteria, glycolipids from mycobacteria, β-glycan from yeast, and RNA strands from viruses (Boman, 2000; Kim *et al.*, 2009). Consequently, AMPs can be harnessed to effectively target specific microbial contaminants as needed. The abundance of diverse AMPs offers a wide array of choices, allowing for alternative selections in case the initially chosen AMP proves ineffective. However, even though AMPs are promising agents for combatting microbial infections, their development and industrial use face several challenges.

The natural synthesis rate of AMPs is low, and they are susceptible to degradation by proteases (Da Cunha *et al.*, 2017). AMPs can be synthesized chemically, however it is an expensive process. Alternatively, they can be synthesized by a recombinant expression host. The recombinant approach is relatively low cost and scalable, resulting in a more attractive methodology for large-scale production of AMPs (Li, 2009). This study focused on the recombinant approach of AMP production in a yeast host strain.

One of the most significant issues in recombinant AMP synthesis is PTM, since the expression host must perform precise modifications for the peptide to be active (Eckert, 2011). AMP secretion also poses a challenge since mature AMPs need to be expelled from the cell to effectively target contaminants. Moreover, the metabolic impact of AMP synthesis on the expression host yeast is not yet fully understood. The metabolic cost of AMP expression should thus also be considered (Poulsen *et al.*, 2002; Johnston and Rolff, 2015). AMP effectiveness may be compromised by gradual degradation over time, which means continuous secretion is required to counteract contaminant growth. Certain AMPs are sensitive to harsh environmental conditions and degradation by proteases (Sieprawska-Lupa *et al.*, 2004).

In vitro studies have highlighted that microorganisms are more susceptible to combinations compared to individual AMPs (Rahnamaeian and Vilcinskas, 2015). When multiple AMPs are expressed, they often act synergistically, exhibiting a stronger inhibitory effect against sensitive strains (Garneau, Martin and Vederas, 2002; Limonet *et al.*, 2004; Martin *et al.*, 2004). However, they may also act antagonistically (Mulet-Powell *et al.*, 1998). Additionally, the combination of unrelated bacteriocins with different MOA reduces the likelihood of sensitive bacteria developing resistance. (Horn *et al.*, 1999; Reviriego *et al.*, 2005). Therefore, it is crucial to explore synergistic combinations of AMPs that exhibit potent efficacy in eradicating contaminating organisms within fermentation environments.

1.7. Bacteriocin classification

Similar to AMPs overall, there exists a diverse array of bacteriocins, which further complicates their classification. The first bacteriocin classification system was proposed by Klaenhammer (1993), after which it has been a subject of ongoing revision (Klaenhammer, 1993; Chen and Hoover, 2003). Bacteriocins are classified based on their molecular weight, structure, and MOA (Cotter, Ross and Hill, 2013). Bacteriocins can be split into Gram-negative targeting and Gram-positive targeting, with the latter being the focus of this study, since the LAB contaminants of industrial fermentations are Gram-positive. Furthermore, they can be divided into membrane disrupters and non-membrane disruptors, both of which initially target the plasma membrane through electrostatic charges (Huerta-Cantillo and Navarro-García, 2016). Gram-positive targeting bacteriocins can be further subdivided into different classes, whereas Gram-negative bacteriocins are categorized into mainly colicins or microcins. Figure 1.4 illustrates other groups of Gram-negative bacteriocins, not included among the two main categories previously mentioned. It is important to note that the classification of bacteriocins is a dynamic and evolving field of research, and new discoveries may lead to further categorization or re-evaluation of their significance.

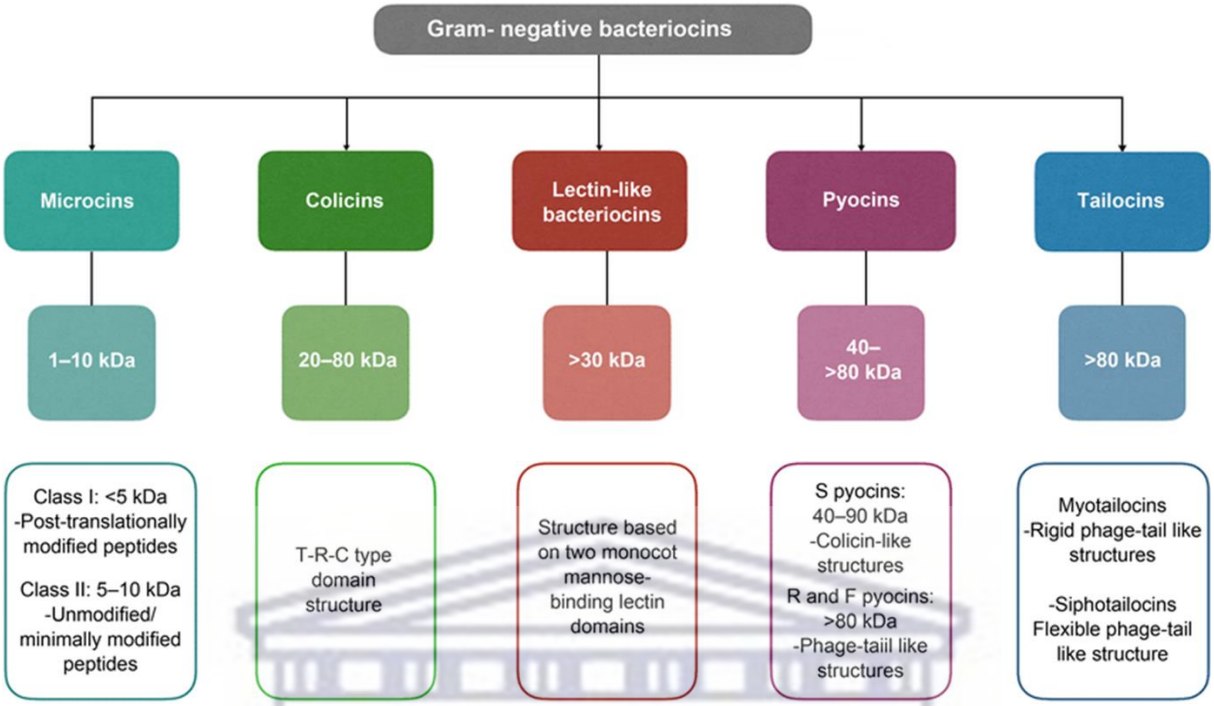

Figure 1.4: Classification of Gram-negative bacteriocins (Cesa-Luna *et al.*, 2021).

Colicins are high molecular weight molecules (25-80 kDa) encoded on plasmids (Huerta-Cantillo and Navarro-García, 2016). Colicins are divided into three categories depending on their MOA, which include: pore-forming colicins, nuclease type colicins and peptidoglycanase type colicins (Cascales *et al.*, 2007). In contrast, microcins are low molecular weight molecules (< 10 kDa) produced as precursor peptides (Huerta-Cantillo and Navarro-García, 2016). Microcins are divided into two classes depending on their molecular weight and their PTM, which include: Class I (< 5 kDa) that requires PTM, and Class II (5-10 kDa) where some types require PTM, but not all (Huerta-Cantillo and Navarro-García, 2016). Microcins are hydrophobic, low molecular weight molecules that are pH- and heat-stable (Cesa-Luna *et al.*, 2021).

Gram-positive targeting bacteriocins can be subdivided into three primary classes (Figure 1.5). Class I bacteriocins are small peptides that requires PTM, class II are small (<10 kDa) thermostable peptides (Cui *et al.*, 2020), and Class III are larger (>30 kDa), heat labile peptides (Garneau, Martin and Vederas, 2002).

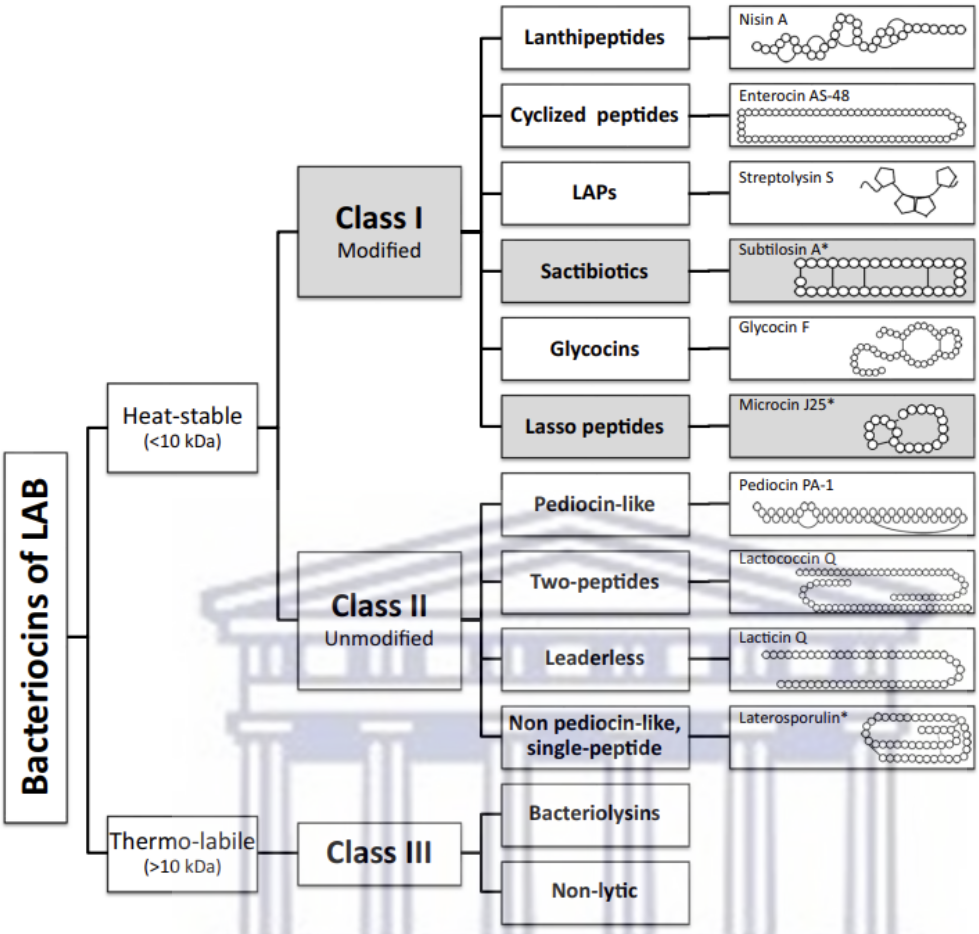

Figure 1.5: Classification of Gram-positive bacteriocins (Alvarez-Sieiro *et al.*, 2016).

Class I are small peptides (<10kDa) that undergo enzymatic modifications during biosynthesis (Alvarez-Sieiro *et al.*, 2016). It can be subdivided into Class Ia or lanthipeptides (types I, II, III, and IV), Class Ib or head-to-tail cyclized peptides, class Ic or sactibiotics, class Id or linear azol(in)e-containing peptides, class Ie or glycocins and class If or lasso peptides (Alvarez-Sieiro *et al.*, 2016).

Class II bacteriocins are membrane-active, heat-stable (Balandin, Sheremeteva and Ovchinnikova, 2019), and include unmodified bacteriocins (Alvarez-Sieiro *et al.*, 2016). The high diversity, strong specific activity, and relative abundance of class II bacteriocins favour their use as effective agents against multidrug-resistant strains (Gradisteanu Pircalabioru *et al.*, 2021). Cotter and coworkers (2005) suggested dividing class II bacteriocins into subclasses: class IIa (Pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and class IIc (circular bacteriocins).

Class IIa bacteriocins are potent inhibitors of *Listeria spp.*(Cintas *et al.*, 1997). They are named after a Pediocin PA-1, which was the first characterized bacteriocin representative of the group (Van Reenen *et al.*, 2003). This group does not require enzymes for their maturation other than a leader peptidase and/or a transporter (Alvarez-Sieiro *et al.*, 2016). This makes them ideal candidates for recombinant expression since the host doesn't require complex PTM machinery. Class IIa bacteriocins use the membrane disruption method to cause the dissipation of transmembrane potential, adenosine triphosphate depletion, and the loss of inorganic ions, amino acids, and other low molecular weight substances (Balandin, Sheremeteva and Ovchinnikova, 2019; Jeckelmann and Erni, 2020). Species belonging to the following genera have been reported to be sensitive to class IIa bacteriocins: *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Carnobacterium, Enterococcus, Micrococcus, Staphylococcus, Streptococcus, Clostridium, Bacillus* and *Brochothrix* (Holck *et al.*, 1992; Klaenhammer, 1993; Larsen, Vogensen and Josephsen, 1993; Jack, Tagg and Ray, 1995; Bhugaloo-Vial *et al.*, 1996; Fimland *et al.*, 1996; Fleury *et al.*, 1996; Cintas *et al.*, 1997). Given that LAB are the primary contaminants in biofuels fermentations, bacteriocins from Class IIa present a relevant and appropriate option to focus on in this study.

Class IIb bacteriocins consist of two very different peptides, and full activity requires the presence of both peptides in about equal quantities (Nissen-Meyer *et al.*, 2010). Despite the synergistic effect of their combined presence, individual antimicrobial activity has been observed in certain cases. (Alvarez-Sieiro *et al.*, 2016). Class IIc bacteriocins are circular bacteriocins formed by covalent connection of the N-terminus and C-terminus (Kawai *et al.*, 2009; Rohrbacher, Zwicky and Bode, 2017). Class IId are single, unmodified, linear, leaderless, non-Pediocin-like bacteriocins without any conservative sequence or structural feature (Cotter, Hill and Ross, 2005; Nissen-Meyer *et al.*, 2009).

Class III bacteriocins are large, heat-labile, composed of different domains and have a distinct MOA that differs from other Gram-positive bacteriocins (Cotter, Hill and Ross, 2005; Alvarez-Sieiro *et al.*, 2016). Class III bacteriocins are unmodified peptides larger than 10 kDa with bacteriolytic or non-lytic MOA (Alvarez-Sieiro *et al.*, 2016).

Class IV bacteriocins are complex peptide structures associated with lipid and carbohydrate moieties forming glycoproteins and lipoproteins and Class V includes cyclic peptide structures (Cesa-Luna *et al.*, 2021). The classification of AMPs plays an important role in narrowing down the search for specific bacteriocins. Thus, as new bacteriocins are discovered, it is important to refine this classification system.

1.8. Bacteriocin structure

Structural features such as size, charge, hydrophobicity, amphipathicity, and solubility are all crucial physiochemical properties in the antimicrobial activities and target specificity of AMPs (Tossi, Sandri and Giangaspero, 2000). Knowing the structure of bacteriocins can also serve as a basis for the rational design of bacteriocin analogues with optimized properties. (Acedo *et al.*, 2016). Even AMPs with very similar structures can have drastically different MOA, and even have a different range of targeted cells (Jenssen, Hamill and Hancock, 2006). Hence, when genetically modifying any AMP gene, there is a risk of rendering the peptide inactive due to structural changes.

The antimicrobial activity of bacteriocins is specifically related to their amino acid composition and physical chemical properties, such as positive net charge, flexibility, size, hydrophobicity, and amphipathicity (Nguyen, Haney and Vogel, 2011; Malanovic and Lohner, 2016). The electrostatic force of a bacteriocin, which is cationic in most cases, and the polyanionic surface of bacteria is the driving force of interactions between the bacteriocin and its target bacteria (Nguyen, Haney and Vogel, 2011; Malanovic and Lohner, 2016). This electrostatic force between the cationic bacteriocin and the negatively charged bacterial surface is critical for peptide-membrane interaction (Yeaman and Yount, 2003; Yeung, Gellatly and Hancock, 2011; Ebenhan *et al.*, 2014).

Most bacteriocins do not show a particular structure when free in solution and are only folded to their final conformation when they bind directly to the membrane (Zhang, Zhao and Zheng, 2014). Bacteriocins show different secondary structures, such as α-helix, β-pleated sheet, a mix of β-sheets and α-helices and lineal or random (Figure 1.6). Many AMPs are amphipathic, allowing the binding to both hydrophobic and hydrophilic regions such as lipid components and phospholipid groups, respectively (Jenssen, Hamill and Hancock, 2006). The hydrophobic portion of the AMP molecule facilitates its insertion into the cell membrane (Madani *et al.*, 2011). The positive charge of the bacteriocin is essential for its initial binding

to the membrane surface, whereas the hydrophobicity is necessary for insertion into, and perturbation of, the membrane (Lohner and Blondelle, 2005; Henderson and Lee, 2013).

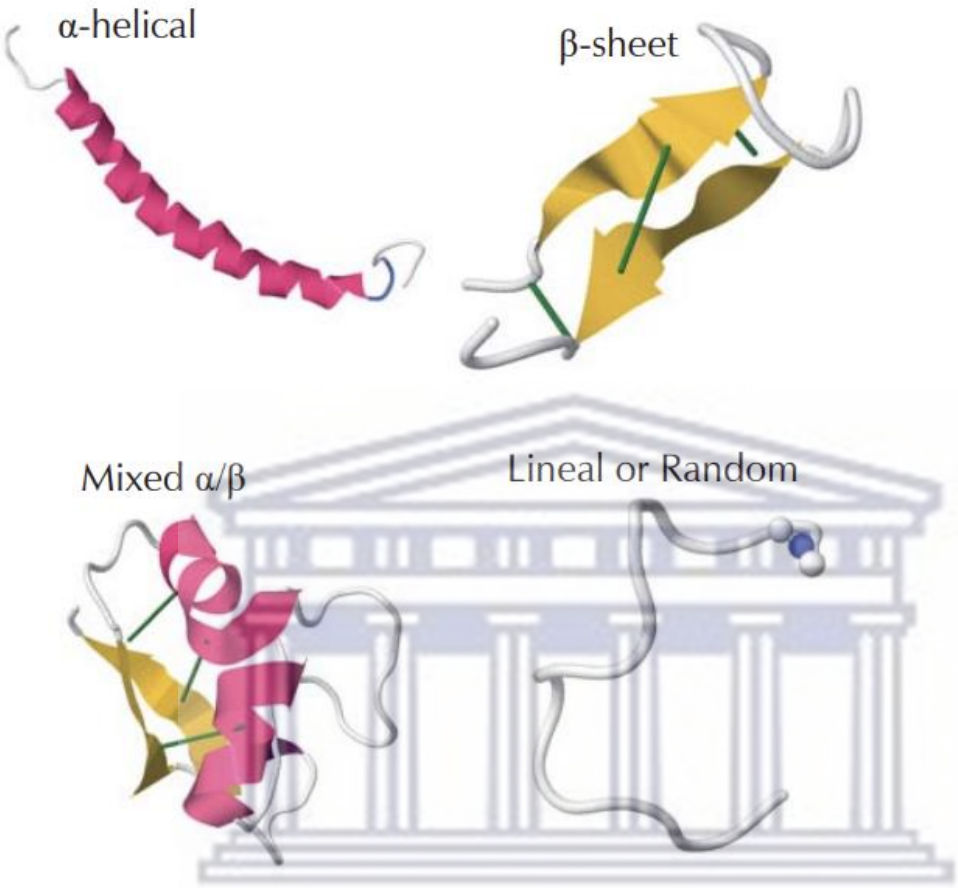

Figure 1.6: Representative secondary structures of AMPs. α-helix structures are shown in magenta, β-sheet in yellow, and green lines represent the disulfide bridges (Huerta-Cantillo and Navarro-García, 2016).

The structure of Class IIa peptides can be divided into two distinct regions, the N-terminal and C-terminal regions, separated by a flexible hinge (Haugen *et al.*, 2008). The flexible hinge between the N- and C-terminal regions allow the two domains to move in relation to each other, which is necessary for N-terminal recognition and C-terminal membrane penetration (Zhu *et al.*, 2022). The cationic N-terminal half contains two cysteine residues joined by a disulfide bridge (Cui *et al.*, 2012). This N-terminal half facilitates nonspecific binding to the target bacterial surface (Kazazic, Nissen-Meyer and Fimland, 2002). Most class IIa peptides contain a conserved YGNGV (Tyr-Gly-Asn-Gly-Val) amino acid sequence (Yamazaki *et al.*, 2005; Belguesmia *et al.*, 2011), as illustrated in Figure 1.7. This region is commonly referred to as the "Pediocin box" which renders the N-terminus hydrophilic and cationic (Eijsink *et al.*, 1998). The N-terminus adopts a three-stranded anti-parallel β-sheet-

like structure, with hydrophobic residues on one side and hydrophilic residues on the other and is further stabilized by a conserved disulfide bridge (Zhu *et al.*, 2022). This "Pediocin box" is a recognition site for the membrane-bound protein receptor (protein IIC (MptC)), found on the target organism, which is part of the mannose phosphotransferase system (Man-PTS) (Alvarez-Sieiro *et al.*, 2016; Wu *et al.*, 2020). This interaction between the AMP and the docking molecule disrupts the Man-PTS which prevents sugar transport required for growth, causing cell membrane permeabilization and cell death (Kjos *et al.*, 2010; Yi *et al.*, 2022).

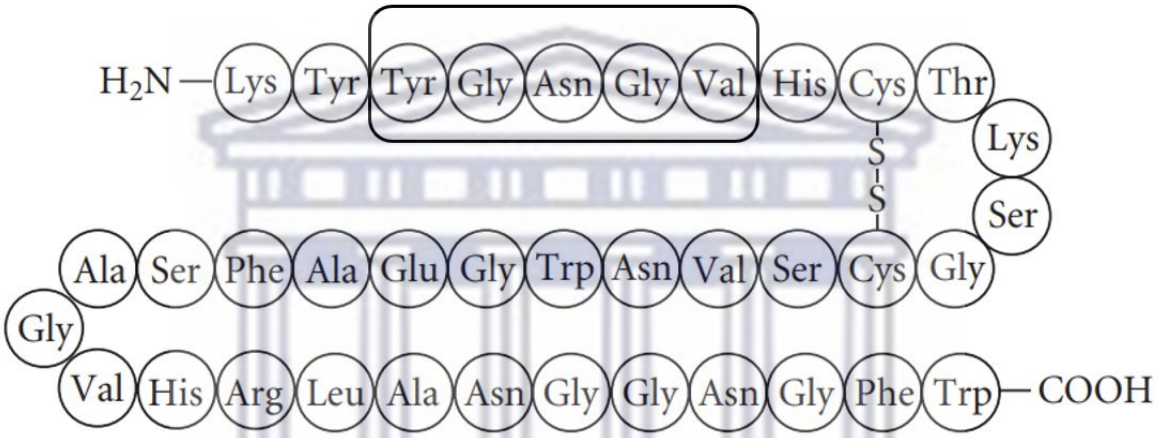

Figure 1.7: A representation of a class IIa bacteriocin. This is leucocin A, with the YGNGV conserved sequence and an N-terminal disulfide bridge (Lohans and Vederas, 2012).

The C-terminal domain is less conserved and seems to be involved in target specificity (Cotter, Hill and Ross, 2005; Cui *et al.*, 2012). The hydrophobic C-terminal region usually forms a hairpin-like structure consisting of an amphiphilic α-helix followed by an extended C-terminal tail that folds back onto the central α-helix (Nissen-Meyer *et al.*, 2009). Figure 1.8 illustrates the structure and orientation of a class IIa bacteriocin in a membrane.

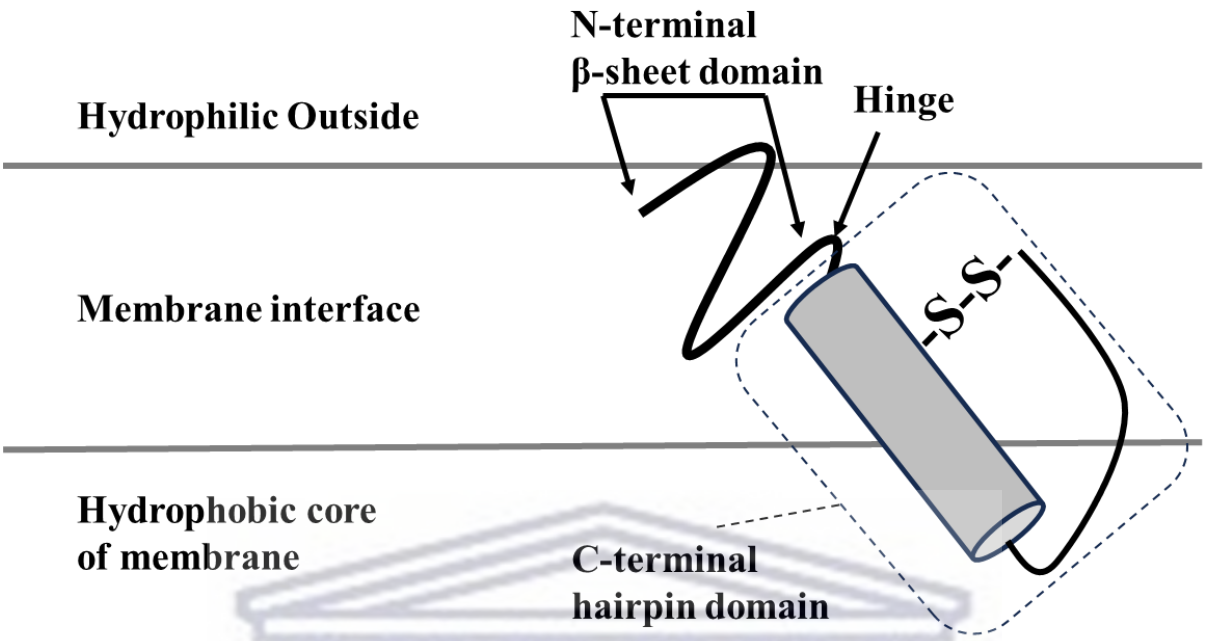

Figure 1.8: A schematic depiction of the structure and orientation in membranes of class IIa bacteriocins, adapted from (Belguesmia *et al.*, 2011). In class IIa bacteriocins, the C-terminal hairpin structure is stabilized by a disulfide bridge indicated by by -S-S-.

In class IIa bacteriocins, the C-terminal hairpin structure is stabilized by a disulfide bridge, indicated by -S-S- (Belguesmia *et al.*, 2011). The N-terminal β-sheet-like domain mediates binding of class IIa bacteriocin to the target cell surface (Kazazic, Nissen-Meyer and Fimland, 2002), which allows the hydrophobic C-terminal hairpin-like domain to penetrate into the hydrophobic core of target membranes, mediating membrane leakage (Belguesmia *et al.*, 2011). The angle between these domains can be altered by the hinge region joining the conserved hydrophilic N-terminus and variable C-terminus (Johnsen *et al.*, 2005).

PTMs involved in the processing of naturally forming AMPs include: (i) phosphorylation (Goumon *et al.*, 1996), (ii) addition of D-amino acids (Kreil, 1997), (iii) methylation (Hancock and Chapple, 1999), (iii) amidation (Rifflet *et al.*, 2012), (iv) glycosylation (Oman *et al.*, 2011), (v) formation of disulphide linkage(s) (Mangoni *et al.*, 1996), and (vi) proteolytic cleavage (Zasloff, 2002; Shinnar, Butler and Park, 2003). This becomes a challenge when using complex AMPs that require sophisticated PTMs. Bacteriocins that do not require PTMs apart from disulphide bridge formation, such as those from class IIa (Majchrzykiewicz *et al.*, 2010), are of particular interest in this study.

1.9. Mechanism of action

The MOA of individual AMPs varies depending on factors such as peptide concentration, bacterial target species, tissue localization and the growth phase of the bacteria (Yeaman and Yount, 2003; Jenssen, Hamill and Hancock, 2006). AMPs exhibit diverse MOA, including the disruption of membrane integrity, intracellular interactions involving inhibition of proteins and protein synthesis, as well as interference with DNA and RNA synthesis. (Bahar and Ren, 2013). Enzymatic mechanisms are typically not associated with the antimicrobial activities of AMPs (Marr, Gooderham and Hancock, 2006).

The MOA of AMPs can be classified into either membrane disruptive mechanisms, associated with membrane lysis, or membrane undisruptive mechanisms, focussing on intracellular targets (Malanovic and Lohner, 2016). Membrane interaction is essential for both mechanisms, even when dealing with intracellular targets, which requires translocation (Jenssen, Hamill and Hancock, 2006; Nguyen, Haney and Vogel, 2011; Yeung, Gellatly and Hancock, 2011). In both cases, sensitive bacteria possess docking receptors that play a crucial role in the bacterial membrane-bacteriocin mediated binding (Cotter, 2014; Benfield and Henriques, 2020; Negash and Tsehai, 2020; Lozo, Topisirovic and Kojic, 2021).

Bacterial membrane proteins serve numerous critical functions that are essential for cellular processes, including nutrient transportation, respiration, maintenance of proton motive force, adenosine triphosphate generation, and intercellular communication (Zhang and Rock, 2009). The disruption of these critical proteins leads to depolarization of the transmembrane potential, subsequently resulting in membrane dysfunction, and ultimately, membrane rupture and rapid lysis of microbial cells (Yeaman and Yount, 2003; Brogden, 2005).

AMPs can also compromise the host internally by inhibiting protein, DNA or RNA synthesis, or by interacting with certain intracellular targets (Bahar and Ren, 2013). This leads to failure of metabolic pathways, and subsequent cell death. To interact with intracellular targets, bacteriocins need to gain access to the intracellular environment of the cell. They do this by either spontaneously translocating across the cell membrane or by having a secondary structure that allows membrane permeabilization (Da Cunha *et al.*, 2017).

Most Gram-positive targeting bacteriocins are unable to kill Gram-negative pathogens (Helander, von Wright and Mattila-Sandholm, 1997; Chen and Hoover, 2003; Cotter, Hill and Ross, 2005; Deegan *et al.*, 2006; Gillor, Etzion and Riley, 2008), since these have an outer

membrane, which acts as an effective barrier (Cao-Hoang *et al.*, 2008; Gyawali and Ibrahim, 2014). Gram-positive bacteria, in contrast, lack the outer membrane and are characterized by having a thicker peptidoglycan layer (Nguyen, Haney and Vogel, 2011). Figure 1.9 illustrates the different characteristics of Gram-positive and Gram-negative cell walls. The target specificity of bacteriocin activity is influenced by these differences.

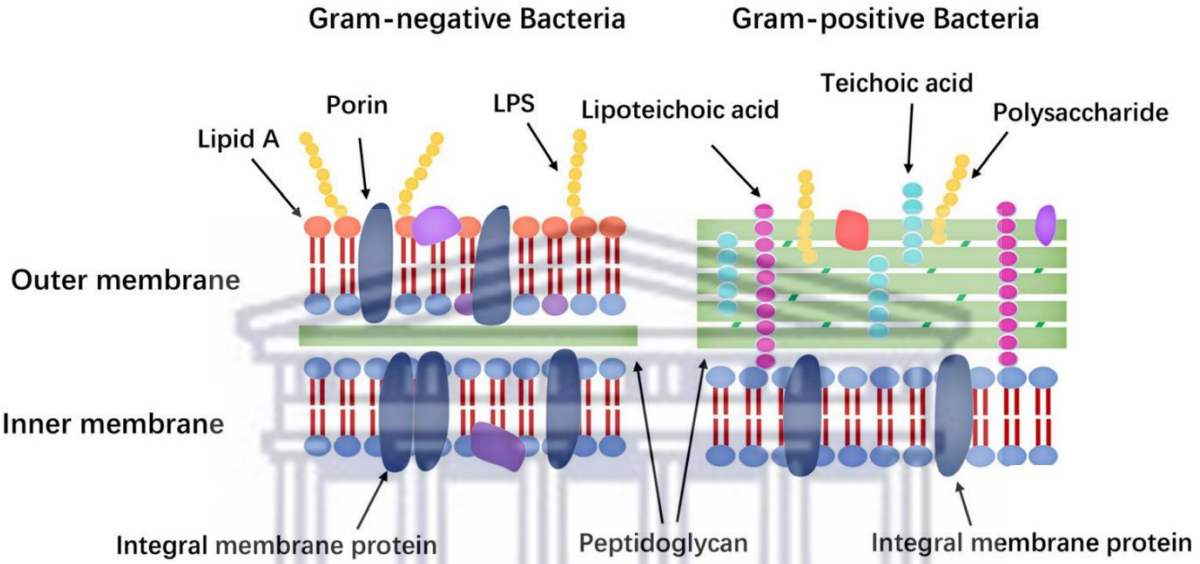

Figure 1.9: Comparison of Gram-negative and Gram-positive bacterial cell walls, adapted from (Huan *et al.*, 2020).

A docking receptor molecule such as lipid II or mannose permease of the Man-PTS is essential for the interaction between certain bacteriocins and the target microbial membranes (Héchard and Sahl, 2002). The Man-PTS is the membrane-specific receptor for many class IIa bacteriocins (Ramnath *et al.*, 2000), including Pediocin PA-1 (Diep *et al.*, 2007; Lohans and Vederas, 2012). When the receptor is obstructed, the bacteriocin's ability to bind to the microbial membrane is hindered, resulting in the inhibition of antimicrobial activity.

Gram-positive and -negative bacteria have certain bacteriocin receptors in common (Peng *et al.*, 2022). However, the outer membrane found in Gram-negative bacteria prevents bacteriocins from gaining access to their respective receptors and therefore they lose the ability to elicit antimicrobial activity (Peng *et al.*, 2022). Nevertheless, agents or treatments that destabilize the outer membrane enable these peptides to bind to their receptor and effectively target Gram-negative bacteria (Martin-Visscher *et al.*, 2011; Chalón *et al.*, 2012). However, implementing this approach on an industrial scale becomes impractical due to the need for additional destabilization agents. The use of such agents adds complexity and

introduces challenges that make large-scale applications less feasible. Nevertheless, given that the main bacterial contaminants during fermentation are LAB due to the prevailing conditions of temperature and pH, the primary focus can be directed towards Gram-positive targets.

According to our current understanding, the MOA can be described as follows: after undergoing electrostatic interactions, hydrophobic interactions, or other interactions with the cellular surfaces of sensitive bacteria, bacteriocins directly or subsequently bind to their receptors (Peng *et al.*, 2022). This is followed by the formation of the α-helix motif in the C-terminal region, enabling the bacteriocin to penetrate the membrane and exhibit its antibacterial activity (Jacquet *et al.*, 2012). Subsequently, pore formation occurs, leading to membrane destabilization and the leakage of intracellular fluids.

Classically, there are three main models for pore formation, including "barrel-stave", "carpet" and "toroidal-pore" (Hoskin and Ramamoorthy, 2008; Kościuczuk *et al.*, 2012b; Benfield and Henriques, 2020). As research continues, more models have been added such as the aggregate model. Figure 1.10 illustrates a graphical representation of these models.

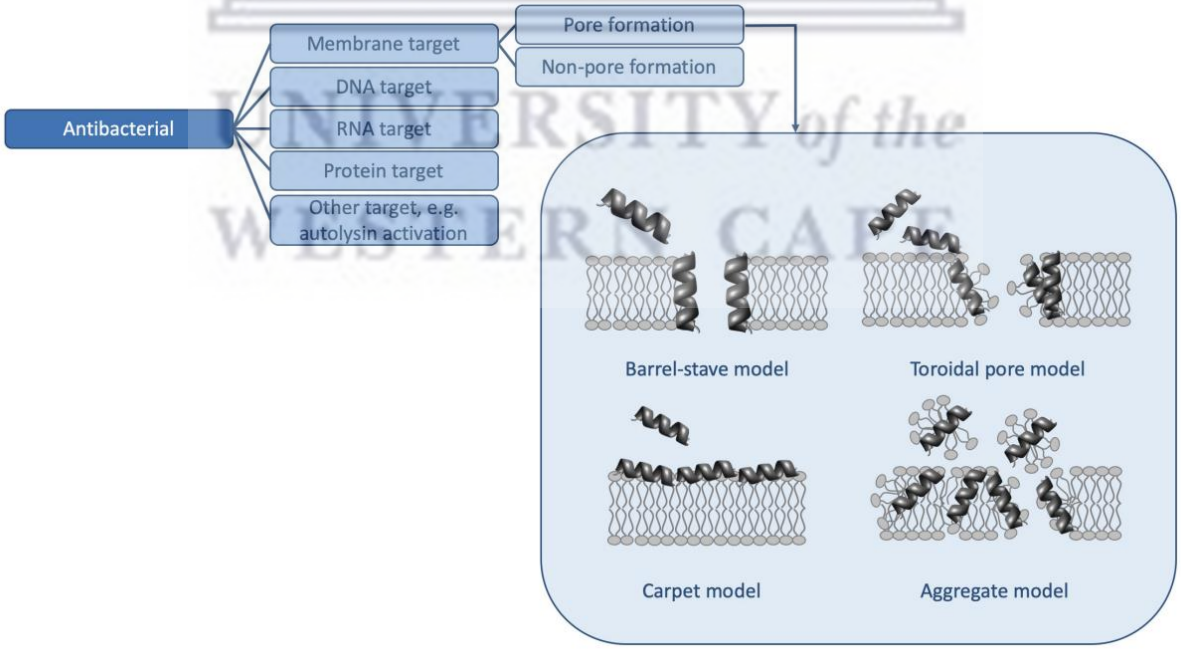

Figure 1.10: MOA of bacteriocins (Raheem and Straus, 2019).

Class II bacteriocins use the barrel stave or carpet model (Cesa-Luna *et al.*, 2021). The barrel model cause pore formation in the lipid bilayer by interacting with hydrophobic parts of the membrane via its hydrophobic side chains, which allows the internalization of the hydrophilic part of the bacteriocin that faces the internal region of the membrane (Nguyen, Haney and Vogel, 2011). In the carpet like model, bacteriocins cover the membrane surface and destroy the cell membrane in a "detergent" like manner (Oren and Shai, 1998). In the toroidal model, bacteriocin binds to the membrane while remaining closely associated with the lipid head groups, forming a "flip-flop" translocation channel that opens the membrane vertically (Nguyen, Haney and Vogel, 2011). The aggregate model, also known as the detergent model, does not lead to membrane rupture; rather, it facilitates the creation of a narrow channel, enabling the translocation of various molecules, including bacteriocins, into the cell. (Nguyen, Haney and Vogel, 2011).

1.10. Heterologous AMP production

Bacteria and yeast are the most widely used host systems for the production of recombinant products (Gupta and Shukla, 2017), and both have demonstrated successful expression of bacteriocins. Pediocin, as an example, has been expressed in bacteria such as *Escherichia coli* (Halami and Chandrashekar, 2007; Liu *et al.*, 2011), as well as in yeast species such as *S. cerevisiae* (Schoeman *et al.*, 1999), and *Pichia pastoris* (Beaulieu *et al.*, 2005). The selection of an ideal expression host for bacteriocins is complex, as each host possesses unique characteristics that can yield advantages or disadvantages depending on the specific application. The frequent use of certain expression hosts does not mean that they represent the optimal choice. Traditional hosts are often used, not because they are the best, but because they were the first to be available and characterized on a molecular level, or the first to be approved for pharmaceutical production processes (Andryushkova and Glieder, 2009).

E. coli is the most utilized microorganism for heterologous AMP production (Ingham and Moore, 2007; Li *et al.*, 2010). Expression of AMPs in *E. coli* can be challenging since the peptides' antibacterial nature makes them potentially fatal to the expression host, and the peptides' small size and high cationic property makes them highly susceptible to proteolytic degradation (Li, 2011). In cases where the host is susceptible to the selected AMP, additional genes conferring immunity against the particular bacteriocin can be integrated into the bacterial host. However, this is a labour-intensive and time-consuming process (Vermeulen,

Van Staden and Dicks, 2020). Additionally, *E. coli* lacks the natural capability to secrete heterologous peptides into the extracellular environment, resulting in the formation of insoluble inclusion bodies (Rosano and Ceccarelli, 2014). *Bacillus subtilis* is another bacterium used in AMP expression (Feng *et al.*, 2012). AMP Scygnodin expression in *P. pastoris* was 1.3 times higher than that of *E. coli* (Peng *et al.*, 2012). For more examples of AMP expression in bacteria and yeast, please refer to (Parachin *et al.*, 2012).

Each type of expression host has their own advantages and drawbacks, all of which should be carefully considered before making the final decision. When choosing the recombinant host strain, it's important to consider the following criteria: total yield, space time yield, specific productivity, quality, type and efficiency of folding and PTM, cost of media and downstream processing, and compatibility of the product with the desired application (Rieder *et al.*, 2019). Furthermore, it is important to take into consideration that the expression of bacteriocins come with a metabolic cost (Poulsen *et al.*, 2002; Johnston and Rolff, 2015).

In this study, yeast was chosen as the recombinant host instead of a bacterial host, since they combine the ease and lower cost of a prokaryotic recombinant expression, such as *E. coli*, with the added benefit of eukaryotic PTM pathways. Traditional and well-established yeast species for recombinant expression are *Saccharomyces cerevisiae*, *Hansenula polymorpha, Klyveromyces lactis, P. pastoris and Schizosaccharomyces pombe* (Andryushkova and Glieder, 2009). Given that the class IIa bacteriocin Pediocin PA-1 has been successfully expressed in both *S. cerevisiae* (Schoeman *et al.*, 1999), and *P. pastoris* (Beaulieu *et al.*, 2005), the choice of expression host falls on these two candidates. *S. cerevisiae* does have a few advantages over *P. pastoris*. One notable advantage is its well characterized and stable multicopy plasmid system, the 2μm plasmid, which typically exhibits copy numbers ranging from 10 to 40 copies per cell and can easily be transformed (Strathern, Hicks and Herskowitz, 1981). Another notable advantage of *S. cerevisiae* is its established dominance as the primary microorganism for ethanol fermentation (Patinvoh and Taherzadeh, 2019). This makes *S. cerevisiae* the ideal candidate for this study.

Hyper glycosylation of proteins, low protein yield and plasmid instability are some of the reasons that limited the number of commercial heterologous protein products from *S. cerevisiae* (Xie, Han and Miao, 2018). Enhancing heterologous expression efficiency can result in a greater product yield. One approach to achieve this is through codon optimization, which modifies the codon usage pattern by utilizing synonymous codons to match the codon

usage bias of the expression host organism (Fox and Erill, 2010). Codon optimization is a good strategy to be used to enhance the heterologous expression of target AMPs (Deng *et al.*, 2017). Another optimization is by using the most efficient promoter and terminator. A strong constitutive promoter and terminator could ensure gene transcription throughout the cultivation process, resulting in higher levels of recombinant peptide (Myburgh, Rose and Viljoen-Bloom, 2020). Gene copy number can also affect the level of gene expression (Redden, Morse and Alper, 2014). Cultivation conditions, including pH, oxygen density, temperature, and aeration can extensively affect the heterologous protein yields of yeast (Bonander and Bill, 2012).

As mentioned, several bacteriocins must be expressed together with specialized secretory machinery and immunity proteins (Ingham and Moore, 2007). For protein secretion, signal peptides are required to direct proteins from cytosol to extracellular matrix (Bolhuis *et al.*, 1999; Owji *et al.*, 2018). Selecting the appropriate signal peptide is crucial for efficient secretion of these peptides and directly influences the yields of targeted proteins in the fermentation broth. (Aw *et al.*, 2018). This study used the alpha mating factor (MFα1) secretion signal from *S. cerevisiae*, which has previously been used for the secretion of small peptides in yeast (Rossouw *et al.*, 2023). By specifically selecting bacteriocins that lack antimicrobial activity against *S. cerevisiae*, the need for immunity proteins was eliminated.

Most of the heterologous AMP expression in yeast have been in *P. pastoris*, whereas only a limited number have been reported in *S. cerevisiae* (Parachin *et al.*, 2012). To our knowledge, only a few AMPs have been expressed in *S. cerevisiae*, including Pediocin PA-1 (Schoeman *et al.*, 1999), Plantaricin 423 (Van Reenen *et al.*, 2003), human β-defensin-1 (Cipakova and Hostinova, 2005), Enterocin L50 (Basanta *et al.*, 2009) and Mundticin ST4SA (Rossouw *et al.*, 2023). Hence, there is substantial potential for exploring the expression of additional AMPs in *S. cerevisiae*.

Schoeman and coworkers (1999) were the first to express a bacteriocin in *S. cerevisiae*, specifically Pediocin PA-1, and observed its activity against *L. monocytogenes* B73. Van Reenen and coworkers (2003) expressed Plantaricin 423 in *S. cerevisiae* L5366h and observed its activity against *L. monocytogenes* LM1. Neither of these studies provided information regarding the yield. Cipakova and Hostinova (2005) expressed the human β-defensin-1 in *S. cerevisiae* AH22 and observed its activity against *E. coli* ML-35p, with a yield of 55 mg/L, purified by cation exchange chromatography. Each of these studies used the

yeast alcohol dehydrogenase I promoter (*ADH1*$_P$) and terminator (*ADH1*$_T$). Basanta and coworkers (2009) cloned Enterocin L50A and Enterocin L50B into *S. cerevisiae*. All these studies mentioned employed plasmid-based expression and the MFα1 secretion signal. Rossouw and coworkers (2023) experimented with the *Trichoderma reesei* xylanase 2 (XYNSEC) secretion signal, nevertheless, they concluded that the MFα1 was a superior secretion signal for the purpose of secreting a small peptide in yeast. Pediocin PA-1 has also been expressed on the cell surface of *S. cerevisiae* W303, inhibiting the growth of *Shigella boydii* and *Shigella flexneri* (Nguyen, Haney and Vogel, 2011).

1.11. AMP database

To effectively leverage AMP studies, data must be collected and stored in databases and connected to pipelines for analysis, modelling, and design of novel peptides (Piotto *et al.*, 2012; Sundararajan *et al.*, 2012; Lee *et al.*, 2015; Pirtskhalava *et al.*, 2016; Singh *et al.*, 2016; Kang *et al.*, 2019). Advances in genome sequencing and transcriptomics have enabled the identification and development of a large AMP library (Javan *et al.*, 2018). This library serves as a valuable tool for identifying an AMP possessing specific characteristics for the intended research purpose. In the context of this study, the objective was to discover bacteriocins that may effectively eliminate LAB contaminants while exhibiting minimal or no PTM requirements and have mature forms that are encoded by a single gene.

The AMP database, known as the "database of Antimicrobial Activity and Structure of Peptides" (https://dbaasp.org/), and BACTIBASE (bactibase.hammamilab.org/main.php) are online databases that provide valuable resources for antimicrobial and bacteriocin research. As of June 2023, the open-access BACTIBASE database (http://bactibase.hammamilab.org/about.php) provides descriptions of 206 bacteriocins derived from Gram-positive bacteria and 19 bacteriocins from Gram-negative bacteria.

Various computational tools, including molecular dynamics simulations, machine learning, and AMP databases serve as valuable aids in enhancing the likelihood of achieving successful peptide modifications (Pirtskhalava *et al.*, 2021). When none of the numerous available AMPs meet the research requirements, or when the objective is to enhance the original peptide, synthetic AMP creation or modification becomes a viable option. AMPs, being composed of amino acids, offer relative ease in modifying their structure compared to

chemical modifications. These modifications have the potential to alter the targets of AMPs or enhance their stability against proteases (Papo *et al.*, 2002). However, there are inherent risks associated with alterations, which may lead to undesirable outcomes, such as peptide inactivation.

1.12. Aim and objectives of this study

Based on the literature reviewed, significant progress has been made in heterologous expression of AMPs. However, little progress has been made in heterologous expression of AMPs in *S. cerevisiae* strains. In the context of biofuel production, LAB represent the predominant contaminant, which adversely affects ethanol yield. With the rise of antibiotic resistance and reduced number of antibiotics available, it is imperative to find alternative strategies to reduce LAB contamination in industrial biofuel fermentations.

Therefore, in this study we aimed to create an antimicrobial *S. cerevisiae*. By endowing these strains with basic antimicrobial properties, it was hoped that these strains could grow better in non-sterile environments by inhibiting microbial contamination. To attain this aim we pursued the following objectives:

- Identify and select candidate antimicrobial peptides from online AMP databases.
- Clone each synthetic codon optimized AMP gene in the laboratory strain *S. cerevisiae* Y294, respectively.
- Screen antimicrobial activity of constructs against a panel of organisms.
- Identify AMPs with antimicrobial activity and clone them into the industrial strain *S. cerevisiae* Ethanol Red.
- Screen antimicrobial activity of industrial constructs against a panel of test organisms.
- Lab scale evaluation of yeast strains in co-culture with representative contaminant bacteria in laboratory media.
- HPLC analysis evaluate ethanol production of yeast strains in co-fermentation with representative contaminant bacteria in laboratory media.

CHAPTER 2: MATERIALS AND METHODS

2.1. AMPs selected for this study

Through the utilization of online AMP databases and published literature, we conducted an extensive search for potential bacteriocins that met our specific criteria. These included (i) non-toxicity to the host, (ii) the requirement of simple PTM that the host could readily perform, (iii) novelty in terms of expression in yeast, (iv) activity against LAB, (v) a single gene encoding the peptide, and (vi) availability of the corresponding amino acid sequence.

In our search, we identified seven potential bacteriocins that met our specified criteria. These include Garvieacin Q, Hiracin JM79, Carnobacteriocin BM1, Piscicolin 126, and Aureocin A53, all of which belong to the class IIa bacteriocins. Two additional AMPs selected were Nisin-A and Pyrrhocoricin. Although Nisin-A does not fall under the category of Class IIa bacteriocins, it was selected due to its extensive research history, which has led to comprehensive characterization. Pyrrhocoricin was selected due to its efficacy against a broad spectrum of Gram-negative bacteria, which could be beneficial in preventing opportunistic contaminant growth. However, it is essential to note that Pyrrhocoricin is derived from an insect and thus does not fall into any described bacteriocin class category. Pediocin PA-1 of class IIa was selected as a positive control since it has already been successfully expressed in *S. cerevisiae* (Schoeman *et al.*, 1999). Table 2.1 displays the origin organism of each AMP, along with their corresponding amino acid (AA) length, peptide size in Daltons (Da), and microbial target.

Table 2.1: AMP selection for this study

AMP	Origin	Length (AA)	Size (Da)	Microbial target	Reference
Garvieacin Q	*Lactococcus garvieae* BCC 43578	70	5339	*Bacillus spp., Enterococcus spp., Lactococcus spp., Listeria spp.,* and *Pediococcus spp.*	Tosukhowong *et al.*, 2012
Hiracin JM79	*Enterococcus hirae* DCH5	74	5093	*Lactobacillus spp., Enterococcus spp., Listeria spp.,* and *Staphylococcus spp.*	Sánchez *et al.*, 2008
Carnobacteriocin BM1	*Carnobacterium maltaromaticum*	43	4524	*Carnobacterium spp., Enterococcus spp.,* and *Listeria spp.,*	Mathieu *et al.*, 1993; Quadri *et al.*, 1994; Afzal *et al.*, 2010
Piscicolin 126	*Carnobacterium piscicola* JG126	44	4400	*Carnobacterium spp., Enterococcus spp., Lactobacillus spp., Leuconostoc spp., Listeria spp., Pediococcus spp.,* and *Streptococcus spp.*	Jack *et al.*, 1996
Aureocin A53	*Staphylococcus aureus* A53	51	6012	strains of *M. luteus, streptococci spp., staphylococci sp.* involved in bovine mastitis, and *L. monocytogenes.*	Giambiagi-Marval *et al.*, 1990; Netz, Bastos and Sahl, 2002; Netz *et al.*, 2002; Nascimento *et al.*, 2006; Fagundes *et al.*, 2016
Nisin A	*Lactococcus lactis*	34	3352	*Listeria spp., Staphylococcus spp., Mycobacterium spp., Bacillus spp., Micrococcus spp., Lactococcus spp., Lactobacillus spp.,* and *Clostridium spp.*	Gross and Morell, 1971; Chen and Hoover, 2003; Asaduzzaman *et al.*, 2009; Field *et al.*, 2012)
Pyrrhocoricin	European fire bug *Pyrrhocoris apterus*	20	2341	*E. coli* strains, *Agrobacterium spp., Salmonella spp., Klebsiella spp., Pseudomonas spp., Staphylococcus spp., micrococcus spp., bacillus spp.,* and *Listeria spp.*	Cociancich *et al.*, 1994; Kragol *et al.*, 2001; Rosengren *et al.*, 2004
Pediocin PA-1	*Pediococcus acidilactici*	44	4629	*Listeria spp., Enterococcus spp., Lactobacillus spp., Leuconostoc spp., Streptococcus spp. & Weissella spp.*	Henderson, Chopko and van Wassenaar, 1992; Fimland *et al.*, 2000; Johnsen *et al.*, 2000; Loessner *et al.*, 2003; Bari *et al.*, 2005; Makhloufi *et al.*, 2013; Naghmouchi *et al.*, 2013

2.2. Bioinformatics approaches for peptide structure prediction and sequence alignment

Seven candidate AMPs were identified and selected from curated online databases. The bioinformatic tool AlphaFold, using the deep learning-based algorithm, was used to predict the tertiary structure of the selected AMPs (Jumper *et al.*, 2021; Varadi *et al.*, 2022). The Clustal Omega tool from the UniProt website was used to construct both a phylogenetic tree and a percentage identity matrix from the AMP amino acid sequences (Table 2.2) (Sievers *et al.*, 2011; Sievers and Higgins, 2018; Baxevanis, Bader and Wishart, 2020). The SNAP2 tool was used to generate a heatmap illustrating the predicted functional effects of mutations within the selected AMPs (Bromberg and Rost, 2007; Hecht, Bromberg and Rost, 2013, 2015).

Table 2.2: Amino acid sequence of the selected AMPs

AMP	Accession number	Sequence
Pediocin PA-1	P29430	MKKIEKLTEKEMANIIGGKYYGNGVTCGKHSCSVDWGKATT CIINNGAMAWATGGHQGNHKC
Garvieacin Q	H6U5Y1	MENKNYTVLSDEELQKIDGGEYHLMNGANGYLTRVNGKYV YRVTKDPVSAVFGVISNGWGSAGAGFGPQH
Hiracin JM79	Q0Z8B6	MKKKVLKHCVILGILGTCLAGIGTGIKVDAATYYGNGLYC NKEKCWVDWN QAKGEIGKIIVNGWVNHGPW APRR
Carnobacteriocin BM1	P38579	MKSVKELNKKEMQQINGGAISYGNGVYCNKEKCWVNKAEN KQAITGIVIGGWASSLAGMGH
Piscicolin 126	P80569	MKTVKELSVKEMQLTTGGKYYGNGVSCNKNGCTVDWSKAI GIIGNNAAANLTTGGAAGWNKG
Aureocin A53	Q8GPI4	MSWLNFLKYIAKYGKKAVSAAWKYKGKVLEWLNVGPTLEW VWQKLKKIAGL
Nisin A	P13068	MSTKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMGCN MKTATCHCSIHVSK
Pyrrhocoricin	P37362	VDKGSYLPRPTPPRPIYNRN

2.3. Microbial strains, plasmids, and primer sequences

All yeast strains and plasmids utilised in this study are listed in Table 2.3 and Table 2.5. The *S. cerevisiae* Y294 +y*ENO1*::fur was included as a reference strain for comparison of strain background diversity. *S. cerevisiae* Y294 + p*ENO1*_PedA, was included as a positive control for comparison of known antimicrobial activity against *L. monocytogenes* EDG-e.

Table 2.3: Yeast strains used in this study

Microbial strain	Abbreviation	Description	Source
Parental strains			
S. cerevisiae Y294	Y294_WT	Haploid yeast strain; auxotrophic for ura, leu, his, trp; generated by random mutagenesis and selected for its high levels of secreted proteins.	ATCC 201160
S. cerevisiae YI13	YI13_WT	Natural yeast strain isolate.	Davison *et al.*, 2019
S. cerevisiae YI13 + pCAS9-Nat	YI13_pCAS9	*S. cerevisiae* YI13 with pCAS9-Nat.	Davison *et al.*, 2019
Ethanol Red version 1	ER_WT	First generation biofuel industry standard, rapid fermentation, temperature-tolerant strain of yeast that displays high alcohol yields.	Prof. M. Bloom (SU)
Reference strains			
S. cerevisiae Y294 + y*ENO1*::fur	Y294_REF	Y294_WT Reference strain that contains a plasmid with $ENO1_{P/T}$ but no heterologous gene.	This study
S. cerevisiae Y294 + p*ENO1*_PedA	Y294_EP	Y294_WT containing a plasmid with the Pediocin PA-1 encoding gene under control of $ENO1_{P/T}$.	Rossouw *et al.*, 2023
S. cerevisiae Ethanol Red, $ENO1_{P/T}$, Enterocin A	ER_ EntA	ER_WT with the Enterocin A encoding gene integrated into δ-sequences under control of $ENO1_{P/T}$.	Prof. M. Bloom (SU)
Recombinant strains			
S. cerevisiae Y294 + pYes2_*GAL1*_AMP1	Y294_G1	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP1 to express the Garvieacin Q encoding gene under control of $GAL1_P$-$CYC1_T$.	This study
S. cerevisiae Y294 + pYes2_*GAL1*_AMP2	Y294_G2	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP2 to express the Hiracin JM79 encoding gene under control of $GAL1_P$-$CYC1_T$.	This study

S. cerevisiae Y294 + pYes2_*GAL1*_AMP3	Y294_G3	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP3 to express the Carnobacteriocin BM1 encoding gene under control of $GAL1_{P}$-$CYC1_{T}$.	This study
S. cerevisiae Y294 + pYes2_*GAL1*_AMP4	Y294_G4	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP4 to express the Piscicolin 126 encoding gene under control of $GAL1_{P}$-$CYC1_{T}$.	This study
S. cerevisiae Y294 + pYes2_*GAL1*_AMP5	Y294_G5	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP5 to express the Aureocin A53 encoding gene under control of $GAL1_{P}$-$CYC1_{T}$.	This study
S. cerevisiae Y294 + pYes2_*GAL1*_AMP6	Y294_G6	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP6 to express the Nisin A encoding gene under control of $GAL1_{P}$-$CYC1_{T}$.	This study
S. cerevisiae Y294 + pYes2_*GAL1*_AMP7	Y294_G7	Y294_WT transformed with plasmid pYes2_*GAL1*_AMP7 to express the Pyrrhocoricin encoding gene under control of $GAL1_{P}$-$CYC1_{T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP1	Y294_E1	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP1 to express the Garvieacin Q encoding gene under control of $ENO1_{P/T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP2	Y294_E2	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP2 to express the Hiracin JM79 encoding gene under control of $ENO1_{P/T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP3	Y294_E3	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP3 to express the Carnobacteriocin BM1 under control of $ENO1_{P/T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP4	Y294_E4	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP4 to express the Piscicolin 126 encoding gene under control of $ENO1_{P/T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP5	Y294_E5	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP5 to express the Aureocin A53 encoding gene under control of $ENO1_{P/T}$.	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP6	Y294_E6	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP6 to express the Nisin A encoding gene under control of $ENO1_{P/T}$	This study
S. cerevisiae Y294 + pMU1531_*ENO1*_AMP7	Y294_E7	Y294_WT transformed with plasmid pMU1531_*ENO1*_AMP7 to express the Pyrrhocoricin encoding gene under control of $ENO1_{P/T}$.	This study

S. cerevisiae Ethanol Red version 1 + pCas9NAT	ER_pCAS9	ER_WT transformed with the pCas9NAT plasmid	This study
S. cerevisiae Ethanol Red, CRISPR δ integrated, *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	ER_δ_E3	ER_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$.	This study
S. cerevisiae Ethanol Red, CRISPR CH11 integrated, *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	ER_CH11_E3	ER_WT with the Carnobacteriocin BM1 encoding gene integrated into chromosome 11 under control of *ENO1*$_{P/T}$.	This study
S. cerevisiae YI13, CRISPR δ integrated, *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	YI13_δ_E3	YI13_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$.	This study
S. cerevisiae YI13, CRISPR CH11 integrated, *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	YI13_CH11_E3	YI13_WT with the Carnobacteriocin BM1 encoding gene integrated into chromosome 11 under control of *ENO1*$_{P/T}$.	This study
S. cerevisiae Ethanol Red, δ integrated *PGK1*$_{P/T}$ (Pediocin PA-1)	ER_Gδ_PP	ER_WT with the Pediocin PA-1 encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Ethanol Red, δ integrated *PGK1*$_{P/T}$ AMP1 (Garvieacin Q)	ER_Gδ_P1	ER_WT with the Garvieacin Q encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Ethanol Red, δ integrated *PGK1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	ER_Gδ_P3	ER_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Ethanol Red, δ integrated *ENO1*$_{P/T}$ (Pediocin PA-1)	ER_Gδ_EP	ER_WT with the Pediocin PA-1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Ethanol Red, δ integrated *ENO1*$_{P/T}$ AMP1 (Garvieacin Q)	ER_Gδ_E1	ER_WT with the Garvieacin Q encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study

S. cerevisiae Ethanol Red, δ integrated *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	ER_Gδ_E3	ER_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *PGK1*$_{P/T}$ (Pediocin PA-1)	Y294_Gδ_PP	Y294_WT with the Pediocin PA-1 encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *PGK1*$_{P/T}$ AMP1 (Garvieacin Q)	Y294_Gδ_P1	Y294_WT with the Garvieacin Q encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *PGK1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	Y294_Gδ_P3	Y294_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *PGK1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *ENO1*$_{P/T}$ (Pediocin PA-1)	Y294_Gδ_EP	Y294_WT with the Pediocin PA-1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *ENO1*$_{P/T}$ AMP1 (Garvieacin Q)	Y294_Gδ_E1	Y294_WT with the Garvieacin Q encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study
S. cerevisiae Y294, δ integrated *ENO1*$_{P/T}$ AMP3 (Carnobacteriocin BM1)	Y294_Gδ_E3	Y294_WT with the Carnobacteriocin BM1 encoding gene integrated into δ-sequences under control of *ENO1*$_{P/T}$ and antibiotic G418 selection.	This study

The DNA sequences of the seven selected AMPs were codon optimised by GeneArt (Thermo Fisher Scientific, USA) using the Gene Optimizer algorithm (Graf, Deml and Wagner, 2004; Raab *et al.*, 2010) for expression in *S. cerevisiae*. The plasmids were engineered to contain the native *S. cerevisiae* MFα1 secretion signal and the Kex2 and two Ste13 sites for protease cleavage at the N-termini of the peptides. Plasmids containing these genes, as listed in Table 2.4, were synthesized by GeneArt Invitrogen (Thermo Fisher Scientific, USA). Table 2.5. also lists plasmids containing gene cassettes for homology repair, Cas9 expression, and gRNA expression, respectively. The Y294_EP and ER_EntA reference strains constructed by the research group of Prof. Marinda Bloom from (SU), also used the MFα1 as the secretion signal.

Table 2.4: Plasmids used in this study

Plasmid	Description	Source
Episomal AMP expression plasmids		
pYes2_*GAL1*_AMP1	Plasmid containing the Garvieacin Q encoding gene (509 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP2	Plasmid containing the Bacteriocin Hiracin-JM79 (521 bp) encoding gene under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP3	Plasmid containing the Bacteriocin Carnobacteriocin BM1 encoding gene (482 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP4	Plasmid containing the Bacteriocin Piscicolin 126 encoding gene (485 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP5	Plasmid containing the Bacteriocin Aureocin A53 encoding gene (452 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP6	Plasmid containing the lantibiotic Nisin-A encoding gene (470 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pYes2_*GAL1*_AMP7	Plasmid containing the Pyrrhocoricin encoding gene (359 bp) under control of $GAL1_P$ and $CYC1_T$ with the MFα1 secretion signal.	GeneArt (Invitrogen)
pMU1531_*ENO1*_AMP1	Plasmid containing the Garvieacin Q encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP2	Plasmid containing the Bacteriocin Hiracin-JM79 encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP3	Plasmid containing the Bacteriocin Carnobacteriocin BM1 encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP4	Plasmid containing the Bacteriocin Piscicolin 126 encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP5	Plasmid containing the Bacteriocin Aureocin A53 encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP6	Plasmid containing the Lantibiotic Nisin-A encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
pMU1531_*ENO1*_AMP7	Plasmid containing the Pyrrhocoricin encoding gene under control of $ENO1_{P/T}$ with the MFα1 secretion signal.	This study
CRISPR integration plasmids		
pCas9-Nat	CRISPR plasmid containing the Cas9 expression cassette.	ADDGENE
pRS52_G_DELTA	CRISPR plasmid containing the gRNA scaffold gene cassette, targeting yeast δ-sequences, G418 resistance.	Jacob, van Lill and den Haan, 2022

pRS52-G-ChXI	CRISPR plasmid containing gRNA scaffold gene cassette targeting Chromosome 11 intergenic region, G418 resistance.	Kruger and den Haan, 2022
Integrating expression plasmids		
pBKD1	An expression vector for integration of genes under control of *PGK1*$_{P/T}$ into *S. cerevisiae* δ-sequences, G418 resistance.	McBride *et al.*, 2008
pBKD2	An expression vector for integration of genes under control of *ENO1*$_{P/T}$ into *S. cerevisiae* δ-sequences, G418 resistance.	McBride *et al.*, 2008
pBKD1_*PGK1*_PedA	pBKD1 plasmid for integration of the Pediocin PA-1 gene cassette and G418 resistance into yeast δ-sequences under control of *PGK1*$_{P/T}$ with the MFα1 secretion signal.	This study
pBKD1_*PGK1*_AMP1	pBKD1 plasmid for integration of the Garvieacin Q gene cassette and G418 resistance into yeast δ-sequences under control of *PGK1*$_{P/T}$ with the MFα1 secretion signal.	This study
pBKD1_*PGK1*_AMP3	pBKD1 plasmid for integration of the Carnobacteriocin BM1 gene cassette and G418 resistance into yeast δ-sequences under control of *PGK1*$_{P/T}$ with the MFα1 secretion signal.	This study
PBKD2_*ENO1*_PedA	pBKD2 plasmid for integration of the Pediocin PA-1 gene cassette and G418 resistance into yeast δ-sequences under control of *ENO1*$_{P/T}$ with the MFα1 secretion signal.	This study
pBKD2_*ENO1*_AMP1	pBKD2 plasmid for integration of the Garvieacin Q gene cassette and under control of *ENO1*$_{P/T}$ and G418 resistance into yeast δ-sequences with the MFα1 secretion signal.	This study
pBKD2_*ENO1*_AMP3	pBKD2 plasmid for integration of the Carnobacteriocin BM1 gene cassette and G418 resistance into yeast δ-sequences under control of *ENO1*$_{P/T}$ with the MFα1 secretion signal.	This study

The primers used for the amplification of the gene cassettes and confirmation of genomically integrated genes are detailed in Table 2.6. For all polymerase chain reaction (PCR) amplifications performed, RedTaq™ DNA polymerase (Ampliqon, Odense, Denmark) was used according to the manufacturer's instructions, in an Applied Biosystems Thermocycler.

Table 2.5: Primers used for the amplification and confirmation of gene cassettes

Primer name	Sequence (5'- 3')	Application
CRISPR integration primers		
DELTA-*ENO1*-L	CTTAAGATGCTCTTCTTATTCTATTAAAAATA GAAAATGACTTCTAGGCGGGTTATCTACTG	To amplify genes between *ENO1*$_{P/T}$ as homology repair template DNA for genomic integration in δ-sequences.
DELTA-*ENO1*-R	GTTTGTTTGCGAAACCCTATGCTCTGTTGTT CGGATTTGACGTCGAACAACGTTCTATTAG G	

CH11int-ENO-L	ACATCTCTAAGCTGAAACTGAGAATACTGT TGTAAAACAGGTATTGGCTGCTTCATAGTAC ACCCAATTGCCCACAACCCCTTCTAGGCGG GTTATCTACTG	To amplify genes between *ENO1*$_{P/T}$ as homology repair template DNA for genomic integration in chromosome 11 intergenic region.
CH11int-ENO-R	ACATCTCTAAGCTGAAACTGAGAATACTGT TGTAAAACAGGTATTGGCTGCTTCATAGTAC ACCCAATTGCCCACAACCCCTTCTAGGCGG GTTATCTACTG	
Confirmation of promoter and terminator		
PGK-L	CTAATTCGTAGTTTTTCAAGTTCTTAGATGC	Confirm gene integration under *PGK1*$_{P/T}$.
PGK-R	ACTATTATTTTAGCGTAAAGGATGGGG	
ENO1-L	GTA ACA TCT CTC TTG TAA TCC CTT ATT CCT TCT AGC	Confirm gene integration under *ENO1*$_{P/T}$.
ENO1-R	GCA ACC CTA TAT AGA ATC ATA AAA CAT TCG TGA	
Confirmation of transformants		
AMPCheck-L	TTAATTAAAATGAGATTTCCTTCAATTTTTA CTGCTG	Left primer used in combination with primers AMPCheck-R(1-7) to check presence of AMP encoding genes in transformed strains.
AMPCheck-R1	GGCGCGCCTTAATGTTGTGG	Confirm presence of Garvieacin Q encoding gene.
AMPCheck-R2	GGCGCGCCTTATCTTCTTGGAG	Confirm presence of Hiracin JM79 encoding gene.
AMPCheck-R3	GGCGCGCCTTAATGACCC	Confirm presence of Carnobacteriocin BM1 encoding gene.
AMPCheck-R4	GGCGCGCCTTAACCTTTGTTC	Confirm presence of Piscicolin 126 encoding gene.
AMPCheck-R5	GGCGCGCCTTACAGGCC	Confirm presence of Aureocin A53 encoding gene.
AMPCheck-R6	GGCGCGCCTTACTTAGAAACATG	Confirm presence of Nisin A encoding gene.
AMPCheck-R7	GGCGCGCCTTAGTTCCTATTATAGATAGG	Confirm presence of Pyrrhocoricin encoding gene.
synMFalpha-L	TAGCTTAATTAAAATGAGATTTCCTTCAATT TTTACTGCTGTTTTATTCG	Confirm presence of Pediocin PA-1 encoding gene with MFα1 secretion signal.
AMPCheck-RPedA	CGATGGCGCGCCCTAGCATTTATGATTACCT TGATGTCCACC	

2.4. Microbial strain cultivations

All chemicals and media components used were of laboratory grade and purchased from Merck or Sigma-Aldrich (Darmstadt, Germany), unless otherwise stated. Microbial yeast strains (Table 2.3), were streaked from 15% (v/v) glycerol stocks, stored at -80°C, onto yeast peptone dextrose (YPD) agar media (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar), or SC^{-URA} agar (1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate [Difco laboratories, Detroit, MI, USA], 5 g/L $(NH_4)_2SO_4$, 20 g/L glucose or 20 g/L galactose, 20 g/L agar, and amino acids pool without uracil (Sigma-Aldrich) supplemented with either 100 µg/mL CloNAT (Werner Bioagents, Cospeda, Germany), and/or 200 µg/mL Geneticin G418 (Invitrogen, Waltham, MA, USA), or without selection as required, followed by incubation at 30°C for 48-72 hours.

Following cultivation on agar media, YPD broth supplemented with 100 µg/mL CloNAT and/or 200 µg/mL Geneticin G418, or without selection, or double strength SC^{-URA} (3.4g/l yeast nitrogen base [Difco laboratories, Detroit, MI, USA], 10g/l $(NH_4)_2SO_4$, 20 g/L glucose or 20 g/L galactose, 3g/l amino acids without uracil and 20 g/L succinate [succinic acid] 12 g/L NaOH pellets), as required, was inoculated with the streaked yeast cultures for incubation at 30°C for 72 hours on an orbital shaker at 180 rotations per minute (rpm).

Plasmids, as listed in Table 2.4, were streaked from *E. coli* DH5*α* 40% (v/v) glycerol stocks, stored at -80°C, onto Luria-Bertani (LB) agar plates (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) supplemented with 100 µg/mL ampicillin (Roche; Basel, Switzerland), followed by overnight incubation at 37°C. To prepare cultures for plasmid DNA isolation, single colonies were inoculated in LB broth supplemented with 100 µg/mL ampicillin, followed by overnight incubation at 37°C on a rotary wheel.

2.5. Plasmid DNA isolation, restriction digestion, PCR amplification.

Plasmid DNA isolation from *E. coli* DH5*α* cultures was performed using the Zyppy™ plasmid miniprep Kit (Zymo research, USA). To verify the sizes of each respective gene cassette and or CRISPR gRNA sequences, isolated plasmid DNA was subjected to restriction digestion at 37°C with *Eco*RI or *Pac*I and *Asc*I (Thermo Fisher Scientific Waltham, MA, USA), respectively, followed by separation on a 1% (w/v) agarose gel. Following confirmation (data not shown), gene cassettes detailed in Table 2.4 were PCR amplified with specific primers, listed in Table 2.5, with cycling conditions shown in Table 2.7.

Table 2.6: PCR cycling conditions to amplify gene cassettes used as homology repair templates or to confirm gene integration

Cassettes	Initial denaturation	Cycles (repeat x31)	Final Extension; Hold
Homology repair template	95°C for 5 min	Denaturation (95°C for 30 sec) Annealing (58°C for 30 sec) Elongation (72°C for 1 min)	72°C for 7 min 4°C (∞)
Gene integration check	94°C for 5 min	Denaturation (94°C for 30 sec) Annealing (55°C for 30 sec) Elongation (72°C for 1min)	72°C for 5 min 4°C (∞)

The PCR products were run on a 1% agarose gel, and the appropriate bands were excised, after which the DNA was extracted using the Freeze-and-Squeeze method (Thuring, Sanders and Borst, 1975) and further purified with the use of phenol: chloroform: isoamyl alcohol (PCI; 25:24:1). Following purification, isolated DNA was subjected to dialysis against purified water on a 0.025 µm MCE membrane filter (Merck Millipore; Burlington, MA, USA), followed by quantitative spectrophotometric analysis (NanoDrop2000, ThermoScientific) to determine the DNA concentration for subsequent use in transformation. The pBKD1 and pBKD2 based plasmids were subjected to *Bst*1107I restriction digestion to linearize them prior to transformation.

2.6. Plasmid construction

Subcloning was performed to change the gene promoters where necessary. It was also used to insert the AMP encoding genes into the integration plasmids, pBKD1 and pBKD2. During the subcloning process, the pYes-based plasmids (Table 2.4) were digested with *Pac*I and *Asc*I to excise the AMP encoding genes. The expression plasmids pMU1531, pBKD1 and pBKD2 were digested with the same enzymes to allow cloning of the various AMP encoding genes, creating the plasmids listed in Table 2.4 The T4-ligase kit (Thermo Fischer Scientific) was used as instructed by the manufacturer for all ligations.

2.7. Electro-transformation of yeast strains and screening of putative positive transformants

Transformation of yeast strains with AMP expression plasmids, homology repair template, the pCas9-NAT plasmid, and/or the CRISPR plasmids was conducted as described (Cho, Yoo and Kang, 1999) with minor adaptations to the permeabilization of yeast cells to allow for improved transformation efficiencies (Moriguchi *et al.*, 2016). Briefly, harvested cells were washed with sterile distilled water, followed by resuspension in LiOAc/TE (0.1 M LiOAc, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA). Resuspended cells were then incubated at 30°C for 45 minutes with shaking, followed by addition of 20 µL 1 M dithiothreitol and incubation for another 15 minutes. The mixture was then centrifuged, and cells washed with sterile distilled water, followed by resuspension in electroporation buffer (1 M sorbitol, 20 mM HEPES). These competent cells were transformed with the appropriate plasmid vectors under standard electroporation conditions (1.4 kV, 200 ohms, 25 µF) using a micropulser (BioRad; Hercules, CA, USA). Approximately 1 µg of plasmid DNA was used during transformation. For integration using the CRISPR system, ~10 µg homology repair template DNA and ~1 µg CRISPR plasmid DNA were used during transformation. Following electroporation, cells were suspended in 1 ml YPD broth media supplemented with 1 M sorbitol, followed by overnight incubation at 30°C on an orbital shaker at 180 rpm. The transformation mixture was then plated on YPD agar medium supplemented with CloNAT (100 µg/ml) and/or Geneticin G418 (200 µg/ml), or SC^{-URA} media, depending on the plasmid transformed and incubated for 48-72 hours at 30°C.

Putative positive transformants obtained from transformation plates were then streaked on YPD media supplemented with CloNAT (100 µg/ml) and/or Geneticin G418 (200 µg/ml), or SC^{-URA} plates followed by incubation at 30°C for 24-48 hours, prior to inoculating overnight YPD cultures for quick yeast DNA extractions, as described by Hoffman and Winston (1987). Isolated yeast DNA was used as templates to confirm the presence of the AMP genes with PCR analyses, using specific primers (Table 2.5).

2.8. Screening for antimicrobial activity

The recombinant yeast strains were screened for antimicrobial activity using the soft agar-overlay and agar well-diffusion assays (Holder and Boyce, 1994; Schoeman *et al.*, 1999; Baindara *et al.*, 2016). The Y294_REF and Y294_EP strains served as a negative and positive control, respectively. Assays were performed in triplicate. A visible zone of inhibition indicated antimicrobial activity. Preliminary screening was performed against the *L. monocytogenes* EDG-e strain for all antimicrobial activity assays. This strain was maintained on Brain Heart Infusion (BHI) agar supplemented with 7.5 µg/mL chloramphenicol and incubated at 37°C.

The bacterial strains in Table 2.7 were streaked from 40% (v/v) glycerol stocks stored at -80°C onto BHI agar or Man De Rogosa medium (MRS), as required. Test tubes with 5 ml BHI or MRS broth were inoculated with the streaked bacterial cultures, as required, and incubated at 37°C, for 18-24 hours on a rotary wheel.

Table 2.7: Bacterial strains used in this study

Microbial strain	Description	Source
Enterobacter cloacae	Gram-negative, facultative anaerobe, rod-shaped bacterium, motile.	Dr. C. Jacobs (UWC)
B. subtilis	Gram-positive, facultative anaerobe, rod-shaped bacterium, motile, spore former.	Prof. M. Bloom (SU)
B. subtilis DSM_NO_10 R5S5C1		Prof. M. Bloom (SU)
Bacillus amyloliquefaciens		Prof. M. Bloom (SU)
Bacillus cereus ATCC 10702		Dr. L. van Zyl (IMBM)
Enterococcus faecalis ATCC 29212	Gram-positive, facultative anaerobe, cocci-shaped, non-motile.	Dr. L. van Zyl (IMBM)
E. faecalis ATCC 29212		Prof. P. Gouws (SU)

Klebsiella oxytoca	Gram-negative, facultative anaerobe, rod-shaped, non-motile.	Prof. P. Gouws (SU)
L. monocytogenes EDG-e	*L. monocytogenes* EDG-e with Chloramphenicol resistance	Prof. M. Bloom (SU)
L. monocytogenes ATCC 19114	Gram- positive, facultative anaerobe, rod-shaped, motile.	Prof. P. Gouws (SU)
L. monocytogenes ATCC 23074		Prof. P. Gouws (SU)
L. monocytogenes ATCC 7644		Prof. P. Gouws (SU)
Listeria innocua ATCC 33090		Prof. P. Gouws (SU)
Pseudomonas aeruginosa PAO1	Gram-negative, facultative anaerobic, rod-shaped bacterium, motile.	Dr. L. van Zyl (IMBM)
P. aeruginosa		Dr. C. Jacobs (UWC)
P. aeruginosa ATCC 27853		Prof. P. Gouws (SU)
Pseudomonas putida KT2440		Dr. L. van Zyl (IMBM)
S. aureus ATCC 25923	Gram-positive, facultative anaerobe, cocci-shaped, non-motile.	Dr. C. Jacobs (UWC)
S. aureus ATCC 25923		Prof. P. Gouws (SU)
S. aureus ATCC 33591		Dr. C. Jacobs (UWC)
S. aureus ATCC 43300		Dr. C. Jacobs (UWC)
Staphylococcus epidermidis ATCC 14990		Dr. L. van Zyl (IMBM)
Salmonella enteritus ATCC 13076	Gram-negative, facultative anaerobe, rod-shaped, motile.	Prof. P. Gouws (SU)
Salmonella typhi ATCC 14028		Prof. M. Bloom (SU)
LAB strains		
Lactiplantibacillus plantarum ATCC 8014	Gram positive, facultative anaerobe, rod-shaped, motile.	KwikStik microbiologics
Lactiplantibacillus pentosus DSM no 20314		Prof. M. Bloom (SU)
L. pentosus DSM no 20223		Prof. M. Bloom (SU)
L. lactis lactis 345	Gram positive, facultative anaerobe, cocci-shaped, non-motile.	Prof. P. Gouws (SU)
L. lactis lactis 346L		Prof. M. Bloom (SU)
L. lactis lactis NCFB 277		Prof. M. Bloom (SU)

Antimicrobial activity screening was conducted using the soft agar-overlay method as outlined in Schoeman *et al.* (1999), with a few modifications. Briefly, recombinant strains were grown overnight at 30°C in test tubes containing 5 mL of SC^{-URA} broth, 2 µL of each culture were spotted onto SC^{-URA} plates and incubated at 30°C for 72 h. The plates were subsequently overlaid with BHI 0.7% (w/v) agar seeded with a 1% (v/v) overnight culture of the target organism, as listed in Table 2.7 After incubation at 30°C for 18 h, the plates were examined for inhibition zones, which is indicative of antimicrobial activity against the target organism.

The agar well-diffusion Assay was conducted to screen antimicrobial activity in the cell free supernatant (CFS). Recombinant *S. cerevisiae* strains were inoculated in 20 mL double strength SC^{-URA} broth and grown aerobically at 30 °C for 72h on a rotary shaker at 180 rpm. The CFS was harvested (1500×g, 5 min) and filtered through 0.2 µm pore-size low-protein binding nonpyrogenic membranes syringe filters (Pall Life Sciences, New York, USA). Where necessary, the CFS was lyophilised for 3 days or acetone precipitated, and dissolved in sterile 1X PBS, to achieve a 20-fold concentration. The antimicrobial activity of the CFS was determined using the agar well-diffusion assay (Holder and Boyce, 1994) by spotting 100 µL of each sample in 6-mm wells cut into the surface of BHI 1% (w/v) agar seeded with a 1% (v/v) overnight culture of target organism. All plates were incubated at 37°C for 18 h and examined for inhibition zones. Putative positive recombinant strains obtained were subsequently screened for antimicrobial activity against a variety of bacterial strains listed in Table 2.7.

2.9. Peptide analysis using Tricine-SDS-PAGE

The CFS from the recombinant strains were analysed using Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Tricine-SDS-PAGE) (Schägger, 2006) to confirm the presence of the recombinant AMPs. Tricine-SDS-PAGE analyses was performed in duplicate using the ultra-low range molecular weight marker (Sigma-Aldrich). One gel was subjected to silver staining (O'Connell and Stults, 1997) to visualize the protein bands. The other gel was fixed for 20 min in a 25% (v/v) isopropanol, 10% (v/v) acetic acid fixing solution and rinsed thrice for 15 min with sterile Milli-Q water. The gel was then cast in a BHI 0.8% (w/v) agar bilayer (supplemented with 7.5 µg/ mL chloramphenicol) seeded with an overnight

culture of *L. monocytogenes* EDG-e and incubated overnight at 37°C to assess antimicrobial activity (Baindara *et al.*, 2016).

2.10. Plate count method to generate co-culture growth curve

To measure microbial growth, 250 ml flasks containing 20 ml YPD were inoculated with overnight yeast and/or *L. monocytogenes* EDG-e cultures and grown at 30°C for 48 hours on an orbital shaker at 180 rpm. The *S. cerevisiae* strains, and *L. monocytogenes* EDG-e were inoculated at a final OD_{600} value of 0.1 and 0.005, respectively. The colony forming units per ml (CFU/ml) readings were determined every 8 hours. To determine CFU/ml, a hundred-fold serial dilution was made from each growth sample at every time interval, which was subsequently plated on appropriate agar plates, and grown at 30°C or 37°C, as required. To count yeast colonies, 100 µl of each dilution was plated on YPD agar plates supplemented with 100 µg/ml Streptomycin. To count *L. monocytogenes* EDG-e colonies, 100 µl of each dilution was plated on BHI agar plates supplemented with 100 µg/ml cycloheximide. The plates containing between 30 to 300 colonies, were selected to be counted. To calculate the CFU/ml, the following formula was used: CFU/ml = (number of colonies x dilution factor) / volume of sample plated.

2.11. HPLC analysis

To facilitate ethanol production, 20 mL rubber stoppered glass bottles (Lasec, Cape Town, South Africa) were filled with 20 ml YPD. A glass bead was added to the media to allow for improved mixing of the fermentation broth. The bottles were co-inoculated with the *S. cerevisiae* strains and *L. monocytogenes* EDG-e at a final optical density $(OD)_{600}$ value of 0.1 and 0.005, respectively. The bottles were incubated on a shaker at 30°C for 72 h at 180 rpm. Thereafter, 1 mL samples were taken from each bottle at regular time intervals. The fermentations samples were subjected to High Performance Liquid Chromatography (HPLC).

Samples collected during fermentation were subjected to centrifugation at 13 000 rpm for 10 min, after which the CFS was acidified by addition of 10% (v/v) sulfuric acid (H_2SO_4) solution. Samples were then filtered through a 0.22 µm filter into 2 mL HPLC vials. Ethanol, cellobiose, acetic acid, glucose, and glycerol concentrations were determined in each sample by HPLC equipped with a BioRad guard (part # 125-0129) and refractive index (RI) detector.

Compound separation was achieved on a BioRad Aminex HPX-87H (part # 125- 0140) 7.8x300 mm column at a temperature of 65°C, with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.7 mL/min. Values obtained for each respective compound were presented as the mean of triplicates, in g/L, with their standard deviations.

2.12. Statistical analysis

Significant differences between AMP antimicrobial activities, growth data and/or metabolite concentrations attained were investigated using two-tailed T-tests, assuming unequal variance. A *p*-value lower than 0.05 was deemed significant.

CHAPTER 3: RESULTS AND DISCUSSION

To establish *S. cerevisiae* as an industrial platform for non-sterile bioprocesses, its antimicrobial capabilities were enhanced by expressing AMPs. As the main bacterial contaminants of industrial ethanol fermentations are LAB and enteric bacteria, candidate AMPs that are active against bacterial strains belonging to these groups were selected. AMPs featuring a variety of antimicrobial spectrums were selected. While most of the selected AMPs belonged to the bacteriocin class IIA (Garvieacin Q, Hiracin JM79, Carnobacteriocin BM1, Piscicolin 126 and Aureocin A53), a bacteriocin from class IA (Nisin A), as well as an AMP derived from an insect (Pyrrhocoricin from a firebug) were also included to broaden the range of testing. Furthermore, unique AMP candidates that were not previously expressed in *S. cerevisiae* were also selected.

3.1. Bioinformatic analysis of selected AMPs

Bioinformatic tools were used to analyse the amino acid sequence of the selected AMPs. AlphaFold (https://alphafold.ebi.ac.uk/) was used to predict the tertiary peptide structure, Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to create a phylogenetic tree and an identity matrix, and SNAP2 (https://rostlab.org/services/snap2web/) was used to predict the functional effects of mutations. A protein's amino acid sequence dictates its three-dimensional structure, which in turn influences its function (Liberles *et al.*, 2012; Bahar and Ren, 2013; Bastolla, Dehouck and Echave, 2017). Hence it is reasonable to hypothesize that by looking at an amino acid sequence, it should be able to infer protein function. Nonetheless, this proves challenging due to unpredictable factors, including environmental influences, PTM and the adaptive nature of protein behaviour. Three-dimensional protein structure determination is important to elucidate the function of a protein (Bertoline *et al.*, 2023). Experimentally, tertiary protein structures are resolved by X-ray crystallography, nuclear magnetic resonance, and electron cryomicroscopy, which are complex, time consuming, expensive, and often the structure is not in its native form (Bertoline *et al.*, 2023). AlphaFold was recently created by DeepMind and employs deep learning artificial intelligence (AI) technology to accurately predict 3D protein structures from amino acid sequences. While groundbreaking and promising for the scientific community, it comes with inherent limitations. AlphaFold has difficulty in predicting intrinsically disordered protein regions

(Ruff and Pappu, 2021) and loops (Stevens and He, 2022), and only allows prediction of a single conformer, not identifying the apo and holo forms (Saldaño *et al.*, 2022). It is currently unable to predict structures with metal ions, cofactors and other ligands, complexes with DNA or ribonucleic acid (RNA), or PTM, such as glycosylation, methylation and phosphorylation (Perrakis and Sixma, 2021). The application of AI tools, such as AlphaFold, for protein prediction is a relatively recent development and as such, we believe that ongoing advancements in AI technology has the potential to mitigate some of these limitations. Figure 3.1 illustrates the AlphaFold predicted tertiary structure of the AMPs selected in this study, as well as Pediocin PA-1 for comparison.

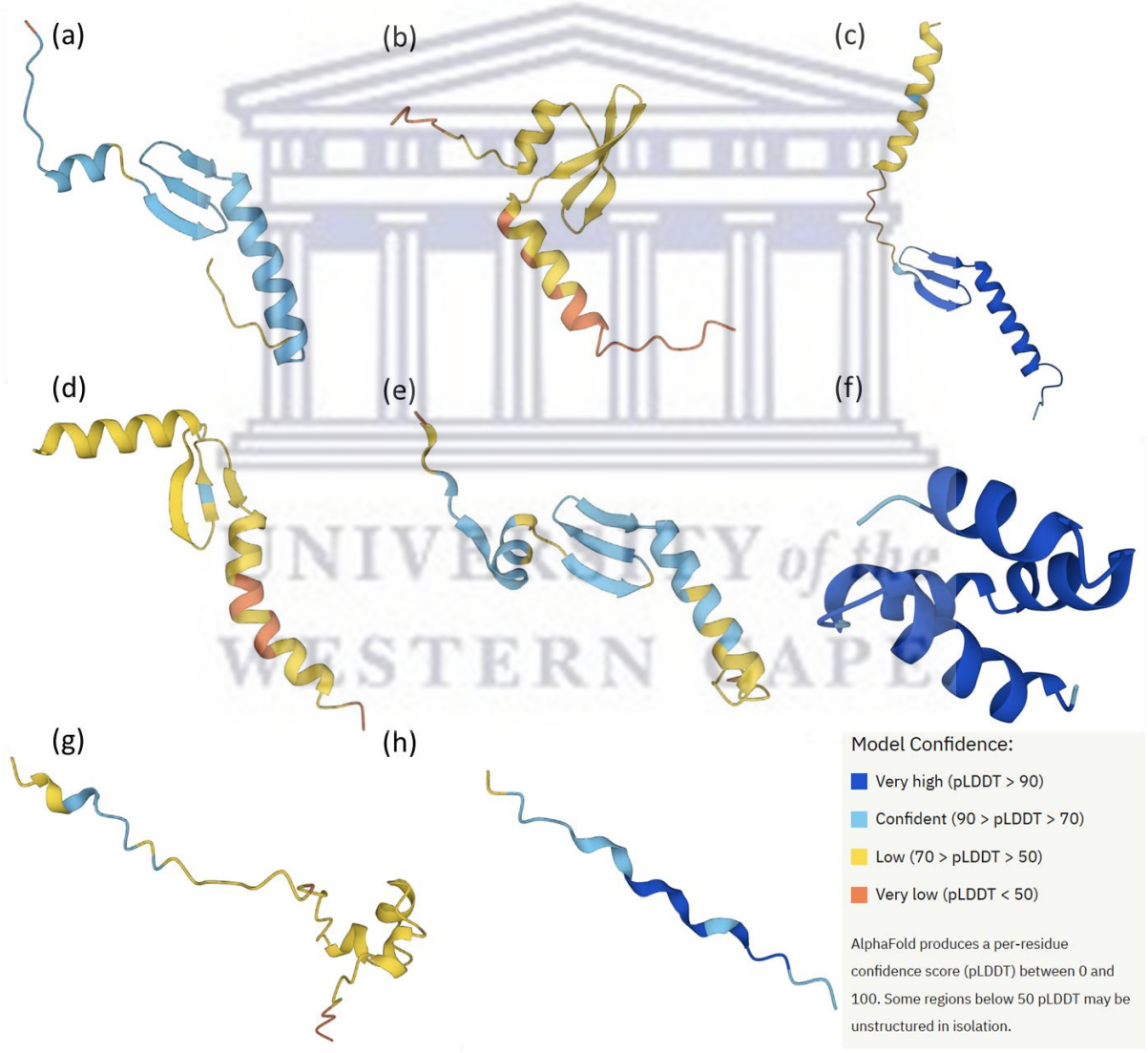

Figure 3.1: AlphaFold predicted tertiary peptide structures of the selected AMPs (Jumper *et al.*, 2021; Varadi *et al.*, 2022). (a) Pediocin PA-1; (b) Garvieacin Q; (c) Hiracin JM79; (d) Carnobacteriocin BM1; (e) Piscicolin 126; (f) Aureocin A53; (g) Nisin A; (h) Pyrrhocoricin.

To our knowledge, none of the selected AMPs have been expressed in *S. cerevisiae* thus far. Given the successful expression of Pediocin PA-1 (Schoeman *et al.*, 1999), we hypothesized that the more closely related the selected AMPs are to Pediocin PA-1, the more likely they would successfully express in *S. cerevisiae*. The Clustal Omega program was used to create the phylogenetic tree (Figure 3.2A) and percent identity matrix (Figure 3.2B) for each selected AMP and Pediocin PA-1 as a reference. Although a protein exists in different conformations, the chances that two closely related sequences will fold into distinctly different structures are so small that they are often neglected in research practice (Krissinel, 2007). When comparing Figure 3.1 to Figure 3.2A, it is clear that the more closely related peptides share a similar predicted structure, which in turn could show similar function. Piscicolin 126 and Pediocin PA1 may share a similar spectrum of antimicrobial activity since they are on the same branch (Figure 3.2A), indicating close relatedness. Pyrrhocoricin, on the other hand, is less likely to have a similar spectrum of antimicrobial activity spectrum compared to Pediocin PA1, given its placement on a different branch (Figure 3.2A). However, these predictions still require laboratory testing for confirmation.

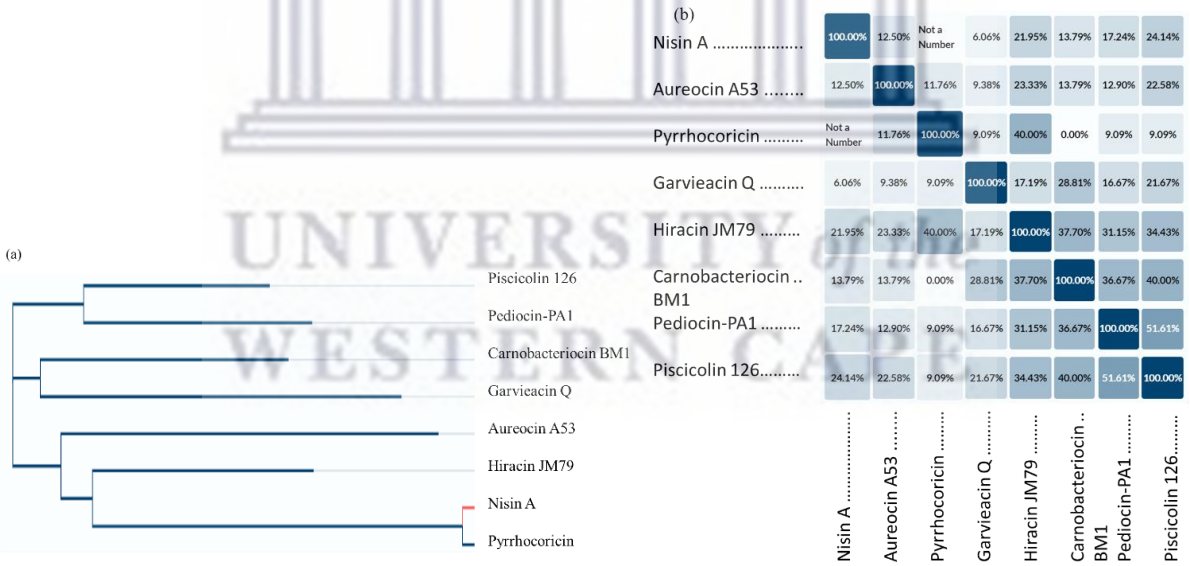

Figure 3.2: (a) Phylogenetic tree and (b) Percent identity matrix of the selected AMPs, created using the Clustal Omega (Sievers *et al.*, 2011; Sievers and Higgins, 2018; Baxevanis, Bader and Wishart, 2020). (a) Neighbour-joining tree without distance corrections. A branch containing negative values is shown in red, representing negative distances as measured by the algorithm. (b) The shading intensity in each block corresponds to the respective AMP's higher percentage match.

Protein pairs with a sequence identity higher than 35% - 40% are very likely to be structurally similar (Rost, 1999; Kinjo and Nishikawa, 2004). Structural similarity in pairs with a sequence identity of 20% - 35% is considerably less common (Rost, 1999). Many studies have found that that it is highly unlikely for proteins pairs sharing below 20% sequence identity to have similar structures (Chothia and Lesk, 1986; Hubbard and Blundell, 1987; Chothia, 1992). This is evident when comparing protein pairs from the percent identity matrix from Figure 3.2B to the tertiary structures in Figure 3.1. Pediocin PA-1 and Piscicolin 126 for example, showed a score of 51.61% relatedness, and share similar predicted structures, (Figures 3.1A; Figure 3.1E). Pyrrhocoricin showed the lowest score range compared to the other AMPs (Figure 3.2B), which is clearly reflected in its distinct predicted structure (Figure 3.1).

When class IIa bacteriocins are synthesized in their host of origin, they are often produced as a precursor which appears not to be biologically active, containing an N-terminal leader sequence (15 to 30 amino acid residues), which is removed by site-specific proteolytic cleavage during export, to yield the mature bacteriocin (Havarstein, Diep and Nes, 1995; Ennahar *et al.*, 2000; Drider *et al.*, 2006; Yount *et al.*, 2020). Synthesizing the bacteriocin as a precursor protects the host by keeping the bacteriocin inactive and acts as a recognition signal during export (Drider *et al.*, 2006). This mature bacteriocin is then secreted and exported through the dedicated transport system involving an ABC-transporter and an accessory protein (Havarstein, Diep and Nes, 1995; Ennahar *et al.*, 2000). Most class II bacteriocins are secreted by an ABC transporter (Drider *et al.*, 2006). Some bacteriocins lack the double-glycine motif, which acts as recognition sequence, in their leader sequence and are exported by the sec-dependent translocation system (Tomita *et al.*, 1996; Cintas *et al.*, 1997; Cui *et al.*, 2012). Hiracin JM79 is secreted by the general sec-dependent export system (Tomita *et al.*, 1996; Cintas *et al.*, 1997; Kalmokoff *et al.*, 2001; Doi *et al.*, 2002; Herranz and Driessen, 2005; Sánchez *et al.*, 2007). Class IIa bacteriocins that depend on the sec-dependent exporters have very different N-terminal propeptide (also lacking the double-Gly motif) and are thus not recognized by the bacteriocin ABC transporters (Nes *et al.*, 2002).

The selected AMPs (excluding Aureocin A53 and Pyrrhocoricin) are expressed as precursors, having both the propeptide and mature amino acid chain, as seen in (Figure S1, Appendix A). This propeptide sequence, can be seen as the extra α-helix on the top left of the predicted structures (Figure 3.1). Predicted mutations in the secretion signal sequence do not seem to affect protein function, as seen in the heatmap (Figure S1, Appendix A). This is expected since the signal sequence is removed during peptide maturation.

3.2. Strain construction and antimicrobial activity testing of a *S. cerevisiae* laboratory strain

The synthesized plasmids pYes2_*GAL1*_AMP(1-7) (Table 2.4), were transformed into Y294_WT to create the Y294_G(1-7) strains (Table 2.3), confirmed by PCR (Figure S2, Appendix A). These recombinant strains were then subjected to antimicrobial activity testing. This required growing them in SC^{-URA} media with galactose as the main sugar source, since any glucose inhibits the $GAL1_P$. We attempted to grow strains on a range of between 20 g/L – 40 g/L galactose, however the growth rate was significantly lower compared Y294_WT, even at higher galactose concentrations. Upon achieving substantial growth, none of the recombinant strains exhibited activity in either the soft agar-overlay, or the well-diffusion assays against *L. monocytogenes* EDG-e (data not shown). We performed Tricine-SDS-PAGE analysis, and confirmed that the AMPs were not present in the CFS (Figure 3.3). This meant that the AMPs were either not expressed or not secreted by the *S. cerevisiae* Y294 strain, under control of the $GAL1_P$.

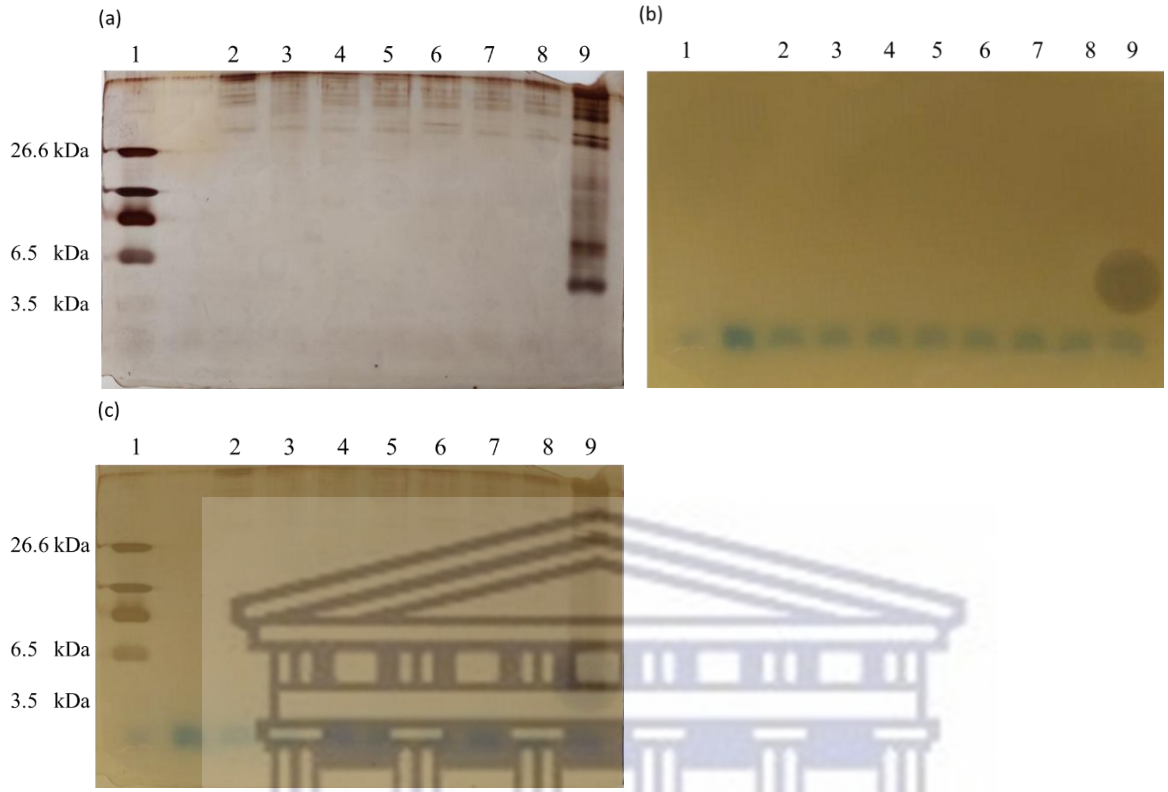

Figure 3.3: Tricine-SDS-PAGE analysis of 20-fold concentrated CFS of the recombinant strains Y294_G(1-7). (a) silver stained gel; (b) overlay gels with *L. monocytogenes* EDG-e; (c) represent the superimposed gels. Lane 1: ultra-low range molecular weight marker (Sigma-Aldrich); Lanes 2-8: Y294_G(1-7) CFS; Lane 9: Y294_EP CFS.

The gels were run in duplicate during Tricine-SDS-PAGE analysis, so that one could be stained to show the position of the heterologous expressed AMP, while the other gel was overlayed with *L. monocytogenes* EDG-e to confirm that it is the AMP that causes inhibition. The CFS from the Y294_EP strain, showed a visible band at the expected size range in the first gel (Figure 3.3A) and a zone of inhibition against *L. monocytogenes* EDG-e in the second gel (Figure 3.3B). When the two images were overlayed it was clear that the Pediocin PA-1 was expressed and responsible for inhibition of *L. monocytogenes* EDG-e growth (Figure 3.3C). A limitation of this method is that if the AMP is secreted in low concentrations it will not show a visible band, however this can be overcome by concentrating the CFS via either lyophilization or acetone precipitation. Another limitation is that two bands of similar size can be indistinguishable from an overexpressed single band.

We experimented with supplementing the SC^{-URA} growth media with a range of 10 g/L - 20 g/L glucose alongside galactose. This approach aimed to enhance the growth rate by providing an initial boost from glucose, with the remaining galactose serving as a sufficient inducer for heterologous expression once the glucose was depleted. Despite a substantial increase in the growth rate, there was still no observable antimicrobial activity, and no visible band within the expected size range was observed in the CFS (data not shown).

Because of inefficient growth and the absence of antimicrobial activity at low glucose concentrations in strains cultivated on galactose, we opted to substitute the inducible $GAL1_P$ with the constitutive $ENO1_{P/T}$. This was done by subcloning the AMP encoding genes to pMU1531, a yeast episomal expression vector with the $ENO1_{P/T}$. The $ENO1_{P/T}$ is not inhibited by glucose; thus, strains can be grown in glucose containing media. The pMU1531_*ENO1*_AMP(1-7) plasmids were transformed into Y294_WT creating the Y294_E(1-7) recombinant strains. The successful transformation was confirmed via PCR, indicated by the presence of a band of approximately 500 bp (Figure 3.4A: Lanes 11-13; Figure 3.4B: Lanes 8-9; Figure 3.4C: Lanes 8-9), when using the appropriate primer sequences (Table 2.5). The growth of these strains was significantly faster compared to strains cultivated on galactose media. A limitation of yeast colony PCR confirmation is that the metabolites might interfere with the PCR amplification. To overcome this, we used the quick yeast DNA extraction method, to extract the DNA within the yeast cell, and using dilutions of this extraction as the template for PCR confirmation.

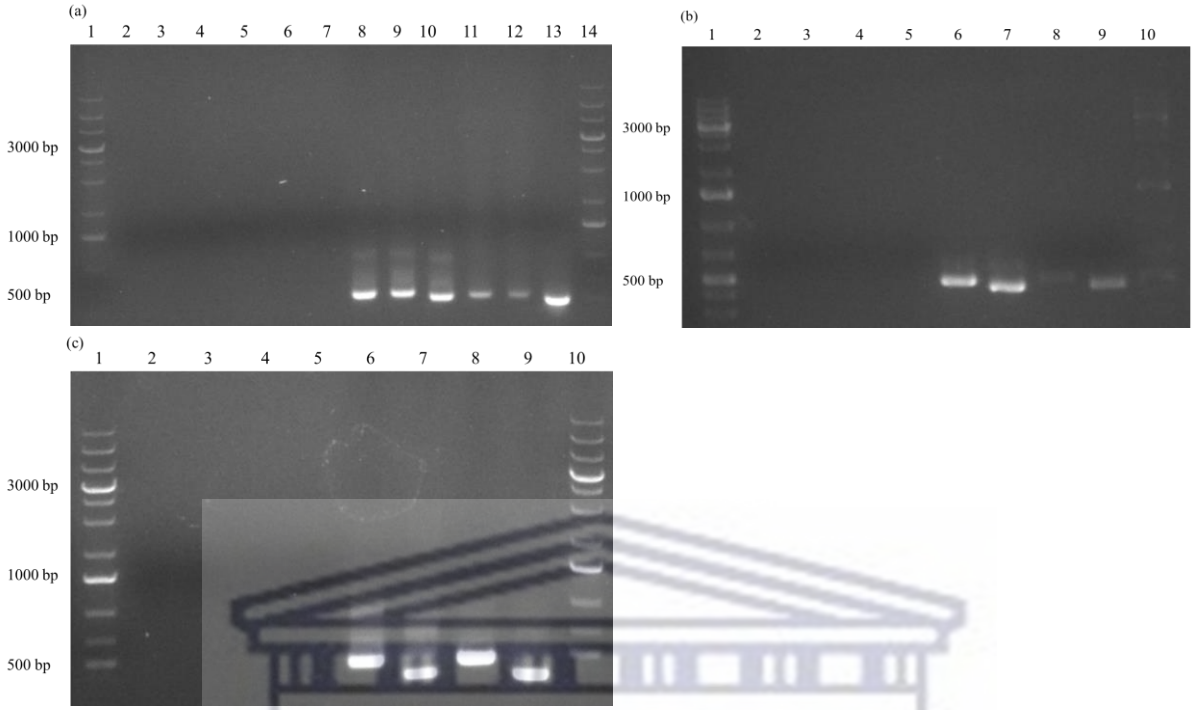

Figure 3.4: AMP gene PCR confirmation: Episomal AMP plasmids were transformed into the yeast strain Y294_WT to create the strains Y294_E(1-7). (a) Lane 1: 1 kb DNA ladder for Safe Stains (NEB); Lanes 2-4: primers only; Lanes 5-7: Y294_WT; Lanes 8-10: pMU1531_*ENO1*_AMP(1-3); Lanes 11-13: Y294_E(1-3); Lane 14: 1kb DNA ladder for Safe Stains (NEB). (b) Lane 1: 1 kb DNA ladder for Safe Stains (NEB); Lanes 2-3: primers only; Lanes 4-5: Y294_WT; Lanes 6-7: pMU1531_*ENO1*_AMP(4-5); Lanes 8-9: Y294_E(4-5); Lane 14: 1kb DNA ladder for Safe Stains (NEB). (c) Lane 1: 1 kb DNA ladder for Safe Stains (NEB); Lanes 2-3: primers only; Lanes 4-5: Y294_WT; Lanes 6-7: pMU1531_*ENO1*_AMP(6-7); Lanes 8-9: Y294_E(6-7); Lane 14: 1kb DNA ladder for Safe Stains (NEB).

The recombinant Y294_E(1-7) strains under control of the *ENO1*$_{P/T}$, underwent antimicrobial activity screening. Figure 3.5 illustrates an example of the soft agar-overlay, and the well-diffusion method. Strains Y294_E1, Y294_E3 and Y294_E4 engineered to express Garvieacin Q, Carnobacteriocin BM1 and Piscicolin 126, respectively, showed zones of inhibition for agar-overlay assays (Figure 3.5A). This presents the successful engineering of antimicrobial strains of *S. cerevisiae*. To our knowledge, none of the selected AMPs have been expressed in *S. cerevisiae* before. Therefore, this study is the first to engineer an *S. cerevisiae* strain capable of inhibiting bacteria via the expression of either Garvieacin Q, Carnobacteriocin BM1 or Piscicolin 126, respectively. It should however be noted that the zone of inhibition size was significantly smaller compared to the Y294_EP Pediocin PA-1 expressing strain. This indicates a reduced potency, which could be due to presence of the native propeptide sequences encoded by the AMP genes.

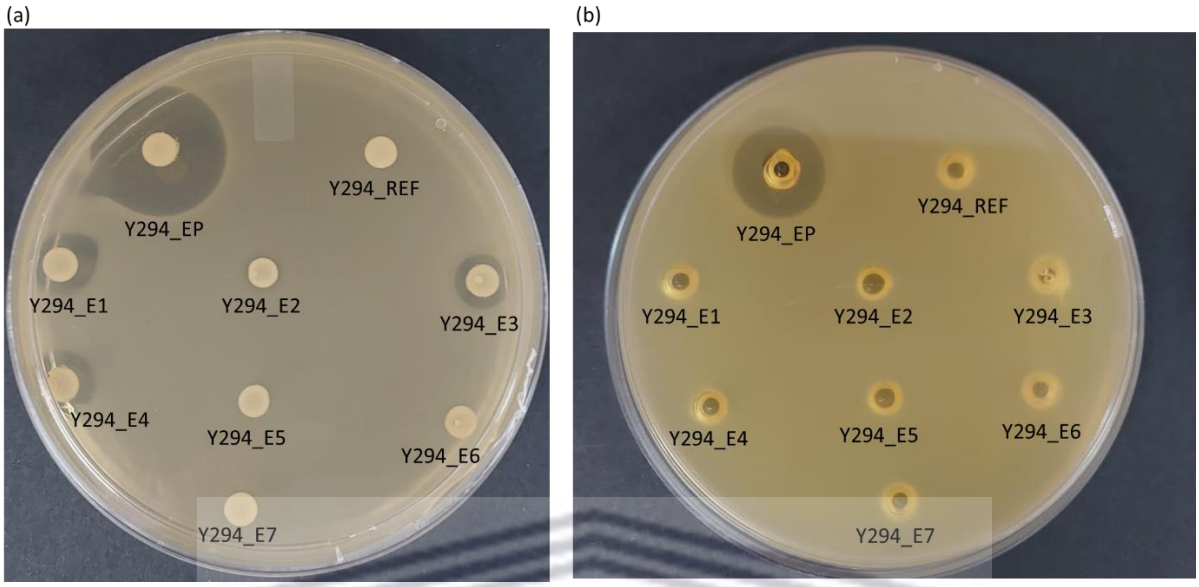

Figure 3.5: Antimicrobial activity screening of recombinant Y294_E(1-7) strains against *L. monocytogenes* ATCC 23074. (a) Agar-overlay assay. (b) Agar well-diffusion assay. The negative and positive controls are Y294_REF and Y294_EP, respectively.

The well-diffusion assays did not yield any observable zone of inhibition for the Y294(1-7) strains (Figure 3.5B). Upon Tricine-SDS-PAGE analysis of the CFS from these strains, no expression bands could be found in the expected size range nor any zones of inhibition against *L. monocytogenes* EDG-e observed, even following attempts to enhance sensitivity through concentration methods, including lyophilization and acetone precipitation (data not shown). This indicates that the AMPs were either not present or inactive in the CFS and were likely not efficiently secreted from the cells which matches with the small zones of inhibition observed (Figure 3.5A).

Schoeman *et al.*, (1999) and van Reenen (2003) both observed little antimicrobial activity in the supernatant. Similar to our results, Schoeman *et al.*, (1999) found relatively low levels of antimicrobial activity in the CFS but the activity was readily detected when intact yeast colonies were used during soft agar-overlays (Schoeman *et al.*, 1999). Schoeman *et al.*, (1999) attributed this low level of activity to the possibility of the bacteriocin remaining associated with the fungal cell wall. When evaluated in the agar well-diffusion assays, the transformants producing the mature Pediocin PA-1 peptide rendered larger zones of inhibition than those produced by the immature form of the peptide (Schoeman *et al.*, 1999). This could explain the smaller zone of inhibition of our strains. A limitation of the soft agar-overlay method is that the colonies spot plated have variable growth rates, which leads to a

larger zone of inhibition for faster growing strains. Our recombinant strains only showed slight variations in growth rate. Both the soft agar-overlay method and the well-diffusion method are qualitative methods. Although it can clearly be seen that the Y294_EP produced a larger zone of inhibition, the increase in AMP potency cannot me measured by only using these methods.

Antimicrobial activity screening of the *S. cerevisiae* Y294_E(1-7) recombinant strains was performed via the soft agar-overlay method and the well-diffusion method against all the organisms listed in Table 2.7. This was done in triplicate. The results from the soft agar-overlay method revealed that the Y294_E1 (Garvieacin Q), Y294_E3 (Carnobacteriocin BM1) and Y294_E4 (Piscicolin 126) strains displayed antimicrobial activity against strains from *Listeria spp.* and *Enterococcus spp.* Table 3.1 illustrates the antimicrobial spectrum of the recombinant Y294_E(1-7) strains, which exclusively shows soft agar-overlay results where antimicrobial activity was observed (Figure S3, Appendix A). Although these species do not represent the main biofuel contaminants, this study showcased yeast's AMP expression potential. Finding the optimal AMP for the biofuel production environment remains a future prospect.

Table 3.1: Antimicrobial activity range of the recombinant Y294_E(1-7) strains: Soft agar-overlay method; ✓ indicates a visible zone of inhibition

Target organism	Pediocin PA-1 (+)	Garvieacin Q	Carnobacteriocin BM1	Piscicolin-126	Hiracin JM79; Aureocin A53; Nisin-A or Pyrrhocoricin
L. monocytogenes EDG-e	✓	✓	✓		No Activity
L. monocytogenes ATCC 23074	✓	✓	✓	✓	
L. monocytogenes ATCC 7644	✓	✓	✓	✓	
L. monocytogenes ATCC 19114	✓	✓	✓		
L. innocua ATCC 33090	✓	✓			
E. faecalis ATCC 29212	✓	✓	✓		
L. pentosus DSM no 20314	✓				
L. pentosus DSM no 20223	✓				

No antimicrobial activity was detected against the remaining organisms listed in Table 2.7, which belong to diverse range of bacteria, including *Enterobacter spp., Lactococcus spp., Lactiplantibacillus spp., Escherichia spp., Staphylococcus spp., Bacillus spp.* and *Pseudomonas spp.* (data not shown). In the well-diffusion assay, none of the Y294_E(1-7) strains exhibited antimicrobial activity in the CFS against any of the organisms listed in Table 2.7 (data not shown). An additional limitation of the technique is the zone size variability when screened against different organisms. If the inhibition zone size is visibly too small, it could lead to a false negative result. To overcome this, we increased the growth time from three to five days, which should increase AMP production as well as inhibition zone size.

It is difficult to compare the antimicrobial spectra and potencies of the different bacteriocins from literature, since the target strains, the levels of bacteriocin purification, and the antibacterial assays differ between the studies (Drider *et al.*, 2006). The following results are from studies conducted by other researchers, specifically examining antimicrobial activity against the same genera of organisms that we tested.

Carnobacteriocin BM1 is active against Gram-positive bacteria, including, *Enterococcus spp.* and *Listeria spp.* (Mathieu *et al.*, 1993; Quadri *et al.*, 1994). Jack and coworkers (1996) found that Piscicolin 126 showed antimicrobial activity against *Enterococcus spp.* and *Listeria spp.*, while no activity was observed against *Bacillus spp.*, *Escherichia spp.*, *Lactiplantibacillus spp.*, *Lactococcus spp.*, *Pseudomonas spp.*, *Salmonella spp.* and *Staphylococcus spp.* We found similar results.

While our strains did not produce active Aureocin 53 (recombinant strain Y294_E5), previous studies have demonstrated antimicrobial activity of Aureocin 53 against *Staphylococcus spp.*, *Listeria spp.*, and *Enterococcus spp.* (Netz, Bastos and Sahl, 2002; Netz *et al.*, 2002). De Oliveira and coworkers (1998) and Fagundes and coworkers (2016) also found Aureocin 53 to be active against *L. monocytogenes*, while Nascimento coworkers (2006) showed its activity against *Staphylococcus spp*. We screened against various strains from these genera, however none of the organisms we tested against were inhibited by Y294_E5.

Despite their high structural similarity, the class IIa bacteriocins differ markedly in their antimicrobial spectrum of activity (Drider *et al.*, 2006). The bactericidal activity of class IIa bacteriocins seems to be targeting primarily *Listeria* strains, but it is also commonly directed against several other Gram-positive bacteria (Ennahar *et al.*, 2000). We found similar results as all the AMPs that showed antimicrobial activity were active against *Listeria spp.* However certain *Listeria spp.* strains were still resistant to Carnobacteriocin BM1 (Y294_E3) or Piscicolin-126 expressing strains (Y294_E4). Drider and coworkers (2006) stated that class IIa bacteriocins exhibit a relatively limited antimicrobial spectrum compared to other bacteriocins produced by Gram-positive bacteria. Our findings align with this observation.

Our study shares similarities with Schoeman *et al.* (1999) where they transformed an *S. cerevisiae* Y294 strain with a plasmid containing the AMP Pediocin PA-1 gene using the MFα1 secretion signal. However, they used the $ADH1_{P/T}$ and did not codon optimize their genes. They also tested against a series of test organisms and found antimicrobial activity against *L. monocytogenes* strains B73, WS2249 and WS2250; *L. innocua* LMG13568; *L. ivanovii* SLCC4769; and *L. fermentum* LMG13554. We also observed antimicrobial activity of the Pediocin PA-1 expressing strain (Y294_EP) against *Listeria spp.* and *Lactobacillus spp.* Eijsink and coworkers (1998) found very few discrepancies in antimicrobial activity between four class IIa bacteriocins (Pediocin PA-1, Enterocin A, Curvacin A and Sakacin P) when acting against strains of *Listeria spp.*, while within LAB

genera, the results varied considerably within each genus and species. We found similar results in that all *Listeria spp.* strains tested against were inhibited by Y294_EP, however few LAB strains (*L. pentosus* DSM no 20314 and *L. pentosus* DSM no 20223) were inhibited (Table 3.1), even though we tested against various other LAB strains, including some from the same genus (*L. plantarum* ATCC 8014*).*

3.3. Construction and antimicrobial activity testing of a *S. cerevisiae* industrial strain

We proceeded to the engineering of the robust industrial yeast strain *S. cerevisiae* Ethanol Red (ER_WT), which is commonly used in the bioethanol production industry (Dmytruk *et al.*, 2017). The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system were used to integrate the AMP genes into the δ-sequences scattered around the yeast genome, allowing for multiple copy number integrations. While traditional methods exist for genome integration, CRISPR-Cas9 holds a distinct advantage by enabling markerless integration allowing multiple rounds of strain manipulation. Genome integration is ideal since it eliminates the risk of losing heterologous genes during cell division and ensures a constant optimum gene copy number, however only a limited numbers of genes can be inserted (Yamada *et al.*, 2010).

We attempted to integrate the Carnobacteriocin BM1 gene, since we displayed its antimicrobial activity. After multiple transformation attempts and screening multiple transformants, we were unable to find any that showed antimicrobial activity against *L. monocytogenes* EDG-e (data not shown). We decided to target a different site, the intergenic regions of Chromosome 11 (Jacob, van Lill and den Haan, 2022), however we were unable to find any transformants active towards *L. monocytogenes* EDG-e (data not shown), even though the presence of these genes were confirmed (Figure S4, Appendix A). We therefor assessed the activity in an alternative host and selected the natural *S. cerevisiae* YI13 strain for its ease of transformation, and good heterologous enzyme production (Davison *et al.* 2019). Even after targeting δ-sequences and chromosome 11 intergenic sites of the YI13_WT strain, no activity against *L. monocytogenes* EDG-e was observed for any of the transformants (data not shown). This outcome, unexpectedly and regrettably, suggests that the gene is either not expressed, or expressed in inadequate quantities. This could be due to low gene copy number in the transformant strains.

We opted to transition away from the CRISPR-Cas9 integration system and use traditional genome integration methods instead. This required the subcloning of the AMP genes into the pBKD expression vectors that allow genome integration as described in Table 2.5. We subcloned the Garvieacin Q, Carnobacteriocin BM1 and Pediocin PA-1 encoding genes into the integration plasmids pBKD1 (*PGK1*$_{P/T}$) and pBKD2 (*ENO1*$_{P/T}$) plasmids. In addition to the *ENO1*$_{P/T}$ we also incorporated the *PGK1*$_{P/T}$ transcriptional control, to assess its potential impact on gene expression levels.

We confirmed the integration of Garvieacin Q, Carnobacteriocin BM1, and Pediocin PA-1 encoding genes into ER_WT, as seen in (Figure S5, Appendix A). This yielded two distinct sets of recombinant strains under the control of *PGK1*$_{P/T}$ and *ENO1*$_{P/T}$, respectively. In the antimicrobial activity assay, none of the recombinant strains showed activity against *L. monocytogenes* EDG-e (Figure 3.6). Consequently, we used the Y294_WT strain as an alternative host, based on its prior demonstration of antimicrobial activity from the Y294_E(1-7) strains. We integrated the Garvieacin Q, Carnobacteriocin BM1 and Pediocin PA-1 encoding gene cassettes under *PGK1*$_{P/T}$ and *ENO1*$_{P/T}$ into the δ-sequences of Y294_WT strain (Figure S6, Appendix A). In the antimicrobial activity assay, none of these recombinant strains showed activity against *L. monocytogenes* EDG-e (Figure S7, Appendix A).

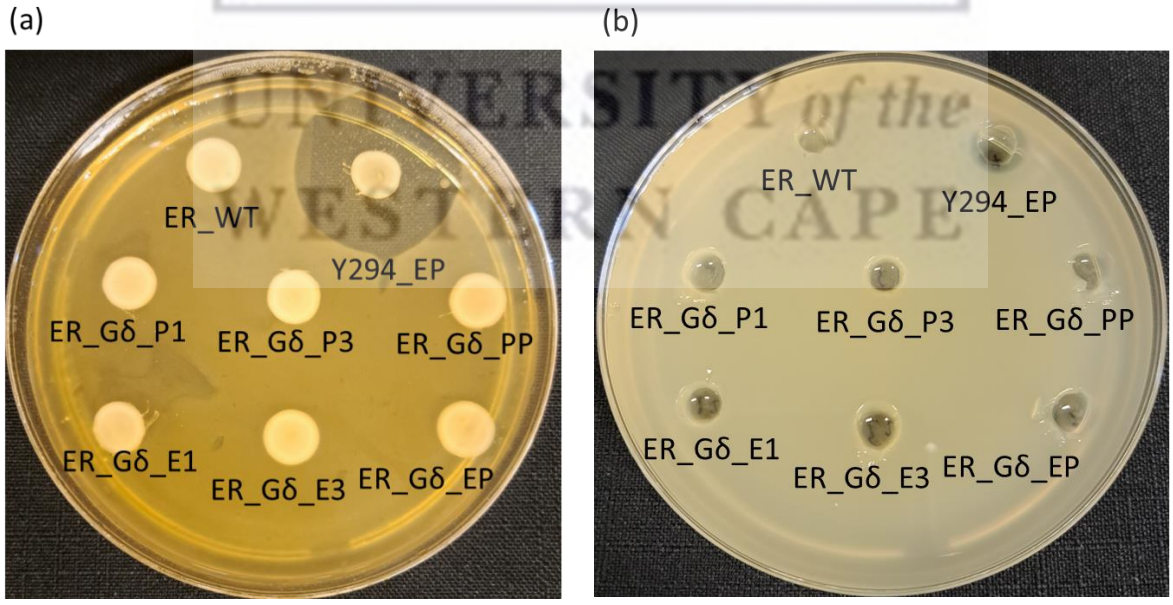

Figure 3.6: Antimicrobial activity screening of ER_Gδ_P(1;3;P) and ER_Gδ_E(1;3;P) strains against *L. monocytogenes* EDG-e. (a) Agar-overlay assay. (b) Agar well-diffusion assay. The negative and positive controls are ER_WT and Y294_EP, respectively.

Therefore, we conclude that the absence of antimicrobial activity cannot be attributed to variations in promoters, host strains, gene position in genome, or integration methods. The reason for this observation could be due to a low gene copy number, low secretion efficiency or another unknown variable yet to be determined. Future testing is required to the determination as to why we could find antimicrobial activity when expressed on a plasmid, compared to no activity when expressed in the genome.

3.4. Co-fermentation of an antimicrobial *S. cerevisiae* industrial strain with a microbial contaminant

The class IIa bacteriocin Enterocin A, produced by *Enterococcus faecium*, is one of the most potent anti-listerial bacteriocins known (Ennahar *et al.*, 2000; Drider *et al.*, 2006). We obtained the ER_EntA strain from Prof. Bloom's LAB (SU), that has the Enterocin A gene genomically integrated at the δ-sequences, since we were unable to find antimicrobial activity from any of our genomically integrated recombinant strains. We evaluated the antimicrobial activity of the ER_EntA strain against *L. monocytogenes* EDG-e and the strains listed in Table 2.7, and found the ER_EntA strain to be active only against *L. monocytogenes* EDG-e. Thus, we used ER_EntA strain and *L. monocytogenes* EDG-e in a yeast-bacteria co-culture (Figure 3.7). This was to ascertain whether the ER_EntA could suppress bacterial growth during culture conditions. The non-logarithmic growth curve of the yeasts from this co-culture can be seen in (Figure S8, Appendix A). When *L. monocytogenes* EDG-e was co-cultured with the antimicrobial ER_EntA strain there was a significantly lower cell density at stationary phase compared to when co-cultured with ER_WT (Figure 3.7C). This clearly shows the antimicrobial properties of the ER_EntA strain. This successfully shows that bacterial contamination could be suppressed without the addition of antibiotics. We did see an initial rise in bacterial growth, however this slowed down and decreased as the yeast count increased. This could be due to the increase in Enterocin A with yeast growth, which has a low initial concentration.

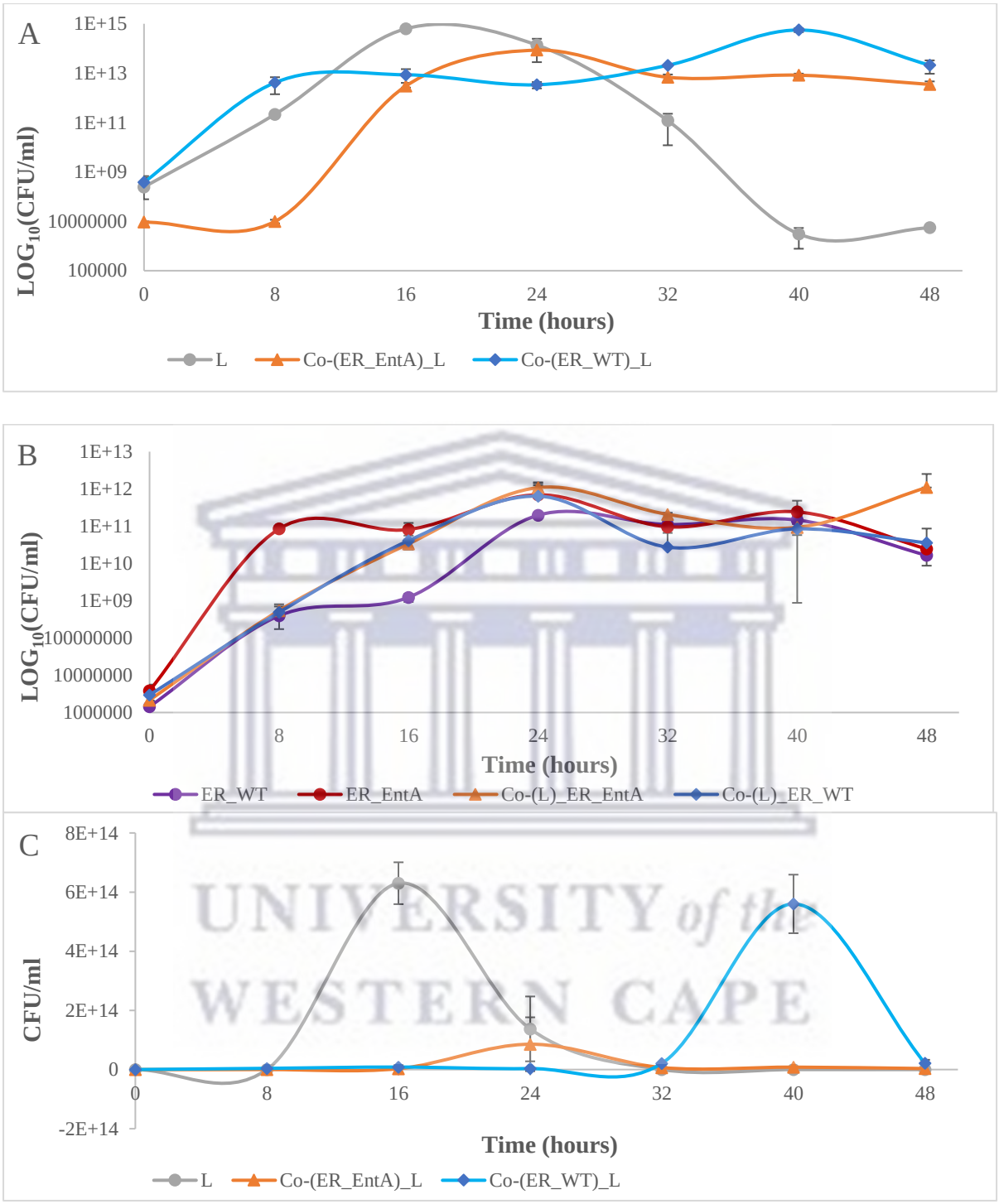

Figure 3.7: Growth curves of ER_EntA and *L. monocytogenes* EDG-e co-cultured in YPD at 30°C for 48h. (a) Logarithmic growth curve of *L. monocytogenes* EDG-e strains; (b) Logarithmic growth curve *S. cerevisiae* strains; (c) Non-Logarithmic growth curve of *L. monocytogenes* EDG-e. L: *L. monocytogenes* EDG-e; Co-(ER_EntA)_L: *L. monocytogenes* EDG-e co-cultured with ER_EntA; Co-(L)_ER_EntA: ER_EntA co-cultured with *L. monocytogenes* EDG-e; Co-(ER_WT)_L: *L. monocytogenes* EDG-e co-cultured with ER_WT; Co-(L)_ER_WT: ER_WT co-cultured with *L. monocytogenes* EDG-e. Data points represent the mean of two biological repeats per respective strain isolate, and error bars indicate mean ± standard deviation.

All the yeast strains reached stationary phase after 24h (Figure 3.7B). It appears that all the yeast strains grew at a relatively similar rate, even when competing against *L. monocytogenes* EDG-e. This outcome was unforeseen, as we anticipated a slightly slower growth of the yeast in the co-cultures. Co-(ER_EntA)_L grew until Co-(L)-ER_EntA reached stationary phase at 24h, after which it decreased (Figure 3.7B; Figure 3.7C). This is likely due to Enterocin A production by Co-(L)-ER_EntA. When *L. monocytogenes* EDG-e grew alone it reached stationary phase at 16 h, followed by a death phase (Figure 3.7A). When *L. monocytogenes* EDG-e was co-cultured with ER_EntA, it took longer to reach stationary phase at 24h and remained in this phase onwards. This observation could be due to the initially low concentration of Enterocin A, which gradually increased as ER_EntA continued to grow.

There are some limitations to this study to keep in consideration. Firstly, at an industrial scale, the likelihood of *L. monocytogenes* EDG-e contamination is minimal, given their lack of adaptation to the fermentation environment. Furthermore, unlike LAB, *L. monocytogenes* EDG-e does not produce substances inhibitory towards yeast. Nevertheless, these findings show the promising inhibitory characteristics exhibited by an AMP producing *S. cerevisiae* strain. Developing an antimicrobial industrial *S. cerevisiae* strain to combat LAB remains a future prospect.

3.5. Ethanol production from co-fermentation of an antimicrobial *S. cerevisiae* industrial strain with a microbial contaminant.

Enhanced ethanol yields are economically advantageous, as they lead to a reduction in the cost of ethanol distillation, which significantly impacts the overall economic evaluation of biofuel production (Kim and Dale, 2005). Even an 1% increase would result in a considerable increase in profit (Della-Bianca *et al.*, 2013; Dmytruk *et al.*, 2017). We tested if the suppression of bacterial growth would translate to a higher final ethanol yield. The ethanol production in a yeast-bacteria co-culture was tested with the ER_EntA strain and *L. monocytogenes* EDG-e. Ethanol production was tested by inoculating pre-cultured ER_EntA and *L. monocytogenes* EDG-e into YPD in oxygen-deficient conditions for 48 h. Samples were taken for HPLC analysis, to determine ethanol yield (Figure 3.8). After 24h, the Co-(L)_EntA strain showed a 4% increase in yield compared to Co-(L)_ER_WT. This study was able to show that an engineered antimicrobial industrial *S. cerevisiae* strain

fermented with a bacterial contaminant, produced a significantly higher ethanol yield compared to the wild type (p = 0.012 and 0.003 at 24 h and 48 h, respectively).

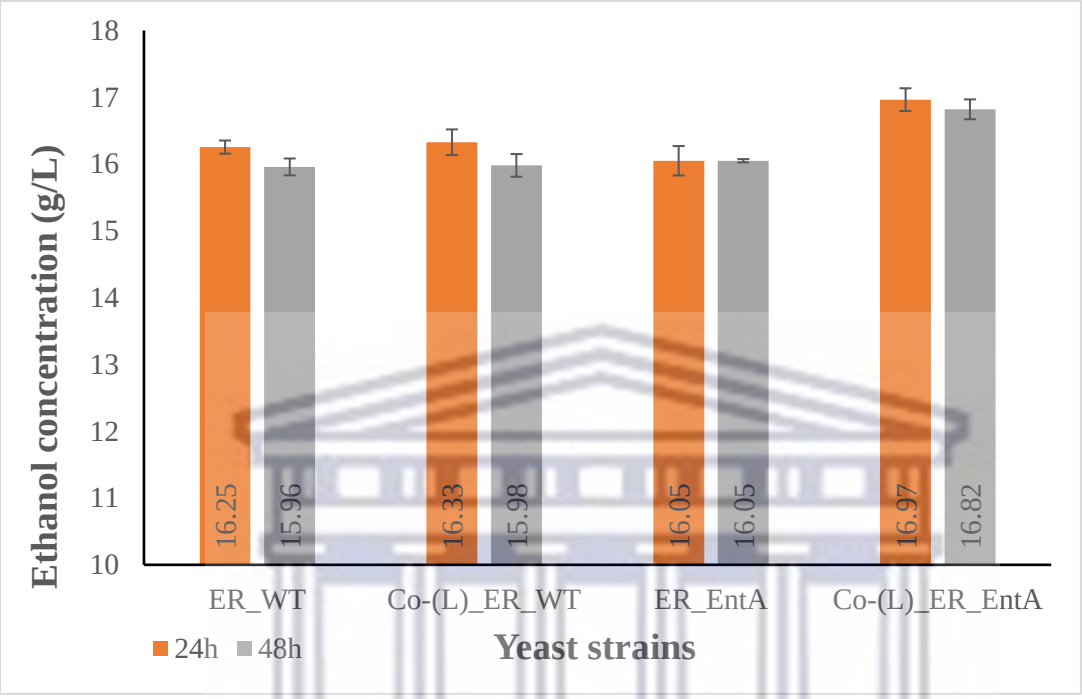

Figure 3.8: Ethanol production analysis via HPLC of ER_EntA and *L. monocytogenes* EDG-e co-fermentation in YPD at 30°C for 48h. ER_WT: *S. cerevisiae* Ethanol Red version 1; ER_EntA: ER_WT expressing Enterocin A; Co-(L)_ER_EntA: ER_EntA co-cultured with *L. monocytogenes* EDG-e; Co-(L)_ER_WT: ER_WT co-cultured with *L. monocytogenes* EDG-e. Data points represent the mean of three biological repeats per respective strain isolate, and error bars indicate mean ± standard deviation.

The theoretical maximum for *S. cerevisiae* during alcoholic fermentation is 0.51 g ethanol per g of consumed glucose (Gombert and van Maris, 2015). The ethanol concentrations of all the strains tested fell within the range of 16 g/L -17 g/L (Figure 3.8), corresponding to 0.43 g - 0.45 g of ethanol per g of consumed glucose. This translates to an ethanol yield, ranging from 84% - 89% of the theoretical maximum. The Co-(L)_ER_EntA was the best ethanol producer, generating ~88,73% of the theoretical maximum ethanol yield. This was expected as *S. cerevisiae* Ethanol Red is an industrial strain known for excellent fermentation capacity and yield (Demeke *et al.*, 2013). Industrial ethanol production operates at >90% of this theoretical yield (Della-Bianca *et al.*, 2013). Although our yields were just below this threshold, we believe optimized fermentation conditions could further enhance ethanol yield. Substrate and product inhibition significantly affect ethanol and biomass yield during ethanol batch fermentation (Thatipamala, Rohani and Hill, 1992).

There is a significant difference in ethanol yield between ER_EntA and Co-(L)_ER_EntA (Figure 3.8; $p < 0.05$). This shows that when *L. monocytogenes* EDG-e was co-cultured with the antimicrobial ER_EntA strain it resulted in a higher ethanol yield compared to when co-cultured with ER_WT. In contrast, there was no significant difference between the ethanol yield of ER_WT and Co-(L)_ER_WT.

Both co-culture and co-fermentation methods share similar limitations. On an industrial scale, the focus is primarily on LAB rather than *L. monocytogenes* EDG-e, which does not produce inhibitory substances affecting ethanol yield. Furthermore, the fermentation conditions are suboptimal for *L. monocytogenes* EDG-e. Additionally, these were controlled co-culture conditions, however this does not consider multiple bacterial contaminations that could have unpredictable outcomes on ethanol yield due to different metabolites they produce. Nevertheless, these findings show that the antimicrobial *S. cerevisiae* strain co-fermented with a bacterial contaminant resulted in a higher ethanol yield. This study was able to show that an engineered antimicrobial industrial *S. cerevisiae* strain grown under fermentation conditions with a bacterial contaminant, produced a higher ethanol yield compared to the wild type.

CHAPTER 4: SUMMARY AND CONCLUSION

Bacterial contamination in the biofuel industry, leads to lower ethanol production which results in economic losses. Antibiotics used to reduce microbial contamination led to AMR development. This work aimed to engineer an industrial *Saccharomyces cerevisiae* strain that produces AMPs active against LAB and *Enterobacteriaceae* which are some of the main bacterial contaminants found in industrial biofuel fermenters. It was hoped that creating an antimicrobial *S. cerevisiae* strain would replace the need for antibiotics and by reducing microbial contamination, ethanol yield should increase due to more available resources otherwise used by the microbial contaminant.

The laboratory *S. cerevisiae* Y294 strain was successfully transformed with seven candidate AMPs, respectively. However, they displayed no antimicrobial activity. After replacing the *GAL1*$_{P}$-*CYC1*$_{T}$ with the constitutive *ENO1*$_{P/T}$ via subcloning, the newly constructed plasmids into were transformed into *S. cerevisiae* Y294. Three of these clones displayed antimicrobial activity. It was found that the clones engineered to express the Garvieacin Q, Carnobacteriocin BM1 and Piscicolin 126 respectively, showed antimicrobial activity against *Listeria spp.* and *Enterococcus spp.* Thus, this study was able to successfully engineer an antimicrobial *S. cerevisiae* strain, although not an industrial strain. To our knowledge, none of the selected AMPs have been expressed in *S. cerevisiae* before. Therefore, this study is the first to engineer an *S. cerevisiae* strain capable of inhibiting bacteria by expressing these AMPs. It should however be noted that the zone of inhibition size was significantly smaller compared to the control. This indicates a reduced potency, which could be due to presence of the native propeptide sequence encoded in the AMP gene of the plasmid constructs.

An attempt was made to create an antimicrobial industrial *S. cerevisiae* strain. Even though the gene presence in the genome of the host was confirmed, no antimicrobial activity was displayed. The Carnobacteriocin BM1 gene was then integrated into the genome of the industrial ER_WT strain targeting either δ-sequences or the intergenic regions of chromosome 11. The same strategy was applied in a different host, YI13_WT, without attaining activity. This study subsequently switched to traditional plasmid-based methods for genome integration, which required the subcloning of our AMP genes. The Garviecin Q and Carnobacteriocin BM1 gene was subcloned to be under control of the *PGK1*$_{P/T}$ and the *ENO1*$_{P/T}$ and these gene cassettes were integrated into both ER_WT as well as Y294_WT, respectively. None of the transformants created showed antimicrobial activity, even though

the presence of the genes were confirmed. Therefore, this inactivity cannot be attributed to variations in promoters, host strains, gene position in genome, or integration methods. It was concluded that this likely indicated a low gene copy number, or the AMPs being stuck inside the host, which means that the AMPs were either not expressed or secreted at a high enough concentration required for antimicrobial activity. Future testing is required for the determination as to why we could find antimicrobial activity when expressed from a plasmid, compared to no activity when genes were integrated into the genome.

Since an industrial antimicrobial *S. cerevisiae* strain couldn't be constructed, the study continued the experiments with an industrial antimicrobial *S. cerevisiae* Ethanol Red strain obtained from Prof. M. Bloom (SU). This strain expressed the AMP Enterocin A, and when screened for antimicrobial activity, it was found to only be active against *L. monocytogenes* EDG-e. Upon generating a growth curve from a co-culture of this strain and *L. monocytogenes* EDG-e, it was found that the ER_EntA successfully suppressed the growth of the bacterial contaminant *L. monocytogenes* EDG-e. This shows that bacterial contamination could be inhibited without the addition of antibiotics. An initial rise in bacterial growth was found, however this slowed down and decreased as the yeast count increased. This could be due to the increase in Enterocin A with yeast growth, which has a low initial concentration.

It was hypothesized that this decrease in bacterial growth would translate to a higher final ethanol yield. Thus, this co-culture was repeated in anaerobic fermentation conditions to reflect industrial fermentation conditions. a 4% higher ethanol yield was found for the antimicrobial industrial *S. cerevisiae* strain compared to the wild type, when co-fermented with *L. monocytogenes* EDG-e. This study was able to show that an engineered antimicrobial industrial *S. cerevisiae* strain grown under fermentation conditions with a bacterial contaminant, produced a higher ethanol yield compared to the wild type. We acknowledge limitations, such as being unable to show antimicrobial activity in the CFS of our constructed strains. As well as not being able to engineer our own antimicrobial industrial *S. cerevisiae* strain in this study, even though we attempted a variety of strategies. For future research we suggest testing a greater variety of AMPs, increasing the gene copy number, and testing different secretion methods. Our study's findings could help develop antimicrobial industrial *S. cerevisiae* strains beyond those tested, potentially replacing antibiotics in industry.

4.1 Future perspectives

Future studies should focus on engineering industrial strains for expression of multiple AMPs. *In vitro* studies have highlighted that the susceptibility of microorganisms is greater to combinations than to individual AMPs (Rahnamaeian and Vilcinskas, 2015; Yu *et al.* 2019). Expressing a variety of AMPs has the potential to broaden its antimicrobial spectrum and enhance potency. Often, when more than one bacteriocin is produced, they act synergistically, exhibiting a stronger inhibitory effect against sensitive strains (Garneau, Martin and Vederas, 2002; Limonet *et al.* 2004; Martin *et al.* 2004), although they may also act antagonistically (Mulet-Powell *et al.* 1998). By combining unrelated bacteriocins with different modes of action, it is also possible to prevent the emergence of resistance to either bacteriocin (Horn *et al.*, 1999; Reviriego *et al.*, 2005). Additionally, a larger variety of AMPs should be screened to find the optimal AMP for expression in *S. cerevisiae*. Furthermore, we recommend using the mature peptide sequence without the propeptide sequence during the engineering of *S. cerevisiae* for higher antimicrobial activity. We would also recommend mRNA analysis to check expression levels in cases where the engineered *S. cerevisiae* show either no or low levels of antimicrobial activity. We recommend looking into the overexpression of native *S. cerevisiae* AMP genes.

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APPENDIX A: SUPPLEMENTARY FIGURES

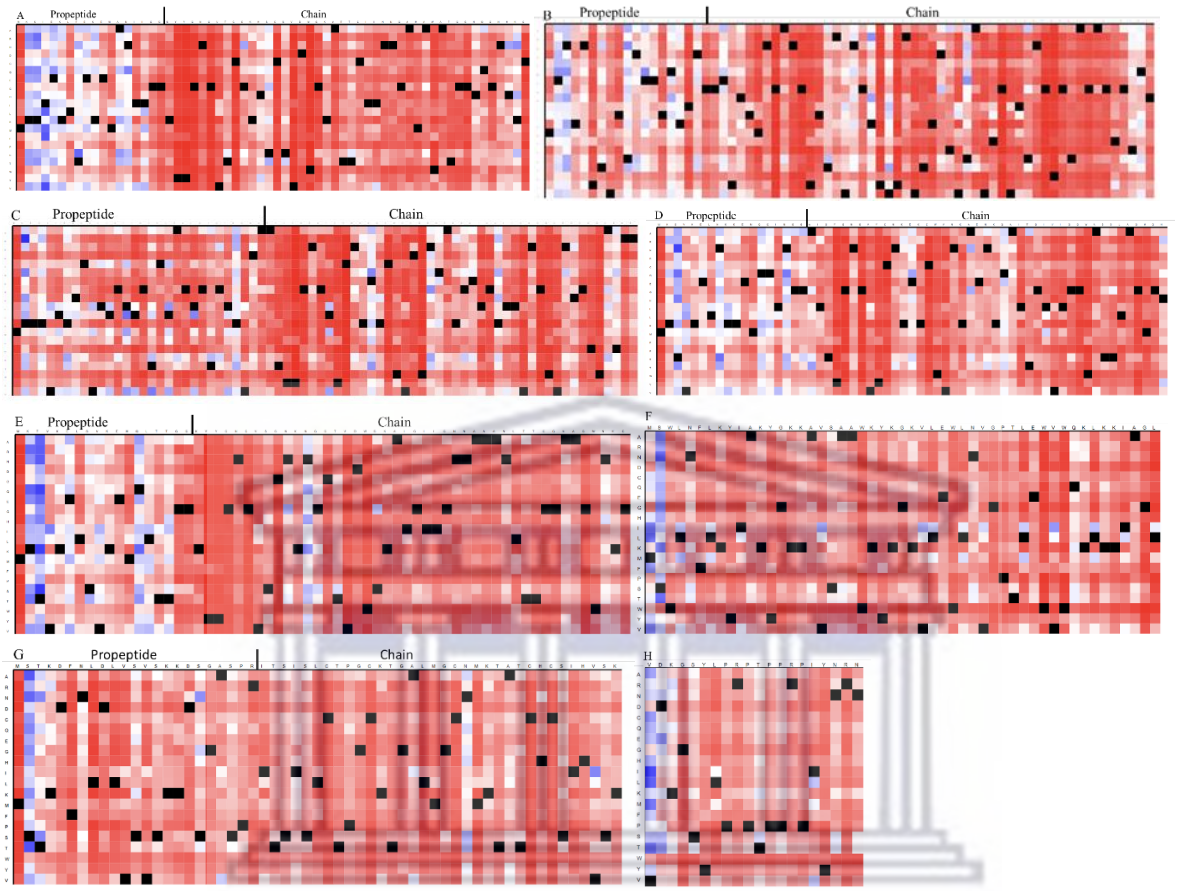

Figure S1: Heatmap of SNAP2 predicted functional effects of mutations on the amino acid sequence of the selected AMPs. (a) Pediocin PA-1; (b) Garvieacin Q; (c) Hiracin JM79; (d) Carnobacteriocin BM1; (e) Piscicolin 126; (f) Aureocin A53; (g) Nisin A; (h) Pyrrhocoricin. Each substitution is represented independently for each position of a protein in the heatmap. Dark red indicates a high score (score > 50, strong signal for effect), white indicates weak signals (-50 < score < 50), and blue a low score (score < -50, strong signal for neutral/no effect). Black marks the corresponding wild-type residues. The first residue in the sequence has index/position 0 (Bromberg and Rost, 2007; Hecht, Bromberg and Rost, 2013, 2015).

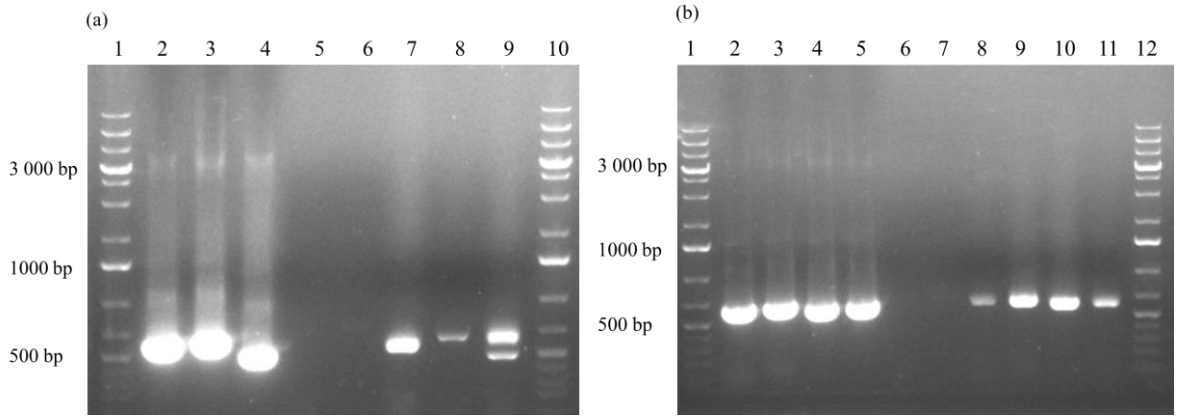

Figure S2: AMP gene PCR confirmation: Episomal AMP plasmids were transformed into the yeast strain Y294_WT to create the strains Y294_G(1-7). (a) Lane 1: 1kb DNA ladder for Safe Stains (NEB); Lanes 2-4: pYes2_*GAL1*_AMP(1-3); Lane 5:Y294_WT; Lane 6: primers only; Lanes 7-9: Y294_G(1-3); Lane 10: 1kb DNA ladder for Safe Stains (NEB). (b) Lane 1: 1kb DNA ladder for Safe Stains (NEB); Lanes 2-5: pYes2_*GAL1*_AMP(4-7); Lane 6:Y294_WT; Lane 7: primers only; Lanes 8-11: Y294_G(4-7); Lane 10: 1kb DNA ladder for Safe Stains (NEB).

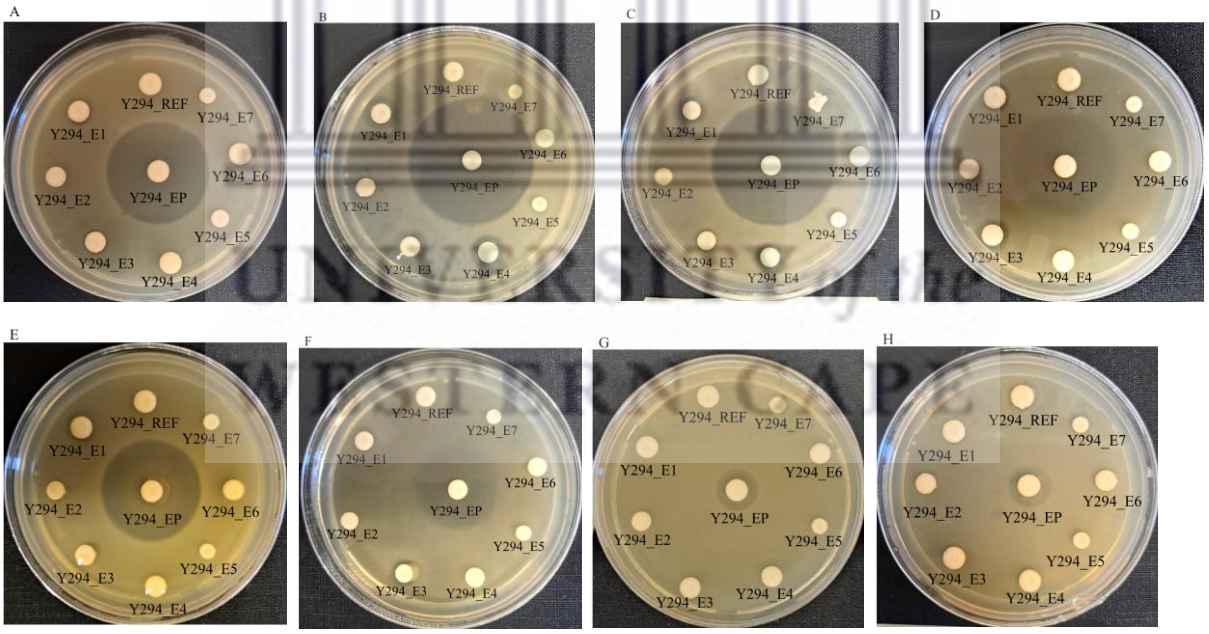

Figure S3: Soft agar-overlay Y294_WT_E(1-7) strains against a variety of bacterial targets. The negative and positive controls are Y294_REF and Y294_EP, respectively. The microbial targets were: (a) *L. monocytogenes* EDG-e; (b) *L. monocytogenes* ATCC 23074; (c) *L. monocytogenes* ATCC 7644; (d) *L. monocytogenes* ATCC 19114; (e) *L. innocua* ATCC 33090; (f) *E. faecalis* ATCC 29212; (g) *L. pentosus* DSM no 20314; (h) *L. pentosus* DSM 20223.

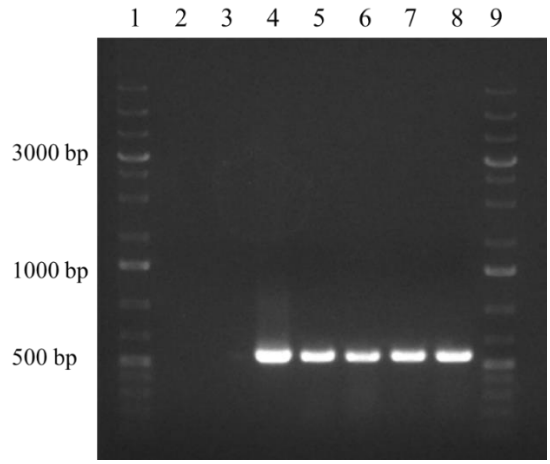

Figure S4: AMP gene PCR confirmation: Genomically integrated Carnobacteriocin BM1 gene using CRISPR-Cas9 system into δ-sequences and chromosome intergenic sites of ER_WT and YI13_WT isolates to create the ER_δ_E3, ER_CH11_E3, YI13_δ_E3 and YI13_CH11_E3 recombinant strains, respectively. Lane 1: 1 kb DNA ladder for Safe Stains (NEB); Lane 2: ER_WT; Lane 3: YI13_WT; Lane 4: pMU1531_*ENO1*_AMP3; Lane 5: ER_δ_E3; Lane 6: ER_CH11_E3; Lane 7: YI13_δ_E3; Lane 8: YI13_CH11_E3; Lane 9: 1 kb DNA ladder for Safe Stains (NEB).

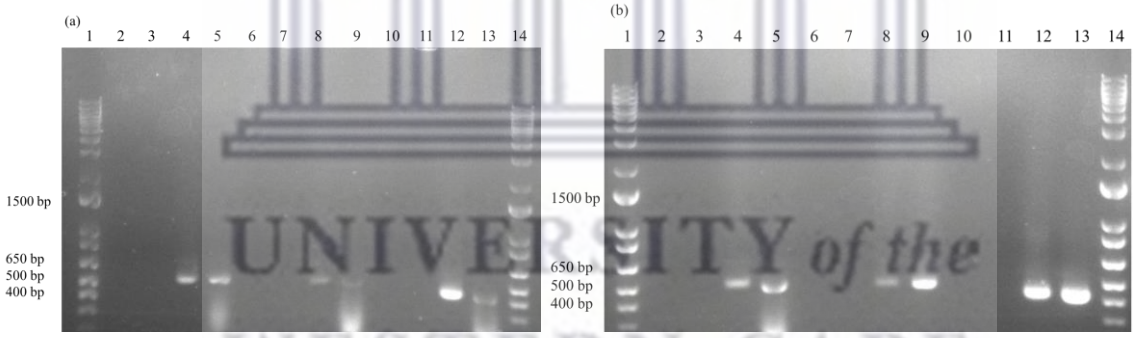

Figure S5: PCR confirmation of Garvieacin Q, Carnobacteriocin BM1 and Pediocin PA-1 integration under control of the (a) *PGK1*$_{P/T}$ or (b) *ENO1*$_{P/T}$ into ER_WT to create the strains (a) ER_Gδ_P(1;3;P) and (b) ER_Gδ_E(1;3;P). (a) Lane 1:1 kb Plus DNA Ladder (Invitrogen); Lane 2: primers only; Lane 3: ER_WT; Lane 4: pBKD1_*PGK1*_AMP1; Lane 5: ER_Gδ_P1; Lane 6: primers only; Lane 7: ER_WT; Lane 8: pBKD1_*PGK1*_AMP3; Lane 9: ER_Gδ_P3; Lane 10: primers only; Lane 11: ER_WT; Lane 12: pBKD1_*PGK1*_PedA; Lane 13: ER_Gδ_PP; Lane 14: 1 kb Plus DNA Ladder (Invitrogen). (b) Lane 1: 1 kb Plus DNA Ladder (Invitrogen); Lane 2: primers only; Lane 3: ER_; Lane 4: pBKD2_*ENO1*_AMP1; Lane 5: ER_Gδ_E1; Lane 6: primers only; Lane 7: ER_WT; Lane 8: pBKD2_*ENO1*_AMP3; Lane 9: ER_Gδ_E3; Lane 10: primers only; Lane 11: ER_WT; Lane 12: pBKD2_*ENO1*_PedA; Lane 13: ER_Gδ_EP; Lane 14: 1 kb Plus DNA Ladder (Invitrogen).

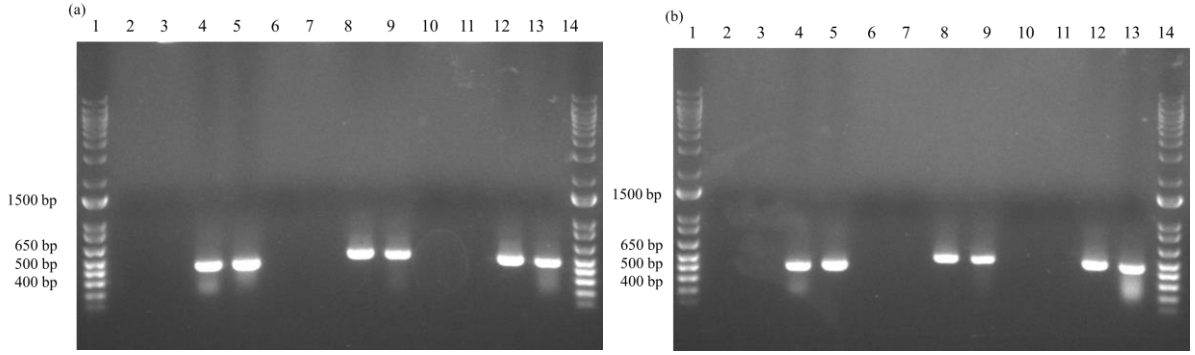

Figure S6: PCR confirmation of Garvieacin Q, Carnobacteriocin BM1 and Pediocin PA-1 integration under control of the (a) *PGK1*$_{P/T}$ or (b) *ENO1*$_{P/T}$ into Y294_WT to create the strains (a) Y294_Gδ_P(1;3;P) and (b) Y294_Gδ_E(1;3;P). (a) Lane 1:1 kb Plus DNA Ladder (Invitrogen); Lane 2: primers only; Lane 3: Y294_WT; Lane 4: pBKD1_*PGK1*_AMP1; Lane 5: Y294_Gδ_P1; Lane 6: primers only; Lane 7: Y294_WT; Lane 8: pBKD1_*PGK1*_AMP3; Lane 9: Y294_Gδ_P3; Lane 10: primers only; Lane 11: Y294_WT; Lane 12: pBKD1_*PGK1*_PedA; Lane 13: Y294_Gδ_PP; Lane 14: 1 kb Plus DNA Ladder (Invitrogen). (b) Lane 1: 1 kb Plus DNA Ladder (Invitrogen); Lane 2: primers only; Lane 3: Y294_; Lane 4: pBKD2_*ENO1*_AMP1; Lane 5: Y294_Gδ_E1; Lane 6: primers only; Lane 7: Y294_WT; Lane 8: pBKD2_*ENO1*_AMP3; Lane 9: Y294_Gδ_E3; Lane 10: primers only; Lane 11: Y294_WT; Lane 12: pBKD2_*ENO1*_PedA; Lane 13: Y294_Gδ_EP; Lane 14: 1 kb Plus DNA Ladder (Invitrogen).

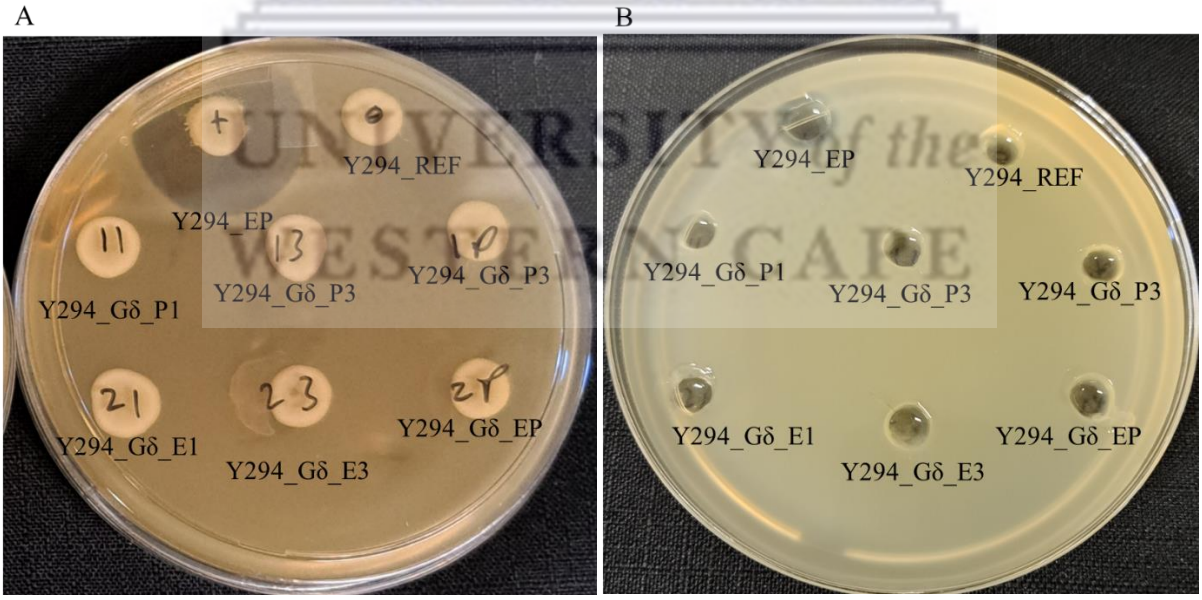

Figure S7: Antimicrobial activity screening of genomically Y294_Gδ_P(1;3;P) and Y294_Gδ_E(1;3;P) strains against *L. monocytogenes* EDG-e. (a) Agar-overlay assays and (b) agar well-diffusion. The negative and positive controls are Y294_WT and Y294_EP, respectively.

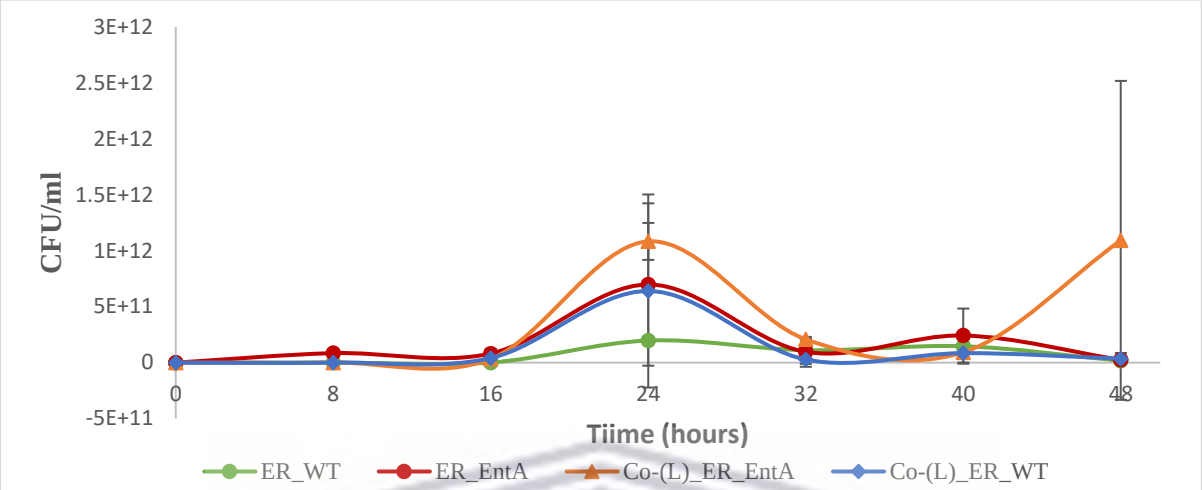

Figure S8: Non-logarithmic growth curve of yeast strains from ER_EntA and *L. monocytogenes* EDG-e co-cultured in YPD at 30°C. ER_WT: *S. cerevisiae* Ethanol Red version 1; ER_EntA: ER_WT expressing Enterocin A; Co-(L)_ER_EntA: ER_EntA co-cultured with *L. monocytogenes* EDG-e; Co-(L)_ER_WT: ER_WT co-cultured with *L. monocytogenes* EDG-e. Data points represent the mean of two biological repeats per respective strain isolate, and error bars indicate mean ± standard deviation.