



Genetic engineering of *Saccharomyces boulardii*: Tools, strategies and advances for enhanced probiotic and therapeutic applications

João Paulo Carvalho^{a,1}, David Sáez Moreno^{a,1}, Lucília Domingues^{a,b,*}

^a CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal

^b LABBELS - Associate Laboratory, Braga, Guimarães, Portugal

ARTICLE INFO

Keywords:

CRISPR-Cas9

Saccharomyces boulardii

Probiotics

Probiotic engineering

ABSTRACT

Saccharomyces boulardii is a probiotic yeast that has been extensively studied in clinical trials, and it is used to treat several gut disorders. Its high survivability and the ability of secreting recombinant proteins, make *S. boulardii* an attractive delivery vessel for molecules of interest in the gut. Despite its natural mechanisms of action still not being fully understood, genetic engineering offers the advantage of rationally design the delivery of molecules of interest to the gut. As a yeast, *S. boulardii* can produce complex proteins with post translational modifications, an advantage when compared with bacterial probiotics. In addition, antibiotics can be co-administered with this yeast, increasing gut residence times and improving the positive effects on human health.

This review aims to cover the genetic engineering advances and applications of genetically engineered *S. boulardii*. The similarity of this yeast with *S. cerevisiae* has made it possible to develop tools like CRISPR-Cas9, plasmid expression systems and gene integration techniques that have allowed the modification of *S. boulardii*. These genetic tools and modifications are discussed in detail according to the state of the art highlighting the most effective ones. Genetic engineering has increased the possible applications of *S. boulardii*, including immune response modulation, enhanced pathogen neutralization, anti-obesity strategies, vitamin production. Others have used these tools to tackle its limitations as probiotic compared to others commonly used such as lactic acid bacteria, increasing its residence times and allowing the development of biocontainment strategies. Current limitations and future directions of the field such as scalability, standardization and the lack of clinical data are also highlighted and discussed.

1. Introduction

Probiotics are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization as “live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host” (Gopalan et al., 2023). Although most currently used probiotics are lactic acid bacteria (LAB) (*Lactobacillus* spp., *Bifidobacterium* spp., *Bacillus* spp., etc.) (Didari et al., 2014), *Saccharomyces boulardii*, stands out as the only probiotic yeast approved by FDA (Abid et al., 2022). Recently, LAB and non-conventional probiotics were reviewed in detail elsewhere (Murali and Mansell, 2024). This review aims to focus exclusively on *S. boulardii*, providing an in-depth discussion on the most effective genetic tools currently available and compiling the modifications made to date to enhance its therapeutic and probiotic properties.

S. boulardii was firstly isolated from peels of tropical fruits (Hossain et al., 2020) and its probiotic activities have been studied in several clinical and non-clinical trials in the last few decades (McFarland, 2017; Moré and Vandenplas, 2018). This yeast shows high tolerance to acidic conditions and grows well at 37 °C (the human host's internal temperature) (Pais et al., 2020), both key factors for surviving the harsh conditions of the gastrointestinal (GI) tract. It is considered Generally Regarded as Safe (GRAS) by the FDA (US Food and Drug Administration) (Douradinha et al., 2014) and is recommended for treating acute gastroenteritis, for prevention of antibiotic-associated diarrhoea and has proven to have positive effects in traveler's diarrhoea, acute infectious diarrhoea, Crohn's disease, ulcerative colitis (McFarland, 2010; Dinleyici et al., 2014; Salazar-Parra et al., 2023; Szajewska and Kołodziej, 2015) due to the inhibition of pathogens and the increase of beneficial probiotic species in the gut (Gao et al., 2023; Roy Sarkar et al., 2021;

* Corresponding author at: CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal.

E-mail address: luciliad@deb.uminho.pt (L. Domingues).

¹ These authors contributed equally to the work.

Spatz et al., 2023). A recent meta-analysis highlights the efficacy of *S. boulardii* for the treatment of paediatric acute diarrhoea, showing that the administration of this yeast reduced its duration and more participants were cured when compared with the control group (McFarland and Li, 2025). Moreover, the use of *S. boulardii* as an adjuvant therapy for combating *Helicobacter pylori* is supported by another recent meta-analysis comprising 19 studies and over 5000 cases, showing both a higher eradication rate and the reduction of adverse effects when *S. boulardii* was used to complement the standard of care (Li and Xie, 2025).

While the positive effects of this yeast have been proven, the exact health effects of the consumption of *S. boulardii* are under constant investigation and have been attributed to several mechanisms of action. This probiotic yeast exerts a broad range of health-promoting activities, attributed to its antimicrobial, immunomodulatory, and gut-support properties. One key mechanism involves the secretion of antimicrobial peptides capable of neutralizing pathogenic toxins (i.e., toxins A and B from *Clostridium difficile*, cholera toxin and pathogenic *Escherichia coli* surface endotoxins) (Terciolo et al., 2019; Pais et al., 2020). Additionally, *S. boulardii* can directly bind to pathogens microbes (a process facilitated by mannose residues on its cell wall), bind to toxins and reduce the availability of pathogens binding sites in intestinal surfaces, preventing their adhesion (Pais et al., 2020).

The mechanisms of action of *S. boulardii* extend beyond pathogen neutralization. *S. boulardii* produces short chain fatty acids (SCFA's) (acetate, propionate, and butyrate) that not only inhibit pathogen proliferation but also contribute positively for the microbiome modulation. This yeast also has shown immune modulation properties (Baek et al., 2024) and reduced levels of pro-inflammatory cytokines such as TNF- α and IL-8 in paediatric acute diarrhoea patients (McFarland and Li, 2025). It has been shown to stimulate the production of anti-inflammatory cytokines IL-10 and to suppress pathways associated with inflammatory response (e.g., NF- κ B-mediated signal transduction pathways) (Terciolo et al., 2019). *S. boulardii* also aids in maintaining the integrity of the intestinal mucosa through stimulation of mucin production, secretion of polyamines such as spermidine and spermine and production of SCFA's (Pais et al., 2020; Cui et al., 2022; Tang et al., 2024a). Moreover, antioxidant effects of *S. boulardii* were shown to protect against oxidative stress in rats, something hypothesized to be related to the enhancement of the blood-brain barrier integrity (Babaei et al., 2024). Animal trials (Chu et al., 2024) and a case-report (Kobliner et al., 2018) suggest its positive influence in mental disorders, via modulation of the gut microbiome. The probiotic yeast also exerts other trophic effects like stimulating the production of brush border membrane digestive enzymes and favouring nutrient absorption (Moré and Vandenplas, 2018; Terciolo et al., 2019). For a more detailed view of the proven health effects and the ongoing investigations on molecular mechanisms driving those, we recommend the reader to turn to (Kelesidis and Pothoulakis, 2012; McFarland, 2010; Terciolo et al., 2019; Pais et al., 2020). *S. boulardii* is not naturally part of human gut microbiota, and competes with the endogenous microorganisms, which leads to low residence times in the gut (3–5 days after stopping dosing) (Blehaut et al., 1989; Elmer et al., 1999). This limitation can be addressed through repeated dosing, co-administration with antibiotics or with genetic engineering, as will be further discussed. In fact, in the clinic, *S. boulardii* is often used to mitigate the side effects of antibiotic therapy related to gut dysbiosis or *C. difficile* infection (Lau and Chamberlain, 2016). *S. boulardii* unlike bacterial probiotics, is not susceptible to antibiotics and can be administered concurrently (Hossain et al., 2020; Kaźmierczak-Siedlecka et al., 2020; Spatz et al., 2023). Recently, a randomized controlled trial showed that *S. boulardii* as adjunct therapy with vancomycin resulted in reduced *C. difficile* infection recurrence without any adverse effects (Chitapanarux et al., 2025). This fact can also enable a more accurate dosage since it allows it to be effectively cleared from the gut (Czerucka et al., 2007; Klein et al., 1993), which is another potential advantage for the use of *S. boulardii* as a transient drug

delivery vessel. There is a growing scientific and clinical interest in *S. boulardii*, Fig. 1 illustrates the increase in the number of publications referring to this probiotic. Moreover, according to ClinicalTrials.gov there are 45 completed clinical trials including the use of *S. boulardii*, and there are several *S. boulardii* probiotic products already in the market.

Despite the interest and efficacy of *S. boulardii*, the global probiotic market is dominated by LAB, particularly *Lactobacillus* and *Bifidobacterium* species, being among the most clinically evaluated probiotics, widely incorporated into yogurt, dairy, and capsule-based formulations. These strains are more commonly used in food and supplement formulations due to their historical association with fermented foods. In fact, over 1000 completed clinical trials have involved LAB strains, compared to only 13 for *S. boulardii* as of 2025 (according to ClinicalTrials.gov) and the number of strains in the market is much superior. In contrast, *S. boulardii* offers certain advantages, such as resistance to antibiotics, lower optimal pH, and auto-aggregation capacity (Czerucka et al., 2007), and its transient behavior which can also be seen as an advantage, for short-term therapeutic actions, such as prevention of antibiotic-associated diarrhoea or traveler's diarrhoea. However, this limited gut colonization can also be seen as a disadvantage, since it requires multiple dosing. This, together with its less diverse strain library (only a few strains have been studied) and lower use than LAB in food fermentations have impacted its use as a probiotic in humans.

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

This review aims to provide first a comprehensive overview of the genetic engineering tools available for *S. boulardii* and present the latest advancements in its applications to enhance its therapeutic potential. These applications, current limitations and future directions of this technology are also discussed in detail in the second part of the review.

2. Heterologous gene expression systems and metabolic engineering

S. boulardii shares more than 99 % of nucleotide identity along its genome with *S. cerevisiae* (Khatrri et al., 2017) and, even though they have different metabolic and physiological characteristics, they both can produce and secrete heterologous proteins. The capacity of *S. boulardii* to survive and colonize the gut, together with the ability to secrete heterologous proteins, offers a strong potential for *S. boulardii* to deliver molecules of interest to the gut by genetic engineering. There are a few sequenced strains of *S. boulardii* (Table 1) among which, the most used strain is *S. boulardii* ATCC-MYA-796.

Yeast can carry out post-translational modifications that, apart from glycosylation, closely resemble those performed by mammalian cells (Garvey, 2022). This is a major advantage of using *S. boulardii* as a potential delivery vessel over commonly used probiotic bacteria, which are unable to express such complex proteins. In fact, many recombinant proteins such as insulin and the Hepatitis B antigens are produced industrially by *S. cerevisiae* (Nielsen, 2013). Moreover, *S. cerevisiae* and *Pichia pastoris* are widely used expression systems for the development of recombinant vaccines, protozoal proteins and tumour antigens (Tippelt and Nett, 2021), highlighting the potential of engineering probiotic

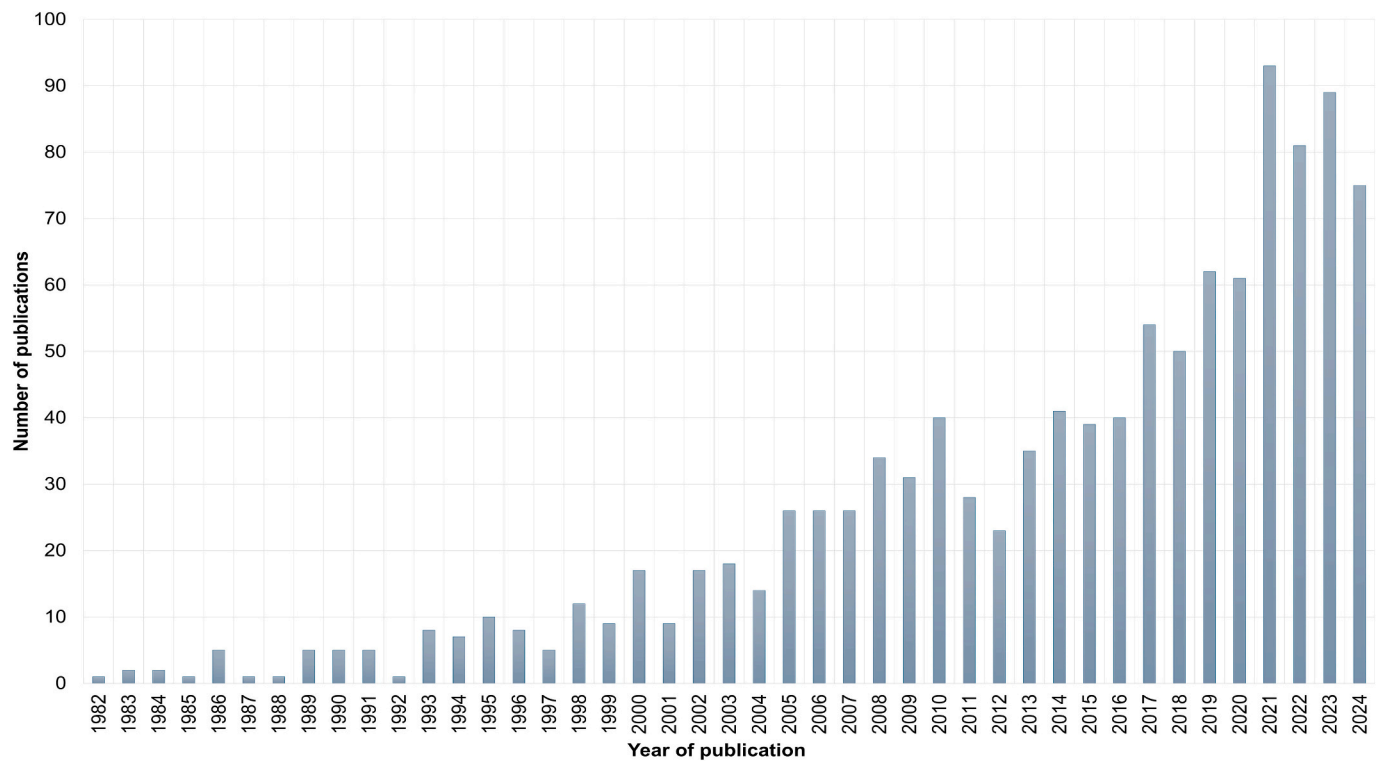


Fig. 1. Number of scientific papers published per year on *Saccharomyces boulardii*, according to PubMed® Total number of publications contemplating *S. boulardii* in the last 42 years (1982–2024). Data were obtained from PubMed® using the queries: “*Saccharomyces boulardii*” and filtered by year (1982 to 2024). <https://pubmed.ncbi.nlm.nih.gov> Accessed in April 2025.

Table 1
Publicly available genome sequences of *S. boulardii* strains.

Strain	GenBank Accession number	Assembly level	Has been genetically engineered?
KCTC 13826BP ^(a)	GCA_026225675.1	Contig	No
BIOCODEX ^(b)	GCA_001298375.2	Chromosome	Yes
ATCC MYA-796	GCA_000769245.1	Chromosome	Yes
ATCC MYA-797	GCA_001625055.1	Scaffold	Yes
lyo (DiarSafe) strain 17	n/a ^(c)	–	Yes
EDRL	GCA_000734875.3	Chromosome	No
kirkman	GCA_000442675.2	Contig	No
unique28	GCA_001545905.1	Contig	No
Unisankyo	GCA_001413975.1	Chromosome	No
CLPY01	GCA_001545975.1	Scaffold	No
	GCA_021216695.1	Scaffold	No

(a)- Reference genome; (b)- This strain is also referred as “CNCM I-745”; (c)- Genome not available.

yeast.

2.1. Plasmid expression systems in *S. Boulardii*

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

With the exception of some studies that employed electroporation, the transformation method used in *S. boulardii* is the PEG/LiAc method

(or some variation) (Gietz and Schiestl, 2007). This method is widely used to transform *S. cerevisiae*. Although this method is proven to work in *S. boulardii*, the transformation efficiency can be up to 100 times lower when compared with *S. cerevisiae* (Chen et al., 2020b). Plasmids, have three major elements that can impact gene expression: the expression cassette, the selective marker and the origin of replication.

The expression cassette is what defines which protein will be over-expressed and the basic structure consists of a promotor, the gene of interest and a terminator (Oliveira et al., 2017).

The selection marker ensures that plasmids are maintained in the cell, otherwise they can be lost after cell replication (Allen et al., 2022). Yeast plasmids normally contain a selective marker for bacteria (for plasmid construction and propagation) and a selective marker for yeast (for transformant selection and plasmid maintenance). This kind of vectors are referred as “shuttle vectors”, as they can replicate in two different host species (Gnügge and Rudolf, 2017) Dominant selective markers like antibiotic resistance genes are widely used but they are not the only type. Auxotrophic markers are also frequently used for selection of positive transformants. These markers eliminate the need of adding extra chemicals to the selective media that can affect cellular function (Pronk, 2002; Ulfstedt et al., 2017). The downside of using this kind of markers is the need of generating auxotrophic strains. This is done by disrupting the function of a gene that is essential for the survival of the microorganism, i.e., enzymes that participate in the synthesis of amino acids or pyrimidines (Pronk, 2002). However, the auxotrophy may impact metabolism, as reported for the uracil mutation ($\Delta ura3$) (Pronk, 2002; Silva et al., 2015). Furthermore, the selection of positive transformants requires the absence of specific nutrients that enable the growth of the mutant strains, thus selecting for the strains that have genetically complemented the mutation.

Regarding types of plasmids and their correspondent replication origins, if the desired objective is to achieve high expression levels, the go-to is a yeast episomal plasmid (YEp) with a 2 μ origin (Lundblad, 1993). This kind of plasmids is also referred to as high-copy number

plasmids since each cell can harbour between 28 and 58 copies (in *S. cerevisiae*) (Karim et al., 2013). However, the metabolic burden can impact cell fitness (Gomes et al., 2018; Rouches et al., 2022; He et al., 2024). Alternatively, there are yeast centromeric plasmids (YCp) whose origin is a combination of an autonomously replicating sequence (ARS) and yeast centromeric sequence (CEN) (Lundblad, 1993). With a CEN/ARS origin, the balance between gene expression and cell growth is maintained, since only 4–8 copies are harboured per cell in *S. cerevisiae* (Karim et al., 2013). There are also yeast-replicating plasmids (YRp) that do not have CEN elements, lacking plasmid stability conferred by CEN, which explains the emergence of YCp (Lundblad, 1993). Finally, yeast-integrating plasmids (YIp) can also be used for heterologous protein expression. In this case, the plasmids must be integrated into a chromosome via homologous recombination since they have no origin of replication (Gnügge and Rudolf, 2017).

There are several examples of heterologous protein expression via plasmid in *S. boulardii* (Table 2). A detailed discussion on the applications of engineered yeast is provided in the second part of the review. The yeast selection markers that are most frequently used are *G418/KanMX* (dominant) and *URA3* (auxotrophic). Plasmid selection markers used for genetic engineering of *S. boulardii* are described in Table 2. Durmusoglu et al. (2021) demonstrated that the choice of the selective marker had an impact in protein expression. They studied the expression of *mRuby* in a 2 μ plasmid and found that the use of auxotrophic markers (*URA3*, *HIS3*, *TRP1*) results in higher expression when comparing with dominant markers (*KanMX*, *ZeoR*, *NatR*, *HygR*) with the 2 μ /*URA3*

pairing achieving the highest overall expression. This study highlights the differences in gene expression resulting from the interaction between origins of replication with auxotrophic and dominant markers (Durmusoglu et al., 2021). The same study also compared the expression using the CEN/ARS origin in the same conditions, demonstrating that the expression was higher when 2 μ plasmids were used. In another study, the expression of Erythromycin destructase (*EreA*) was found to be 17-fold higher when plasmid-mediated, compared to a chromosomal integration. This difference in expression levels was attributed to the high-copy nature (2 μ) of the plasmid used, Table 2 (He et al., 2024).

Apart from the replication origin and the selection marker in a plasmid, the promoter and terminator driving the expression of the gene of interest play a key role in the expression. Durmusoglu et al. (2021), to optimize the expression of *mRuby*, evaluated 18 different *S. cerevisiae* constitutive promoters (combined with the *TDH1* terminator) and 7 terminators (combined with 3 promoters of different strength). Their results show that most of the promoters from *S. cerevisiae* maintain their relative expression levels in *S. boulardii*, and the terminator choice impacts the expression in a promoter-dependent way (Durmusoglu et al., 2021). Previously, several *S. cerevisiae* promoters were confirmed to be present in *S. boulardii* genome with high homology percentages (97–100 % nucleotide identity) indicating that there were probably more examples of promoters shared by the two yeasts (Douradinha et al., 2014). Another example of the use of fluorescent proteins in *S. boulardii* is the expression of *mCherry*, which was used to compare expression levels between a 2 μ plasmids and a single locus integration, indicating that

Table 2
Plasmid expression systems reported in *S. boulardii*.

Study	Strain(s)	Origin(s) of replication	Promotor(s)	Terminator(s)	Marker(s)	Protein(s) expressed
(Pöhlmann et al., 2012)	<i>Perenterol</i> ^(a)	2 μ	<i>GPD</i> ^(b)	<i>ADH I</i>	<i>KanMX (G418)</i>	HCMV IL-10; EBV IL-10
(Michael et al., 2013)	<i>Perenterol</i> ^(a)	2 μ	<i>GPD</i> ^(b)	<i>ADH I</i>	<i>KanMX (G418)</i>	IL-10
(Hamed et al., 2013)	<i>lyo</i> (DiarSafe)	2 μ	Native Promotor	Native Terminator	<i>URA3</i>	Ura3p
(Wang et al., 2014)	<i>CNCM I-745</i>	2 μ	<i>TEF1</i> ; <i>PGK1</i>	n/d ^(c)	<i>KanMX (G418)</i>	EGFP; Eimeria <i>tenella</i> Microneme-2 proteins
(Hudson et al., 2014)	<i>ATCC MYA-797</i>	2 μ	<i>TEF1</i>	n/d ^(c)	<i>URA3</i>	GFP
(Douradinha et al., 2014)	<i>ATCC MYA-796</i>	ARS1	n/d ^(c)	n/d ^(c)	<i>Hygromycin B</i>	Hygromycin B Phosphotransferase
(Liu et al., 2016)	<i>ATCC MYA-796</i>	2 μ	<i>TDH3</i> ^(b)	<i>CYC1</i>	<i>KanMX (G418)</i>	β -galactosidase (<i>lacZ</i>)
(Liu et al., 2016)	<i>ATCC MYA-796Δura3</i>	2 μ	<i>ADH1</i>	<i>ADH1</i>	<i>URA3</i>	<i>mRuby</i>
(Li et al., 2021a)	<i>CNCM I-745</i>	2 μ	<i>TEF1</i>	<i>TPI1</i>	<i>BlastS</i>	Leucocin C
(Liu et al., 2020)	<i>ATCC MYA-796</i>	2 μ	<i>TEF1</i>	n/d ^(c)	<i>Hygromycin B</i>	<i>mCherry</i>
(Chen et al., 2020b)	<i>ATCC MYA-796</i>	2 μ	<i>TEF1</i>	<i>CYC1</i>	<i>URA3</i>	Heavy chain antibodies fusion
(Durmusoglu et al., 2021)	<i>ATCC MYA-796Δura3Δtrp1Δhis3</i>	2 μ ; CEN6/ARS4	18 promoters ^(d)	5 terminators ^(d)	7 markers ^(d)	<i>mRuby</i>
(Durmusoglu et al., 2021)	<i>ATCC MYA-796Δura3</i>	CEN/ARS	9 promoters ^(e)	4 terminators ^(e)	<i>URA3</i>	β -carotene pathway; Violacein pathway
(Jin et al., 2021)	<i>ATCC MYA-796Δura3Δtrp1Δhis3</i>	2 μ	<i>GPD</i> ^(b)	<i>CYC1</i>	<i>URA3</i> ; <i>TRP1</i> ; <i>HIS3</i>	Endo-type β -agarase
(Kim et al., 2022)	<i>ATCC MYA-796Δura3Δleu2Δhis3</i>	2 μ	<i>GPD</i> ^(b)	<i>CYC1</i>	<i>URA3</i> ; <i>LEU2</i> ; <i>HIS3</i>	l-fucose metabolic pathway
(Ling et al., 2023)	<i>CLPY01</i> ^(f)	2 μ	<i>GAL1</i> ; <i>GAL10</i>	<i>CYC1</i> ; <i>ADH1</i>	<i>URA3</i>	Medium chain fatty acids pathway ^(g)
(Nayebhashemi et al., 2023)	<i>CNCM I-745Δura3</i>	2 μ	<i>TEF1</i>	<i>CYC1</i>	<i>URA3</i>	EGFP; Lipase inhibitor peptide 1 and 2
(Durmusoglu et al., 2023)	<i>ATCC MYA-796Δura3</i>	2 μ ; CEN6/ARS4	<i>TDH3</i> ^(b)	<i>TDH1</i>	<i>URA3</i>	<i>C. difficile</i> Toxin A Neutralizing Peptide (NPA)
(He et al., 2024)	<i>ATCC MYA-796Δura3</i>	2 μ	<i>TEF1</i>	n/d ^(c)	<i>URA3</i>	<i>Erythromycin destructase</i> (<i>ereA</i>)
(Rebeck et al., 2025)	<i>ATCC MYA-797</i>	2 μ	<i>TEF1</i>	<i>TPI1</i>	<i>BlastS</i>	haPD-1; rPD-L1nb; Gaussia luciferase
(Xu et al., 2025)	<i>ATCC MYA-797Δura3</i>	2 μ	<i>TDH3</i> ^(b)	<i>CYC1</i>	<i>URA3</i>	yeGFP; <i>Aspergillus aculeatus</i> β -glucosidase

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

only 8.6 % cells carrying the 2 μ plasmids expressed *mCherry*, versus 97.3 % for the single integration (Liu et al., 2020). Some strains of *S. boulardii* have shown the presence of the native 2 μ plasmid with high similarity to the one found in *S. cerevisiae* (Khatiri et al., 2017). This plasmid harbours 4 genes (*FLP*, *REP1*, *REP2* and *RAF1*) that allows self-replication and maintenance through generations. This makes it a selfish genetic element, as its replication is independent of the host yeast cells. Typically, this plasmid is maintained at a high copy number with chromosome like stability and has shown to have some impact in cell fitness (Harrison et al., 2012). Curing the native 2 μ plasmid can improve stability of subsequent transformation with plasmids containing the 2 μ origin (Erhart and Hollenberg, 1981). Conversely, in *S. cerevisiae*, the endogenous 2 μ plasmid has been directly edited to express the dihydroartemisinic acid metabolic pathway and the *TPI1* gene (Zeng et al., 2021). Thus, an auxotroph for *TPI1* was used, showing increased production of dihydroartemisinic acid and higher plasmid stability in a culture with no selective pressure. Curing the endogenous plasmid or using it for genetic engineering have not been explored in *S. boulardii* but it might open new possibilities for genetic engineering in the probiotic yeast.

Beyond being used to quantify expression, fluorescent proteins have a key role in molecular biology since they are reporters for protein localization (Phillips, 2001). The fluorescent protein *mRuby* was also expressed in *S. boulardii* using a plasmid that harboured a mitochondrial targeting sequence. Despite being originally designed for *S. cerevisiae*, the results show that it can also be used in *S. boulardii* (Liu et al., 2016). Green fluorescent protein (GFP) was successfully expressed using *S. boulardii*, delivering it to the immune tissue of the GI tract of mice (Peyer's patches) proving that this yeast can possibly deliver molecules of interest to the gut (Hudson et al., 2014). In *S. boulardii* enhanced GFP (EGFP) was also used as a proof of concept for yeast surface display, again demonstrating the viability this technology in *S. boulardii*, before going on to express other recombinant proteins (Nayebhashemi et al., 2023; Wang et al., 2014). Other reporter genes like *lacZ* or resistance markers (Ura3p and Hygromycin B), have also been used in *S. boulardii* (Table 2) (Douradinha et al., 2014; Hamed et al., 2013; Liu et al., 2016).

2.2. Conventional genome engineering in *S. Boulardii*

Although plasmid expression can be used for achieving high levels of expression, there are some drawbacks associated with these expression systems. Plasmids are not suitable for all applications due to high metabolic burden (associated with high copy number plasmids), plasmid instability without selective pressure and possible antibiotic resistance transmission (Allen et al., 2022; Gomes et al., 2018; Rouches et al., 2022; Svara and Rankin, 2011; Wang et al., 2024). Gene integration methods like YIp, Cre-loxP, Ty (transposons of yeast) elements or the PiggyBac transposon system, offer a stable genome integration without the drawbacks associated with plasmids expression systems. In this section, examples of the application of these engineering strategies in *S. boulardii* will be discussed (Table 3).

As referenced above, YIp vectors lack an origin of replication and rely on integration into the genome via homologous recombination to be stably maintained in recipient cells. Table 3 provides examples of YIp modification in *S. boulardii*. An example of the use of YIp's in *S. boulardii* was the expression of the fusion protein formed by the chicken ovalbumin and *C. perfringens* enterotoxin. Interestingly, authors found that *GAL-1* (one of the 3 promoters tested, Table 3) was not able to drive the expression of this fusion protein, due to the inability of *S. boulardii* to utilize galactose (Bagherpour et al., 2018). Another example of YIp gene integration in *S. boulardii* is the expression of the monomeric streptavidin (mSA) (Heavey et al., 2024).

Not only single expression cassettes, but also metabolic pathways have been introduced to *S. boulardii* via integrative plasmids. The xylose pathway (*XYL1*, *XYL2*, and *XYL3* from *Scheffersomyces stipitis*) was introduced in the *URA3* locus. When compared to *S. cerevisiae* with the same xylose fermentation ability, the modified probiotic yeast achieved higher rates of xylose fermentation (Liu et al., 2016). One other strategy for chromosomal integration of a pathway was used by Ling et al. (2023), where two copies of a medium chain fatty acid pathway were integrated into the *URA3* locus. Due to its large size, this pathway was subdivided in three fragments and assembled using the DNA assembler method (Shao et al., 2009). The 5'-end of the first fragment had a homology region to the targeted locus, while the 3'-end had a homology region that overlapped with the second fragment 5'-end. The third

Table 3

YIp, Cre-loxP and Ty elements integration/gene disruption strategies used in *S. boulardii*. Chr stands for chromosome.

Study	Method	Strain	Chr	Target locus/ Ty element	Promotor	Terminator	Protein(s) expressed/Gene disrupted
(Liu et al., 2016)	YIp	ATCC MYA-796 Δ ura3	V	YEL021W	TDH3; PGK1 PGK1	TDH3; PGK1	Xylose metabolic pathway
(Bagherpour et al., 2018)	YIp	CNCM I-745 Δ ura3 (a)	n/ d (b)	n/d (b)	GAL-1; TEF1	CYC1	Chicken ovalbumin - <i>C. perfringens</i> enterotoxin
(Liu et al., 2020)	YIp	ATCCMYA-796	XII	XII-1; XII-2 XII-4	PGK1; TEF1	n/d (b)	<i>mCherry</i> ; EGFP
(Hedin et al., 2022)	YIp	ATCC MYA-796	X	X-2	TEF1	CYC1	GFP
(Heavey et al., 2024)	YIp	ATCC MYA-796 Δ ura3	n/ d (b)	n/d (b)	TDH3; TPI1	MATa	mSA-AGA1-AGA2
(Hedin et al., 2024)	YIp	ATCC MYA-796	X	X-2	TEF1	CYC1	GFP
(Ling et al., 2023)	YIp	CLPY01 Δ ura3 (c)	V	YEL021W	TDH3; TEF1	CYC1; ADH1	Medium chain fatty acids pathway (d)
(Wang et al., 2015)	Cre-loxP	CNCM I-745 (a)	V	YEL021W	–	–	URA3
(Chen et al., 2020a)	Cre-loxP	CNCM I-745 (a)	V	YEL021W	–	–	URA3
(Dacquay et al., 2024)	Cre-loxP	CNCM I-745 (a)	XV	YOR128C	–	–	ADE2
(Liu et al., 2020)	Ty elements	ATCC MYA-796	–	Ty1.1; Ty1.2; Ty2; Ty3	TEF1	n/d (b)	IL-10; TNFR1-ECD; AP; ANP
(Mohaqiq et al., 2025; Parwez et al., 2025)	Ty elements	ATCC MYA-796 Δ oaz1	–	Ty2	TDH3	CYC1	Overexpression of Spermidine pathway related genes
(Tang et al., 2024b)	PiggyBac Transposon	BIOCODEX Δ ura3 Δ trp1 (a)	–	–	TEF2	n/d (b)	EGFP; l-Ergothioneine pathway

(a)- CNCM I-745 and BIOCODEX are different nomenclatures for the same strain; (b)- Not disclosed (n/d): Information was not available; (c) The strain used was isolated from a commercially available product from Jarrow Formulas and referred as CLPY01 in the study; (d) The Medium chain fatty acids pathway was constituted by the following genes: mutant gene of *Homo sapiens* fatty acid synthase with its native thioesterase domain replaced by the *Rattus norvegicus* thioesterase II gene (mhFAS-rTEII) and *H. sapiens* phosphopantetheinyl transferase gene (hSFP).

fragment was designed to have a 5'-end that overlapped with the 3'-end of the second fragment and a 3'-end that was homologous to the targeted locus. This allowed for the pathway to be assembled in the right order and to be integrated into the targeted locus (Ling et al., 2023). This strategy has the potential to be utilized for assembly of more than three expression cassettes, provided that each fragment has ends that overlap the two flanking expression cassettes and that the first and last cassette have homology for the targeted locus (Shao et al., 2009).

Cre-loxP is another method for gene integration or disruption and it is based on the recognition and binding of specific loxP sites by the Cre Recombinase from the *E. coli* bacteriophage P1. These sites are 34 base pairs (bp) long in which two 13 bp palindromic sequences are separated by 8 bp asymmetric core sequence. Depending on the orientation of the loxP sites, Cre Recombinase can induce a deletion, inversion or translocation. The loxP sites can be added to the genome by flanking them with homologous sequences that will be integrated by homologous recombination. After that, Cre Recombinase is transformed into the cells and the recombination of loxP site occurs. Everything in between the loxP sites is excised from the genome (Gueldener et al., 2002; Güldener et al., 1996; Sengupta et al., 2017; Sternberg and Hoess, 1983). Thus, this system allows for a more targeted engineering approach compared to Yips, as only the intended modification remains in the genome after the selective marker is excised. An added advantage is the ability to perform multiple gene disruptions (Ribeiro et al., 2007) and/or integrations. However, one loxP sequence remains in the genome as a result.

Although gene integration is also possible by using Cre-loxP, in *S. boulardii* this system was used for gene disruption, generating *URA3* auxotrophic strains (Chen et al., 2020a; Wang et al., 2015) as well as disrupting the *ADE2* gene (Table 3). The disruption of this gene makes yeast colonies turn red instead of white and it was used to construct a strain capable of sensing doxycycline, as discussed in section 1.3.8. (Dacquay et al., 2024).

Genetic engineering tools can also derive from innate mechanisms rather than being adapted from other microorganisms. Ty is a type of Class I elements that exist in substantial amount throughout the eukaryotic genome. Ty elements are mobile and can contribute for chromosomal rearrangement, gene duplication or variations in genome size (Dujon, 2006; Feschotte and Pritham, 2007; Maury et al., 2016). The transposition mechanism of Class I elements is known as copy-paste (Wicker et al., 2007). The corresponding mRNA is reverse transcribed back to DNA before transposing in another locus of the genome (Finnegan, 1989). There are 5 sub-families of known Ty elements (Ty1 to Ty5) in *S. cerevisiae* (Carr et al., 2012), and *S. boulardii* shares some of them (Liu et al., 2020). When comparing the consensus Ty elements utilized in the EasyCloneMulti vectors (Maury et al., 2016) and the genome of *S. boulardii* MYA-796, Liu et al. (2020) reported that the Ty elements were highly conserved in *S. boulardii*, finding 140 Ty elements distributed across the Ty1.1, Ty1.2, Ty2, and Ty3 sub-families.

Genome integration using Ty elements allows for multiple copy integration achieving enzymatic activity levels comparable to the ones attained with plasmids (Oliveira et al., 2007). In *S. boulardii*, Ty elements were used to express Interleukin-10 (IL-10), tumour necrosis factor receptor 1 extracellular domain, alkaline phosphatase, and atrial natriuretic peptide (ANP) (Liu et al., 2020). The authors developed this method due to the low efficiency achieved using single-locus integration in *S. boulardii* ATCC MYA-796; which is a common problem encountered and reported for *S. boulardii* in comparison to *S. cerevisiae* (Douradinha et al., 2014; Liu et al., 2020). Using Ty elements, Liu et al. (2020) improved the number of colonies recovered after transformations and show an enhanced expression of EGFP. However, the number of copies integrated was not determined in that study.

Other transposons-based strategies have also been implemented in *S. boulardii* namely, to produce l-Ergothioneine (Tang et al., 2024b). The system used was the PiggyBac transposon, which allows for a stable genome integration with the possibility of footprint-free excision. This

system relies on the enzyme PB transposase, which recognises transposon-specific inverted terminal repeat sequences located on both ends of the transposon vector. Then, the fragment is moved from the vector and integrated in TTAA chromosomal sites. This mechanism is referred as “cut and paste” and, after the integration, the fragment can be precisely excised from the chromosome, with the gap being repaired by ligation. This footprint-free excision is valuable due to the reversibility of the genetic modifications performed with this system (Chen et al., 2020a, 2020b; Zhao et al., 2016).

2.3. CRISPR-CAS genome editing strategies in *S. Boulardii*

CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated endonuclease 9) is a genetic engineering tool that, in the last few years, has been exhaustively studied and developed. Like Cre-loxP, CRISPR-Cas systems are based on mechanisms observed in bacteriophages that were adapted for genetic engineering. CRISPR is the bacterial equivalent of the human immune system, providing defence against phage infection and plasmid transfer (Mojica et al., 2005; Pickar-Oliver and Gersbach, 2019).

This tool is based on a double-strand break performed by the nuclease Cas9, which is guided to a specific location of the genome by a guide RNA (gRNA). The gRNA is a 20-base pair sequence that hybridizes next to a Protospacer Adjacent Motif (PAM) sequence (Karvelis et al., 2013). The specificity of this method relies on the fact that the DNA cleavage is strictly dependent on the presence of a PAM immediately downstream of the target site and, the identity between gRNA and the DNA of the targeted site, must be near perfect (Anders et al., 2016). The recognized PAM sequence depends on the origin of the nuclease. *Streptococcus pyogenes* Cas9 (SpCas9) is the most used endonuclease in CRISPR-Cas systems. The preferred PAM for this endonuclease is 5'-NGG-3' (N = A, T, C, G), which is common in most of the organisms resulting in easier application of the CRISPR-Cas9 tool (Goldberg et al., 2021; Li et al., 2023). After this cut, negative selection takes place: only the yeast able to repair the nuclease cut survives. For this repair to be successful, using homologous recombination, two homologous DNA sequence flanking of the cut site must be given, one upstream and one downstream. Between these homologous regions, the desired DNA sequence is inserted, allowing it to be integrated precisely into the chromosome at the target location.

CRISPR-Cas systems offer several advantages over other integration methods. Using Cre-loxP, along with the integration of the gene of interest, extra genetic material will also be integrated into the genome because one loxP site is always left behind (Gueldener et al., 2002; Güldener et al., 1996). Multiple integrations in the genome using this tool can be harmful as each modification accumulates more undesired scars in the genome. In addition, when integrating genes with Cre-loxP, there is a need to integrate a selective marker in the genome for selection that will be excised after (Gueldener et al., 2002). CRISPR-Cas is a marker-less tool that allows multiple integrations and eliminates the need for integrating selective markers. In addition to its significance in the development of industrial bio-based production processes (Costa et al., 2022), this is also a crucial point for health applications as antibiotic-selective markers are not accepted in clinical trials due to the possibility of spreading the resistance to other microorganisms in the gut microbiome (Vandermeulen et al., 2011). One of the most used toolkits for CRISPR-Cas9 editing is the EasyClone-MarkerFree (Jessop-Fabre et al., 2016; Stovicek et al., 2015), which despite being designed specifically for *S. cerevisiae*, has been used to effectively edit some of the available chromosomal sites (Hedin et al., 2023a; Sands et al., 2024; Gelli et al., 2025). These sites must share identical nucleotide bases in the cut site/PAM sequence between both yeasts. The lower efficiency of *S. boulardii* transformation using this method (Liu et al., 2020) comparing to *S. cerevisiae*, could be related to the higher thickness of the probiotic cell-wall or to the double-strand break repair mechanisms not being as efficient.

Durmusoglu et al. (2021), used *mTurquoise2* and *mRuby2* to compare the expression in different sites of integration and to compare different methods of integration (Table 4). Although, the expression locus showed to have influence (up to 3.4-fold difference) in the expression it had a minor effect when compared with promoter choice (up to 15.3-fold difference). To compare different integration methods, two different Cas endonucleases were used (SpCas9 and LbCas12a) as well as a method without the assistance of the cut performed by the endonuclease. LbCas12a from *Lachnospiraceae bacterium ND2006* (PAM- 5' TTTN-3' (N = A, C, G)) was the most efficient followed by SpCas9 and unassisted DNA (Durmusoglu et al., 2021).

To expand the genetic engineering tools available for *S. boulardii*, in addition to identifying optimal integration sites, it is also essential to characterize promoter strength. Recently, Sands et al. (2024) utilized CRISPR-Cas engineering to express yeast-enhanced green fluorescent protein (yEGFP) and *mKate2* for promoter characterization. The 12 promoters were characterized in media supplemented with different carbon sources (glucose, acetate, fructose, sucrose and inulin) under aerobic and anaerobic conditions. *In vivo* promoter characterization was also performed and showed high correlation with *in vitro* activity (Sands et al., 2024). yEGFP genomic integration was also used as reporter for monitoring rtTA expression. This integration is part of the previously

Table 4
CRISPR-Cas systems used in *S. boulardii*. Chr stands for chromosome.

Study	Strain	Chr	Locus	Promotor	Terminator	Protein (s) expressed/ targeted gene (s)
(Liu et al., 2016)	ATCC MYA-796	–	–	–	–	URA3; HIS3; TRP1; LEU2 (Gene disruption)
(Liu et al., 2016)	ATCC MYA-796	XVI	CS8	TDH3	CYC1	Human lysozyme (Protein expression)
(Liu et al., 2018)	ATCC MYA-796	–	–	–	–	PGM2 (Mutation correction)
(Durmusoglu et al., 2021)	ATCC MYA-796 <i>Δura3</i>	V; XI; XVI; VIII; XV	INT1; INT2; INT3; INT4; INT5	TDH3	TDH1	<i>mTurquoise2</i> ; <i>mRuby2</i> (Protein expression)
(Jin et al., 2021)	ATCC MYA-796	XV	CS5	TDH3	CYC1	BpGH16A (Protein expression)
(Jin et al., 2021)	ATCC MYA-796 <i>Δura3Δtrp1</i>	–	–	–	–	HIS3 (Gene disruption)
(Kwan et al., 2022)	ATCC MYA-796	–	–	–	–	PFK26; PFK27; GDA1 (Gene disruption)
(Hedin et al., 2022)	ATCC MYA-796	–	–	–	–	URA3 (Gene disruption)
(Durmusoglu et al., 2023)	ATCC MYA-796 <i>Δura3</i>	V	INT1	TDH3	TDH1	NPA (Protein expression)
(Durmusoglu et al., 2023)	ATCC MYA-796 <i>Δura3</i>	–	–	–	–	KO of 14 genes involved in protein secretion pathway ^(f)
(Kim et al., 2023)	ATCC MYA-796	VII; XII; XVI	CS6; CS7 CS8	TDH3	CYC1	Human lysozyme (Protein expression)
(Hedin et al., 2023a)	ATCC MYA-796 <i>Δura3</i>	X; XI XII	X-2; X-4 XI-3; XII-5	TDH3	DIT1** ^(e)	GLP-1; Exendin-4 (Protein expression)
(Hedin et al., 2023b)	ATCCMYA-796 <i>Δura3</i>	–	–	–	–	KO of 7 auxotrophic genes and 2 genes involved in temperature resistance ^(g)
(Park et al., 2023)	ATCC MYA-796	VII	CS6	PGK	CYC1	Firefly luciferase (Protein expression)
(Sands et al., 2024)	ATCC MYA-796 <i>Δura3</i>	XI; XII	XI-3; XII-5	12 promoters ^(c)	ADH1	yEGFP; mKate2 (Protein expression)
(Durmusoglu et al., 2024)	ATCC MYA-796	–	–	–	–	URA3; HIS3 (Gene disruption)
(Durmusoglu et al., 2024)	ATCC MYA-796 <i>Δura3Δhis3</i>	–	–	–	–	PGM2 (Mutation correction)
(Durmusoglu et al., 2024)	<i>SbGal</i> ^(a)	XV	INT5	GAL1; TET	n/d ^(c)	NanoLuc (Protein expression)
(Dacquay et al., 2024)	CNCM I-745	n/d ^(c)	n/d ^(c)	TET4; TDH3 MYO2	ADH1	yEGFP; rtTA (Protein expression)
(Van Gaever et al., 2024)	<i>Enterol</i> ; <i>Sb-P</i> ^(b)	II; IV; XII; XVI	II-1; IV-1 XII-1; XVI-1	TDH3; TEF1; RPL18B; SAC6	ENO2; CYC; ADH1	Abscisic acid pathway related genes
(Shin et al., 2025)	ATCC MYA-796	VII	<i>int#1</i>	CCW12	CYC1	EGFP; Endo-inulinase (Protein Expression)
(Mohaqiq et al., 2025; Parweez et al., 2025)	ATCC MYA-796	–	–	–	–	OAZ1 (Gene disruption)
(Gelli et al., 2025)	ATCC MYA-796	XI; XII	XI-3; XII-5	TDH3	DIT1** ^(e)	Production of 4 cell penetrating peptides ^(h)
(Xu et al., 2025)	ATCC MYA-797	–	–	–	–	URA3; CCW12; CCW14; FYV5 (Gene disruption)
(Xu et al., 2025)	ATCC MYA-797 <i>Δura3Δccw12Δccw14Δfyv5</i>	XV	<i>int#11</i>	TDH3	CYC1	yeGFP; <i>Aspergillus aculeatus</i> β-glucosidase (Protein expression)

(a) Strain description- *S. boulardii* ATCC-MYA796*Δura3Δhis3* with PGM2 mutation corrected; **(b)- Enterol-** Commercially available probiotic product which contains the CNCM I-745 strain; **Sb-P-** Natural isolate that accumulates elevated levels of acetic acid in YPD 2 %, sensitive to low pH. (de Carvalho et al., 2024); **(c)- Not disclosed (n/d):** Information was not available; **(d)- Promoters:** ALD6; CYC1; CYC7; DAN1; HSP26; HXT7; JEN1; SSA1; SUC2; TDH3; TEF1 and TPI1; **(e)- Modified version of the DIT1 terminator:** DIT1^(32-34delTGT+74-76delCAT); **(f)- Genes KO:** ROX1; PAH1; DER1; HRD1; GOS1; OCH1; MNN9; VPS5; VPS17; TDA3; YPS1; PRB1; PEP4 and APE1; **(g)- Genes KO:** URA3; HIS3; TRP1; SNO1; SNZ1; THI2; THI6 (auxotrophic) and REI1; BTS1 (temperature resistance); **(h)- Cell penetrating peptides produced:** RRL helix; Shuffle; Penetramax and PN159.

mentioned doxycycline biosensing system. Two promoters (*TDH3* and *MYO2*) were tested and, although *MYO2* achieved lower expression levels when compared with *TDH3*, it was chosen to drive rtTA expression due to lower levels of false positives (i.e. red yeast when doxycycline was not present) (Dacquay et al., 2024). Another biosensing yeast was developed to detect ATP (Adenosine triphosphate) levels in mice with DSS-induced colitis using CRISPR-Cas9 to introduce the Firefly Luciferase expression cassette into an intergenic region between *HIP1* and a gene coding for tRNA in chromosome VII of *S. boulardii* (Park et al., 2023). Lastly, Kim et al., 2023 used a double integration strategy to enhance expression of human lysozyme with a chicken lysozyme secretion signal in *S. boulardii* (Kim et al., 2023). A previous study also expressed this protein but only a single copy was integrated (Liu et al., 2016).

CRISPR-Cas is a versatile tool that can be used with other genetic integration tools like Cre-loxP (Dacquay et al., 2024) but integration is not the only utility for this tool. Previously, the generation of *S. boulardii* auxotrophic strains has been done via UV mutagenesis (Hamed et al., 2013; Hudson et al., 2014) and Cre-loxP (Table 3). However, effective UV mutagenesis can induce undesired mutations (Pfeifer, 2020) and Cre-loxP is not a marker-less tool (Guedener et al., 2002; Güldener et al., 1996). On the contrary, CRISPR-Cas9 can be used for generating auxotrophic strains by only disrupting the function of a specific gene while not leaving scars in the genome. In *S. boulardii*, several auxotrophic strains have been generated by CRISPR-Cas some of them being auxotroph for multiple compounds (Table 4).

Auxotrophic *S. boulardii* strains developed by CRISPR-Cas9 have been used to develop a biocontainment strategy so that the engineered strain would not be able to proliferate in the environment after being excreted. In this regard, 7 auxotroph genes (*URA3*, *HIS3*, *TRP1*, *SNO1*, *SNZ1*, *THI2* and *THI6*) and 2 genes related to temperature tolerance (*REI1* and *BTS1*) were disrupted. The knockout (KO) of *SNO1* and *SNZ1* showed no significant growth defect in media lacking the correspondent nutrients. The simultaneous KO of *URA3*, *THI6* and *BTS1* resulted in a strain that had a growth defect at temperatures lower than 20 °C and in media without uracil and thiamine. This strain could also produce a glucagon-like peptide-1 receptor agonist Exendin-4 at similar levels compared to *S. boulardii* uracil auxotroph meaning that the biocontainment strategy did not impact the target expression (Hedin et al., 2023b).

Gene KO can also be useful to improve the expression of a targeted molecule. In *S. boulardii*, CRISPR-Cas9 was used for the knockout of 14 different proteins involved in secretory pathways. The results showed that KO of some of the genes responsible for vacuolar /extracellular degradation (*YPS1*, *PRB1* and *PEP4*) had the most positive impact on expression of peptide NPA, Table 4. The combination of the KO of *YPS1*, *PRB1*, *PEP4* and *APE1* resulted in a 1004 % increased production of NPA when compared to the strain without these genes disrupted. These tests were done using a 2 µ plasmid expression system but, when combining the quadruple KO strain with NPA genome integration, the expression increased by 13.2-fold when compared with a strain with only NPA integrated (Durmusoglu et al., 2023).

CRISPR-Cas9 can also be used for correction of mutations as demonstrated by the correction of the *PGM2* mutation in *S. boulardii* (Durmusoglu et al., 2024; Liu et al., 2018). This gene is an example of the differences between *S. cerevisiae* and the probiotic yeast. In the latter, *PGM2* transcription is terminated earlier resulting in a truncation that negatively impacts galactose metabolism. In *S. cerevisiae*, the galactose metabolism is more efficient as the *PGM2* is correctly terminated. The truncation of *PGM2* was firstly reported by Liu et al. (2018) and the correction of the mutation resulted in a *S. boulardii* able to utilize galactose (Liu et al., 2018). Durmusoglu et al. (2024), went further and on top of the correction of the *PGM2* mutation, tested several inducible promoters including *GAL*, that was previously reported not to drive any protein expression in the probiotic yeast (Bagherpour et al., 2018). The *GAL1* promoter was able to direct expression in *S. boulardii* with a

corrected *PGM2* and was then used for the surface display of SA1 (protein that binds and inhibits the activity of toxin A from *C. difficile*). Besides, it was also used to control expression of nanoluciferase and to build a genetic logic gate where this protein would only be produced when galactose and anhydrotetracycline were simultaneously present (Durmusoglu et al., 2024).

2.4. Applications of genetically engineered *S. Boulardii*

Regardless of the genetic engineering strategy, improving the probiotic effects of *S. boulardii*, toward a health-related application has been the end goal. The studies that take on a more methodological/characterization approach are essential to better understand how genetic engineering tools can be applied to enhance *S. boulardii* probiotic potential. From the 38 studies related to *S. boulardii* genetic engineering discussed, 29 are directly focused on engineering a probiotic aimed to further improve human health. The applications can be sub-divided in immune system response, pathogen neutralization, retention time improvement, anti-obesity effects, vitamin precursor production, biocontainment, use of alternative carbon sources, use as biosensor and others (Fig. 2). Enhancing the capacity of *S. boulardii* to use alternative carbon sources is the subcategory that attracted more focus, followed by the potentiation of its neutralizing ability by secreting antimicrobial proteins, and the enhancement of the gut residence time.

2.4.1. Immune system response

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are chronic, relapsing inflammatory disorders of the gastrointestinal tract characterized by disturbances in the balance between anti- and pro-inflammatory cytokines in the gut mucosa (Neurath, 2024), epithelial barrier disfunction and altered microbiota composition (Andoh and Nishida, 2023).

Since *S. boulardii* offers a strong potential as a drug delivery vessel, some of the proteins expressed in *S. boulardii* are related to improvement of human health, targeting the immune system response. The first health-oriented heterologous protein expressed by *S. boulardii*, was the Interleukin 10 (IL-10), an anti-inflammatory cytokine that has a role in maintaining the immune homeostasis in the gut. However, mice treated with the modified strain did not show significant difference compared to mice treated with the wild-type strain (Michael et al., 2013) which authors hypothesized could be due to the denaturation of the dimer forming an active protein (Pöhlmann et al., 2012). *S. boulardii* was also engineered to secrete homologues to IL-10 encoded in herpes viruses such as Epstein-Barr virus and human cytomegalovirus, which showed to be active *in vitro* (Pöhlmann et al., 2012).

In fact, wild-type *S. boulardii* has been shown to induce IL-10

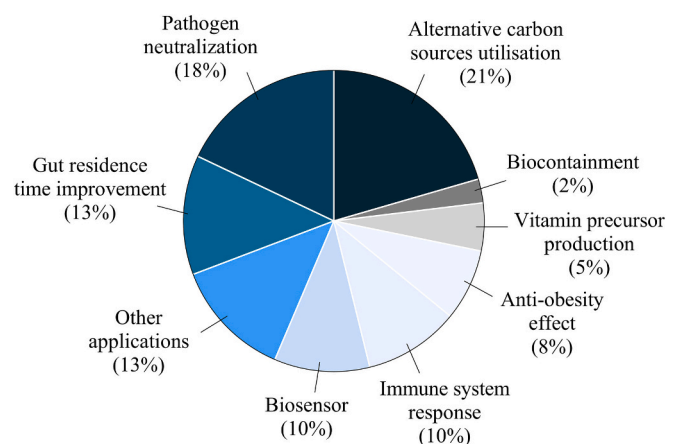


Fig. 2. Overview from the health-related applications of genetically engineered *S. boulardii* (38 studies total published between the years 2013 and 2025).

production upon co-incubation with murine bone marrow-derived dendritic cells comparing to other non-probiotic *S. cerevisiae* (Heavey et al., 2024) and has also been shown to have a superior immune-activation effect, attributed to the thicker mannan layer of the probiotic yeast (Baek et al., 2024). Engineering *S. boulardii* to display monomeric streptavidin on its cell surface, binding biotinylated antibodies against fibronectin, led to a ~ 1000-fold increase in the colonic expression of IL-10, in preclinical models of ulcerative colitis in female mice, compared to the non-treated mice (Heavey et al., 2024).

The administration of *S. boulardii* can also contribute to increase levels of secretory IgA and serum IgG, both playing a key role in immune system regulation (Charles et al., 2001). Administration of the modified *S. boulardii* strain expressing a fusion protein composed of chicken ovalbumin and *C. perfringens* enterotoxin to mice resulted in an enhanced immune response compared to the wild-type strain, as evidenced by elevated levels of IgA and IgG (Bagherpour et al., 2018).

The engineering of *S. boulardii* to modulate immune responses can have direct implications for IBD treatment. Local delivery of immunomodulatory proteins such as IL-10 or surface-displayed antibodies targeting fibronectin or extracellular matrix components, as shown by Heavey et al. (2024), may enhance mucosal healing and reduce inflammation on top of the possible protective probiotic effects of the yeast.

2.4.2. Pathogen neutralization

The yeast *S. boulardii* has been reported to exert antimicrobial activity against pathogens, which has been linked for some strains to the production of acetic acid (de Carvalho et al., 2024; Offei et al., 2019), the secretion of peptides blocking the action of toxin A and B from *C. difficile* (Castagliuolo et al., 1999), of cholera toxin (Czerucka et al., 1994) and dephosphorylating *E. coli* endotoxin (Buts et al., 2006). However, some of the exact mechanisms in which *S. boulardii* neutralizes pathogens remain unknown. In contrast, genetic engineering has opened many ways to rationally improve its antimicrobial capacity.

Clostridioides difficile infection (CDI)—a leading cause of antibiotic-associated diarrhoea and colitis which can lead to death. In fact, patients already suffering IBD are at higher risk of *C. difficile* colonization (Bassotti et al., 2023). CDI is one of the most prevalent nosocomial infections and its incidence has increased globally across all age groups (Xia et al., 2025). In this context, engineered *S. boulardii* strains producing antimicrobial peptides or neutralizing antibodies can play an important role and improve clinical outcomes against CDI by different strategies. To improve the ability of *S. boulardii* to reduce this pathogen and thus improve therapeutic responses, one of the strategies used was to engineer it to secrete a tetra specific heavy chain antibodies fusion that consisted in four toxin neutralizing antibodies. Oral administration of the modified *S. boulardii* in infected mice showed therapeutic efficacy (K. Chen et al., 2020). Another strategy used to enhance the ability of *S. boulardii* to alleviate symptoms of infection with *C. difficile* was to engineer the probiotic yeast to secrete *C. difficile* toxin A neutralizing peptide (Durmusoglu et al., 2023). This peptide inhibits the glucosyl-transferase of *C. difficile* toxin A, a major factor for the pathogenicity of this microorganism (Xiao et al., 2022).

Some of the *S. boulardii* probiotic properties such as pathogen binding and immunomodulatory action have been attributed to the oligosaccharides present in its cell wall ((Pais et al., 2020, Baek et al., 2024). Therefore, a strategy involving cell wall oligosaccharides modulation was implemented by Kwan et al. (2022). In this study, the glycolysis metabolic flux was directed to the production of guanosine diphosphate-mannose and uridine diphosphate-glucose (precursors of mannan and glucan, respectively). The strains with higher mannan showed better adhesion to *Salmonella enterica*, *Typhimurium* and enhanced *Bacteroides thetaiotaomicron* (one of the most abundant bacteria in human gut microbiota) competitiveness in co-culture with *C. difficile* (Kwan et.; al 2022). Further *in vivo* tests could be useful for a better understanding of the underlying mechanisms of this interactions

in a context that better resembles the gut microbiome.

In addition of nosocomial pathogens, others can cause GI disorders and there is need of developing new therapeutic approaches to reduce their impact in human health. Listeriosis is a foodborne disease that is caused by the pathogen *L. monocytogenes*. Upon survival of digestion, *L. monocytogenes* can cross the GI barrier toward the bloodstream, initiating its infection cycle (Luque-Sastre et al., 2018). *Listeria* DNA has been found in human fecal samples, and its abundance has been correlated with a low α -diversity in the gut microbiota, highlighting the importance of the gut microbiome for protection (Hafner et al., 2021). In fact, antibiotic treatment has been shown to decrease α -diversity in the gut microbiota (Huang et al., 2022), which makes the use of enhanced engineered versions of *S. boulardii* a promising approach. In this regard, *S. boulardii* was modified to produce the bacteriocin Leucocin C, showing antimicrobial activity against *L. monocytogenes in vitro*, without the need for antibiotic selective pressure (Li et al., 2021a). This strain was further optimized and used to produce antimicrobial beer, which achieved a stable anti-*Listeria* activity that was maintained for over 6 weeks in refrigeration. While further studies are required, these studies suggest a potential use as functional food, for the treatment of listeriosis (Li et al., 2021a, 2021b).

While the peptides utilized so far to engineer *S. boulardii* are somewhat specific against different pathogens of interest, it is important to consider that the administration of a recombinant *S. boulardii* may have different gut microbiome modulation when compared with the unmodified strain, although not necessarily negative, the different modulation capacities of the developed strains are a subject that needs to be explored to ensure that the modified probiotics do not have undesired side effects.

Another antimicrobial protein expressed by *S. boulardii* was human lysozyme. *In vivo*, the authors evaluated the impact of this engineered probiotic upon sequential dosing in healthy mice. A different gut microbiome modulation was reported: while wild-type *S. boulardii* increased the diversity of the bacterial gut microbiome, the lysozyme-producing *S. boulardii* decreased the levels of *Lactobacillus*, *Blautia*, and *Clostridium*, sensitive to the enzyme (Kim et al., 2023). Recently, *S. boulardii* was engineered to further increase the levels of acetic acid produced, leveraging its antimicrobial potential for broad-spectrum applications (de Carvalho et al., 2024). Another study evaluated the antimicrobial effects of medium chain fatty acids against *Candida albicans*, an opportunistic pathogen whose infections are rising (Ling et al., 2023). *S. boulardii* was engineered for the secretion of medium chain fatty acids which showed anti-biofilm and anti-hyphal formation effects and decreased the expression of virulence-related genes in *C. albicans*. Moreover, the engineered *S. boulardii* showed immunomodulatory properties in a co-culture of *C. albicans* and CACO-2 cells, explained by an upregulation of genes responsible for immune response (*IL-6*, *IL-8*, *CCL20* and *HBD-3*) (Ling et al., 2023). Thus, the modified strain showed great potential for the treatment of *C. albicans* and further *in vivo* tests to validate those findings would be of great value. These broad-spectrum strategies can be useful for certain applications as mentioned, but their impact on the gut microbiome and how this would then impact the overall host health should be carefully considered.

2.4.3. Anti-obesity effect

Obesity is a condition that has raised in prevalence over the past decades. This disease is associated with cardiovascular disease, diabetes, cancer, neurological disorders, chronic respiratory diseases, and digestive disorders (Abbasfati et al., 2020; “WHO Facts Sheet: Obesity and overweight [WWW Document], 2025”). The gut microbiota has also been linked to obesity, due to protective metabolic effects on the intestine and peripheral tissues, influencing body weight by controlling appetite, bile acid metabolism, and the hormonal and immune systems (Van Hul and Cani, 2023). In fact, an imbalance in the gut microbiota, or dysbiosis, has been linked to obesity (Lee et al., 2019). One of the therapeutic approaches against obesity has been to target the secretion

of glucagon-like peptide-1 (GLP-1), which is secreted naturally in the intestine in response to food ingestion. Peptides with anti-obesity potential, agonists of GLP-1R, have demonstrated efficacy, increasing glucose-dependent insulin secretion, decreasing inappropriate glucagon secretion and delaying gastric emptying and increase satiety (Trujillo et al., 2021), factors positively impacting obesity.

Following this principle, Hedin et al. (2023a) engineered *S. boulardii* to deliver GLP-1R agonists. The recombinant strain was shown to produce Exendin-4 in the GI tract of mice: when mice were exposed to low temperatures (8 °C) the Exendin-4 production led to a reduced food intake and higher weight loss when compared with the wild-type *S. boulardii*. Interestingly no difference was observed at 25 °C (Hedin et al., 2023a). The low temperature condition was chosen because when mice are exposed to low temperature food intake increases as coping mechanism to maintain normal body temperature (Ravussin et al., 2014).

Another strategy to address obesity involves reducing fat absorption through the use of lipase inhibitors. These compounds prevent the degradation of dietary fat, thereby reducing its absorption and promoting weight loss. While approved drugs are available for this purpose, Nayebehshemi et al. (2023) proposed a promising alternative: the delivery of novel pancreatic lipase-inhibiting peptides to the GI tract. They demonstrated that engineered *S. boulardii* CNM I-745 can effectively display various lipase inhibitors on its surface via α -agglutinin. In a rat model of obesity, oral administration of engineered *S. boulardii* led to reduced serum lipid levels and weight loss compared to the wild-type strain. Although its efficacy was lower than that of the marketed drug Orlistat, the approach used highlights the potential of *S. boulardii* as a vehicle for delivering therapeutic molecules to the gut to combat chronic, noncommunicable diseases.

L-ergothioneine is an antioxidant molecule that has also been proposed for the prevention and treatment of obesity and other metabolic disorders. To reduce purification and production costs, Tang et al. (2024b) developed an engineered *S. boulardii* strain capable of producing this molecule *in vitro*. They developed a PiggyBac transposon system, which resulted in varying expression levels among different clones, the maximal producer reaching 17.5 mg/L after 120 h.

2.4.4. Vitamin precursor production

Vitamin deficiency is often associated with poor nutrition and vitamin A deficiency has been linked to insufficient intake of vitamin A precursors such as β -carotene (Tan et al., 2023). To prevent this deficiency, *S. boulardii* was engineered to produce β -carotene *in situ* in the intestines of mice (Durmusoglu et al., 2021). This study used a combinatorial approach for pathway assembly and optimization, using the β -carotene production for screening to select the best producers. This study demonstrated the ability of *S. boulardii* to express multi-gene pathways and to produce vitamin precursors. *In vivo*, β -carotene from engineered *S. boulardii* was recovered in mice faeces at 56-fold higher concentration than in the initial dose, implying the successful colonization of the gut (Han et al., 2021).

2.4.5. Gut residence time improvement

To effectively deliver molecules of interest to the gut, a probiotic must colonize and remain in the gastrointestinal tract long enough to exert its intended effect. However, *S. boulardii* has been shown to have a relatively short residence times in the gut, typically persisting for only 3–5 days after dosing is discontinued (Blehaut et al., 1989; Elmer et al., 1999). Different strategies such as multiple dosing (Kim et al., 2023) and co-administration with antibiotics (Hedin et al., 2022) have been used to effectively overcome this limitation. In this regard, *S. boulardii* strains have been engineered to enhance its retention time in the gut. Heavey et al. (2024) engineered *S. boulardii* to display synthetic adhesins using yeast surface display to model pathogen invasion. To improve *S. boulardii* retention time and efficacy against murine colitis, monomeric streptavidin (mSA), was anchored to the α -agglutinin anchorage

subunits AGA1 and AGA2 of the yeast cell membrane. mSA was used to coat the cell membrane with biotinylated antibodies, specific for proteins that accumulate in the extra cellular matrix during colitis. By targeting these proteins, the modified strains were shown to have an additional 24–48 h improved retention time and a therapeutic effect against acute and chronic DSS-induced colitis in mice (Heavey et al., 2024).

2.4.6. Use of alternative carbon sources

Besides the enhancement of the immune response, retention times, the secretion of antimicrobial proteins, or vitamins, *S. boulardii* has also been modified to use alternative carbon sources which can have positive impacts on human health. For instance, a strain capable of degrading agarose was developed by expressing an *endo*-type β -agarase. The degradation of this polysaccharide commonly used as a dietary fiber, generates neogaroooligosaccharides that have prebiotic effects (Jin et al., 2021). Prebiotics are compounds that can help the growth and improve the activity of beneficial microorganisms in the gut which can potentially improve the proliferation of *S. boulardii* in the human gut (Gibson and Roberfroid, 1995; Hutkins et al., 2016). Recently, it was also reported that introducing the erythromycin destructase (*ereA*) gene, *S. boulardii* could degrade the antibiotic erythromycin, reducing residual antibiotics in the gut (He et al., 2024). Another strain was engineered to be able to degrade L-fucose, by transforming the yeast with the corresponding metabolic pathway (*fucA*, *fucI*, *fucU*, *fucK* from *E. coli* K12 MG1655 and *HXT4* from *S. cerevisiae* CEN.PK2-1D). This carbon source is present in mucin secreted by mammalian hosts and is also used by other gut microorganisms (Kim et al., 2022). Other studies have modified *S. boulardii* to utilize additional carbon sources like galactose and xylose (Durmusoglu et al., 2024; Kim et al., 2022; Liu et al., 2018, 2016).

Expanding on the surface display technology, Shin et al. (2025) engineered *S. boulardii* to be able to consume inulin. By testing different anchor proteins (Aga2, Cwp1, Cwp2, Sed1, Pir1, and Tir1), the Sed1 protein, was selected for its higher expression and used to display endo-inulinase (Shin et al., 2025). The surface display strategy was also used to construct a strain capable of degrading cellobiose, by displaying the enzyme β -glucosidase (Xu et al., 2025). Firstly, the surface display technology was optimized for *S. boulardii* by testing several anchor peptides (Aga2, Sed1 and truncated versions of both) in combination with the deletion of genes related to the cell wall (*CCW12*, *CCW14*, and *FYV5*), using a reporter protein (yeGFP) to assess the total protein production. Adding to that, a myc tag was also added to assess the display efficiency by anti-myc fluorescence labelling. The optimization also targeted the AGA1 gene by replacing the native promoter by the strong constitutive promoter *TDH3*. This enhanced the expression of AGA1 to provide more docking sites to the Aga2 peptide and therefore, improve the surface display of the desired protein (Xu et al., 2025). Then, the display of the β -glucosidase allowed the growth of *S. boulardii* on cellobiose as the sole carbon source, showing how the optimized strain had more enzyme displayed validating the optimization strategy (Xu et al., 2025).

2.4.7. Biocontainment strategies

For the development of genetic modified microorganisms, one key aspect to consider is the fact that the modified strains should not have any impact on the environment when released (Lee et al., 2018). Therefore, the biocontainment strategies of the engineered *S. boulardii* strains should also be studied. Currently biocontainment strategies for this yeast are lacking with only one study focused in engineering an *S. boulardii* strain that would not survive after exiting the GI tract (Hedin et al., 2023b). The strategy implemented in this case was a mix of autotrophy and lack of growth at temperatures lower than 20 °C. Some other strategies like CRISPR-based kill switches (Rottinghaus et al., 2022) or synthetic gene circuits that initiate cell death (Callura et al., 2010; Chan et al., 2015) have been implemented in the bacteria *E. coli*. Given that autotrophy can limit the probiotic lifespan *in vivo*

(Rottinghaus et al., 2022) it would be valuable to study the adaptation of these alternative biocontainment strategies in *S. boulardii*.

Although *S. boulardii* can have positive impacts on the treatment or minimize several GI diseases some people might not experience benefits from its ingestion. Administration of *S. boulardii* can cause fungemia (presence of fungi in the bloodstream) especially in elderly people, critically ill patients and patients with a central venous line. Therefore, administration might not be suitable for all group ages or immunocompromised patients (Poncelet et al., 2021). A study from 2021, evaluated 46,729 patients, and the incidence of fungemia attributable to *S. boulardii* administration was reported to be 1.70 cases per 10,000 patient-days. Even though the risk of probiotic bloodstream infection was described as not greater than any other hospital-acquired bloodstream infection (Wombwell et al., 2021), fungemia high-risk factors should be taken into consideration upon administration.

2.4.8. Biosensor

Genetic engineering has also been shown to allow the use of *S. boulardii* as a biosensor, to detect intestinal diseases, by coupling a reporter output to a biomarker of disease. Park et al. (2023) engineered *S. boulardii* to detect intestinal inflammation by monitoring extracellular ATP levels. ATP accumulation is associated with gut inflammation, such as in DSS-induced colitis models. In this study, the firefly luciferase gene was genomically integrated, allowing bioluminescence to be produced in the presence of ATP through the oxygenation of D-luciferin. Dacquay et al. (2024) constructed a doxycycline-responsive strain, using Cre-loxP sites. Expression of Cre was placed under the control of the *TET4* promoter, which activated in the presence of doxycycline. Upon successful induction, Cre recombinase excises *ADE2*, resulting in a red colony phenotype. The authors were able to recover red colonies from mice administered doxycycline, demonstrating both *in vivo* biosensing capability and the genomic stability of the construction. Interestingly, a weaker promoter (*MYO2*) was chosen over a stronger one (*TEF1*) due to its lower background expression in the absence of doxycycline, which reduced false-positive signals (Dacquay et al., 2024).

A notable advance worth considering was reported in probiotic strains of *S. cerevisiae*, where human G protein-coupled receptors (GPCR) were engineered enable controlled expression of proteins in response to stimuli (Scott et al., 2021). Jensen et al. (2022) provided an in-depth study of GPCR biosensing strategies and were able to translate the advances in the model organism *S. cerevisiae* to *S. boulardii*. They show how GPCRs not derived from yeast, enabled a response of *S. boulardii*, which upon detection, activated the mating's pathway. These advances can be expected to be the base for the next generation of *S. boulardii*-based biosensors.

2.4.9. Upcoming applications

Several authors have focused on using *S. boulardii* to deliver other type of molecules to the gut that can have a positive general impact on human health. One example is the engineering of *S. boulardii* to enhance intestinal permeability, a limitation for drug absorption. Gelli et al. (2025) demonstrated that oral dosing of the engineered yeast for 5 or 10 days, was safe and effective in mice, achieving the delivery of cell-penetrating peptides, enhancing intestinal absorption of dextran in comparison with the wild-type yeast.

Another promising area of application for engineered *S. boulardii* is the development of anti-cancer therapies. Rebeck et al. (2025) show that engineered *S. boulardii* can deliver immune checkpoint inhibitors, that can reduce intestinal tumors. Interestingly, in this study the authors demonstrated that despite of not supplementing mice with antibiotics, the plasmid was retained in 95 % of the yeast recovered in mice stool.

Recently, interesting (not yet peer reviewed) applications for *S. boulardii* have been proposed such as the secretion of spermidine, to mitigate the symptoms of gut-related diseases and memory enhancement (Mohaqiq et al., 2025; Parweez et al., 2025). Van Gaeve et al. (2024) reported the successful expression of the phytohormone abscisic

acid, highlighting the advantage of yeast for molecule production over bacterial probiotic strains, since *S. boulardii* allowed the integration of a whole biosynthetic pathway, up to 5 genes from *Botrytis cinerea*, to produce the compound.

2.5. Future directions for engineered *S. Boulardii*: Advances and challenges

Despite the availability and readiness of genetic engineering tools and an increasing number of proof-of-concept studies involving engineered *S. boulardii*, the field is still under development and as of today, there are no clinical trials involving engineered *S. boulardii*. We do have evidence for the capacity of this yeast to produce a wide range of molecules and deliver them in the gut, however, most studies lack translational validation, which is needed for their clinical application.

Several recent studies stand out for their deeper characterization: such as Durmusoglu et al. (2021), who characterized 18 promoters and 7 terminators, and Sands et al. (2024), who complemented the characterization *in vivo*. Meanwhile, expression stability and long-term performance was explored by Dacquay et al. (2024), who demonstrated the use of *S. boulardii* as a biosensor in mice, while Park et al. (2023) used it to track gut inflammation. In terms of safety and containment, the study of Hedin et al. (2023b) stands out for implementing a biocontainment system consisting of auxotrophy coupled with temperature sensitivity. Deeper functional characterizations, while challenging, are essential for advancing engineered *S. boulardii* toward clinical and industrial applications.

One of the most promising areas for future development is the use of microbial cocktails. As research advances and we gain a deeper understanding of the specific functions and interactions within the microbiome, both the probiotic and functional food industries are moving toward the rational design of targeted products. These may include prebiotics and/or engineered probiotics specifically selected to promote or suppress certain microbial species, tailoring interventions to particular health conditions. A key innovation in this field is the development of microbial cocktails—combinations of multiple engineered strains that perform complementary functions. Hedin et al. (2024) demonstrated how engineered microbial consortia can interact dynamically within the host and maintain functional stability in complex environments. In this context, *S. boulardii* could serve as a chassis strain, engineered to secrete immunomodulatory peptides or other beneficial molecules, while a bacterial partner could produce small molecules or detect disease markers, enabling synergistic and multifunctional therapeutic strategies.

The field would also benefit from the development of robust and genetically stable strains, as these must compete with native microbes and maintain their intended genotypic and phenotypic traits within the complex environment of the gut, particularly considering the high variability of the microbiome between individuals. Practically, this underscores the need for standardized strains that perform consistently across different conditions and are scalable for industrial use. This is especially relevant given that large-scale production, in accordance with Good Manufacturing Practices (GMP), is a prerequisite for the clinical trials required before the commercialization of (engineered) probiotics.

Personalized treatments based on individual's microbiome have been proposed in fields such as oncology (Gately, 2019), and could significantly improve the effectiveness of engineered probiotics. These probiotics could be tailored to target specific diseases while accounting for the host's unique microbial environment. A deeper understanding of the factors behind treatment failure or success, combined with the increasing affordability of sequencing technologies, could enhance the reproducibility and precision of these interventions.

The choice of genetic delivery method is an important aspect to consider when aiming to translate these studies into clinical practice. Antibiotic-selectable markers pose a risk of spreading resistance (Vandermeulen et al., 2011). However, in some cases, plasmid loss has

not been observed even in the absence of antibiotic pressure, potentially limiting the risk of resistance dissemination (Rebeck et al., 2025). Nonetheless, marker-free genome editing tools such as CRISPR-Cas (Jessop-Fabre et al., 2016) may further enhance production cost-efficiency, safety, and the robustness and scalability of engineered strains. For instance, Jin et al. (2021) first achieved the production in plasmids and then moved on to the production using genomic integration using CRISPR-Cas, which potentially could serve for more stable modifications upon treatment and scale-up.

Pharmacokinetics and colonization control present significant challenges in the development of engineered probiotics. Heavey et al. (2024) showed that genetic engineering can enhance colonization, but while effective gut colonization is important for therapeutic delivery, it must remain temporary. Engineered strains should be self-limiting to reduce potential risks, such as fungemia associated with *S. boulardii* (Poncelet et al., 2021) and unintended effects from continuous production of the therapeutic molecule. To address this, strategies such as environmental sensing circuits that trigger expression (Scott et al., 2021), conditional expression systems (Durmusoglu et al., 2024) and biocontainment mechanisms (Hedin et al., 2023b) are being developed to strike a balance between colonization control and therapeutic efficacy.

In this regard, an improvement in *S. boulardii* ability to secrete recombinant proteins is one of the factors that can enable better therapeutic outcomes. Higher concentrations of protein, secreted *in situ* at the site of infection or at the drug's target site- specifically in the intestine- can lead to higher local concentrations, increased bioavailability, reduced systemic exposure and has the potential to minimize side-effects. Chen et al., (2020) optimized *S. boulardii* for expression of anti-*C. difficile* antibody production, reporting a 4-fold increase in antibody expression by the yeast, which led to therapeutic effect against primary and recurrent *C. difficile* infection in mice. Durmusoglu et al. (2023) improved the yeast secretory pathway, achieving a 11-fold increase of the therapeutic target peptide. Despite these optimizations, *in situ* performance is likely to be impacted not only by the protein to be produced but also to the residence time of the yeast and host factors such as diet, gut microbiota and colonization resistance.

3. Genetically enhanced probiotic: What are the regulations?

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

Another factor influencing the market are public perception and concerns over genetically modified probiotics despite the accumulation of data suggesting the solution for other growing concerns like obesity, malnutrition, inefficient therapies for GI disorders (Dong et al., 2019) and the ever-growing antibiotic resistance from gut pathogens (Andreescu, 2023; Spigaglia et al., 2018).

The regulation of genetically modified probiotics is reviewed elsewhere (Hoffmann et al., 2014; Kurtz and Calvo, 2024). These regulations differ significantly across regions of the world and lead to different regulatory paths depending on the intended use and claims of the product. Table 5 describes the different regulatory frameworks found in different countries around the world as well as the products marketed to date related to genetically modified probiotics.

Table 5

Regulatory agencies responsible in different countries around the world for the regulation of genetically modified probiotics.

Region	Primary Regulator	Relevant Regulation(s)	Products in the market	Reference
US	FDA, USDA	- Dietary Supplement Health and Education Act (1994) - National Bioengineered Food Disclosure Standard (2016) - Regulation (EC) No 1829/2003 (GMO food and feed) - Directive 2001/18/EC (environmental release)	ZBiotics® Sugar-to-Fiber, ZBiotics® Pre-Alcohol	(“Dietary Supplement Health and Education Act of 1994 [WWW Document], 2025”; The National Bioengineered Food Disclosure Standard, 2025) (“Directive 2001/18 - Deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration - EU monitor [WWW Document], 2025”; “Regulation - 1829/2003 - EN - EUR-Lex [WWW Document], 2025”)
EU	EFSA	- Regulation (EC) No 1829/2006 (health claims) - Natural Health Products Regulations (2004)	None	Canadian Environmental Protection Act, 1999, “Natural Health Products Regulations [WWW Document], 2025”)
Canada	Health Canada, CFIA	- Canadian Environmental Protection Act (1999)	None	(Fsan, n.d.)
Australia & New Zealand	FSANZ, TGA	- Food Standards Code, Standard 1.5.2 (GMOs and novel foods) - Food Sanitation Law;	None	(“Ministry of Health, Labour and Welfare: Food Produced by Recombinant DNA Techniques [WWW Document], 2025”)
Japan	CAA, MHLW	- Standards for Genetically Modified Foods	None	(“Regulations on Administration of Agricultural Genetically Modified Organisms Safety [WWW Document], 2025”)
China	NMPA, MARA	- Agricultural Genetically Modified Organisms Safety Regulation (2001)	None	(“Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011 National Portal of India,” n.d.)
India	FSSAI, GEAC	- Environmental Protection Act (1986) - Food Safety and Standards Regulations (2011)	None	(continued on next page)

Table 5 (continued)

Region	Primary Regulator	Relevant Regulation(s)	Products in the market	Reference
Brazil	ANVISA, CTNBio	- Biosafety Law (Law No. 11.105, 2005)	None	(Lei n. 11.105 de 24/03/2005 - Laws - Comissão Técnica Nacional de Biossegurança\$ &ndash CTNBio [WWW Document], 2025")

Interestingly, in the US, the company Zbiotics, got approval to market a genetically modified nutritional supplement that prevents hangover by breaking down acetaldehydes produced from alcohol, consisting of *B. subtilis* strain expressing acetaldehyde dehydrogenase (Appala Naidu et al., 2019; Hassan-Casarez et al., 2024) (Zbiotics® Pre-Alcohol) and recently, a second product containing an engineered *B. subtilis* strain producing levansucrase, which catalyzes the conversion of sucrose to levan, a prebiotic (ZBiotics® Sugar-to-Fiber) (“How to Turn Sugar Into Fiber | The Science Behind ZBiotics [WWW Document], 2025”). These products adhere to the dietary supplement standards, must comply with Good Manufacturing Practices and its claims focus on structure and function. In regulatory terms, it operates within the framework for probiotics and GMOs but avoids claims that could classify it as a drug or therapeutic product.

4. Conclusions and future perspectives

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

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

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

Informed consent statement

Not applicable.

Institutional review board statement

Not applicable.

Compliance with ethical standards

This article contained no studies incorporating human participants or animals to be performed by any of the authors. None of the authors declares any conflicts of interest. All authors have read and agree to the publication of this manuscript.

Funding

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469/2020 unit and a Ph.D. grant UI/BD/151411/2021 to D.S.M.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David Saez Moreno reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are available within the article and supplementary material.

References

Abbafati, C., Abbas, K.M., et al., 2020. Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the global burden of disease study 2019. *Lancet* 396, 1223–1249. [https://doi.org/10.1016/S0140-6736\(20\)30752-2](https://doi.org/10.1016/S0140-6736(20)30752-2).

Abid, R., Waseem, H., Ali, J., Ghazanfar, S., Ali, G.M., Elsalbi, A.M., Alharethi, S.H., 2022. Probiotic yeast *Saccharomyces*: Back to nature to improve human health. *Journal of Fungi* 8, 444. <https://doi.org/10.3390/JOF8050444>.

Allen, J.R., Torres-Acosta, M.A., Mohan, N., Lye, G.J., Ward, J.M., 2022. Segregationally stabilised plasmids improve production of commodity chemicals in glucose-limited continuous fermentation. *Microb. Cell Fact.* 21, 1–11. <https://doi.org/10.1186/S12934-022-01958-3/FIGURES/4>.

Anders, C., Bargsten, K., Jinek, M., 2016. Structural plasticity of PAM recognition by engineered variants of the RNA-guided endonuclease Cas9. *Mol. Cell* 61, 895–902. <https://doi.org/10.1016/J.MOLCEL.2016.02.020>.

Andoh, A., Nishida, A., 2023. Alteration of the gut microbiome in inflammatory bowel disease. *Digestion* 104, 16–23. <https://doi.org/10.1159/000525925>.

Andrescu, M., 2023. Molecular insights into the role of gut microbiota in antibiotic therapy selection and resistance mitigation. *Cureus* 15. <https://doi.org/10.7759/CUREUS.50318>.

Appala Naidu, B., Kannan, K., Santhosh Kumar, D.P., Oliver, J.W.K., Abbott, Z.D., 2019. Lyophilized *B. Subtilis* ZB183 spores: 90-day repeat dose Oral (gavage) toxicity study in Wistar rats. *J Toxicol* 3042108. <https://doi.org/10.1155/2019/3042108>.

Babaei, F., Navidi-Moghaddam, A., Naderi, A., Ghafghazi, S., Mirzababaei, M., Dargahi, L., Mohammadi, G., Nassiri-Asl, M., 2024. The preventive effects of *Saccharomyces boulardii* against oxidative stress induced by lipopolysaccharide in rat brain. *Heliyon* 10 (9), e30426. <https://doi.org/10.1016/j.heliyon.2024.e30426>.

Back, K.R., Lee, H.J., Jeong, K.H., Park, B.R., Kim, S.J., Seo, S.O., 2024. Parabiotic immunomodulatory activity of yeast cell wall polysaccharides from *Saccharomyces cerevisiae* and *S. boulardii*. *J Funct Foods* 123, 106577. <https://doi.org/10.1016/J.JFF.2024.106577>.

Bagherpour, G., Ghasemi, H., Zand, B., Zarei, N., Roohvand, F., Ardakani, E.M., Azizi, M., Khalaj, V., 2018. Oral administration of recombinant *Saccharomyces boulardii* expressing ovalbumin-CPE fusion protein induces antibody response in mice. *Front. Microbiol.* 9. <https://doi.org/10.3389/fmicb.2018.00723>.

Bassotti, G., Fruganti, A., Stracci, F., Marconi, P., Fettucciari, K., 2023. Cytotoxic synergism of *Clostridioides difficile* toxin B with proinflammatory cytokines in subjects with inflammatory bowel diseases. *World J. Gastroenterol.* 29, 582. <https://doi.org/10.3748/WJG.V29.I4.582>.

Blehaut, H., Massot, J., Elmer, G.W., Levy, R.H., 1989. Disposition kinetics of *Saccharomyces boulardii* in man and rat. *Biopharm. Drug Dispos.* 10, 353–364. <https://doi.org/10.1002/BDD.2510100403>.

- Buts, J.P., Dekeyser, N., Stilmant, C., Delem, E., Smets, F., Sokal, E., 2006. *Saccharomyces boulardii* produces in rat small intestine a novel protein phosphatase that inhibits *Escherichia coli* endotoxin by dephosphorylation. *Pediatr. Res.* 60, 24–29. <https://doi.org/10.1203/01.PDR.0000220322.31940.29>.
- Callura, J.M., Dwyer, D.J., Isaacs, F.J., Cantor, C.R., Collins, J.J., 2010. Tracking, tuning, and terminating microbial physiology using synthetic riboregulators. *Proc. Natl. Acad. Sci. U. S. A.* 107, 15898–15903. https://doi.org/10.1073/PNAS.1009747107/SUPPL_FILE/SM01.AVI.
- Canadian Environmental Protection Act [WWW Document], n.d. URL <https://laws-lois.justice.gc.ca/eng/acts/c-15.31/> (accessed 11.28.24).
- Carr, M., Bensasson, D., Bergman, C.M., 2012. Evolutionary genomics of transposable elements in *Saccharomyces cerevisiae*. *PLoS One* 7, 50978. <https://doi.org/10.1371/JOURNAL.PONE.0050978>.
- Castagliuolo, I., Riegler, M.F., Valenick, L., LaMont, J.T., Pothoulakis, C., 1999. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins a and B in human colonic mucosa. *Infect. Immun.* 67, 302. <https://doi.org/10.1128/IAI.67.1.302-307.1999>.
- Chan, C.T.Y., Lee, J.W., Cameron, D.E., Bashor, C.J., Collins, J.J., 2015. “Deadman” and “passcode” microbial kill switches for bacterial containment. *Nature Chemical Biology* 12, 82–86. <https://doi.org/10.1038/nchembio.1979>.
- Charles, A., Janeway, J., Travers, P., Walport, M., Shlomchik, M.J., 2001. *The Distribution and Functions of Immunoglobulin Isotypes*.
- Chen, Q., Luo, W., Veach, R.A., Hickman, A.B., Wilson, M.H., Dyda, F., 2020a. Structural basis of seamless excision and specific targeting by piggyBac transposase. *Nature communications* 11 (1), 1–14. <https://doi.org/10.1038/s41467-020-17128-1>.
- Chen, K., Zhu, Y., Zhang, Y., Hamza, T., Yu, H., Fleur, A. Saint, Galen, J., Yang, Z., Feng, H., 2020b. A probiotic yeast-based immunotherapy against *Clostridioides difficile* infection. *Sci. Transl. Med.* 12. <https://doi.org/10.1126/scitranslmed.aax4905>.
- Chitapanarux, T., Wiracha, U., Winichakoon, P., Salee, P., Traisathit, P., 2025. Efficacy and safety of *Saccharomyces boulardii* as adjunct therapy with vancomycin in treating *Clostridioides difficile* infection: a randomized controlled trial. *Sci. Rep.* 15, 1–7. <https://doi.org/10.1038/S41598-025-04986-2>.
- Chu, C., Huang, S., Zhao, G., Hao, W., Zhong, Y., Ma, Z., Huang, C., Peng, Y., Wei, F., 2024. Randomized controlled trial comparing the impacts of *Saccharomyces boulardii* and *Lactobacillus rhamnosus* OF44 on intestinal flora in cerebral palsy rats: insights into inflammation biomarkers and depression-like behaviors. *Transl. Pediatr.* 13 (1), 72–90. <https://doi.org/10.21037/tp.23-566>.
- Costa, C.E., Romani, A., Møller-Hansen, I., Teixeira, J.A., Borodina, I., Domingues, L., 2022. Valorisation of wine wastes by de novo biosynthesis of resveratrol using a recombinant xylose-consuming industrial *Saccharomyces cerevisiae* strain. *Green Chem.* 24, 9128–9142. <https://doi.org/10.1039/D2GC02429B>.
- Cui, B., Lin, L., Wang, B., Liu, W., Sun, C., 2022. Therapeutic potential of *Saccharomyces boulardii* in liver diseases: from passive bystander to protective performer? *Pharmacol. Res.* 175, 106022. <https://doi.org/10.1016/J.PHRS.2021.106022>.
- Czerucka, D., Roux, I., Rampal, P., 1994. *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. *Gastroenterology* 106, 65–72. [https://doi.org/10.1016/S0016-5085\(94\)94403-2](https://doi.org/10.1016/S0016-5085(94)94403-2).
- Czerucka, D., Piche, T., Rampal, P., 2007. Review article: yeast as probiotics – *Saccharomyces boulardii*. *Aliment. Pharmacol. Ther.* 26, 767–778. <https://doi.org/10.1111/J.1365-2036.2007.03442.X>.
- Dacquay, L.C., Samman, B., Newman, M.P., Philpott, D.J., McMillen, D.R., 2024. Engineering a colorimetric yeast-based assay to detect small molecules in the gastrointestinal system. *Frontiers in Synthetic Biology* 2, 1265343. <https://doi.org/10.3389/FSYBI.2024.1265343>.
- de Carvalho, B.T., Subotić, A., Vandecruys, P., Deleu, S., Vermeire, S., Thevelein, J.M., 2024. Enhancing probiotic impact: engineering *Saccharomyces boulardii* for optimal acetic acid production and gastric passage tolerance. *Appl. Environ. Microbiol.* 90. <https://doi.org/10.1128/AEM.00325-24.e00325-24>.
- Didari, T., Solki, S., Mozaffari, S., Nikfar, S., Abdollahi, M., 2014. A systematic review of the safety of probiotics. *Expert Opin. Drug Saf.* 13, 227–239. <https://doi.org/10.1517/14740338.2014.872627>.
- Dietary Supplement Health and Education Act of 1994 [WWW Document]. URL https://ods.od.nih.gov/About/DSHEA_Wording.aspx (accessed 11.28.24).
- Dinleyici, E.C., Kara, A., Ozen, M., Vandenplas, Y., 2014. *Saccharomyces boulardii* CNCM I-745 in different clinical conditions. *Expert Opin. Biol. Ther.* 14, 1593–1609. <https://doi.org/10.1517/14712598.2014.937419>.
- Directive 2001/18 - Deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration - EU monitor [WWW Document]. URL <https://www.eumonitor.eu/9353000/1/j4nvk6yhcbeywkj9vvik7m1c3gyxp/vitgbg8ynz6> (accessed 11.28.24).
- Dong, Y., Baumeister, D., Berens, S., Eich, W., Tesarz, J., 2019. High rates of non-response across treatment attempts in chronic irritable bowel syndrome: results from a follow-up study in tertiary care. *Front. Psych.* 10. <https://doi.org/10.3389/FPSYT.2019.00714/FULL>.
- Douradinha, B., Reis, V.C.B., Rogers, M.B., Torres, F.A.G., Evans, J.D., Marques, E.T.A., 2014. Novel insights in genetic transformation of the probiotic yeast *Saccharomyces boulardii*. *Bioengineered* 5, 21–29. <https://doi.org/10.4161/BIOE.26271>.
- Dujon, B., 2006. Yeasts illustrate the molecular mechanisms of eukaryotic genome evolution. *Trends Genet.* 22, 375–387. <https://doi.org/10.1016/J.TIG.2006.05.007>.
- Durmusoglu, D., Al'Abri, I.S., Collins, S.P., Cheng, J., Eroglu, A., Beisel, C.L., Crook, N., 2021. In situ biomanufacturing of small molecules in the mammalian gut by probiotic *Saccharomyces boulardii*. *ACS Synth Biol* 10, 1039–1052. <https://doi.org/10.1021/acssynbio.0c00562>.
- Durmusoglu, D., Al'Abri, I., Li, Z., Islam Williams, T., Collins, L.B., Martínez, J.L., Crook, N., 2023. Improving therapeutic protein secretion in the probiotic yeast *Saccharomyces boulardii* using a multifactorial engineering approach. *Microb. Cell Fact.* 22. <https://doi.org/10.1186/s12934-023-02117-y>.
- Durmusoglu, D., Haller, D.J., Al'Abri, I.S., Day, K., Sands, C., Clark, A., San-Miguel, A., Vazquez-Urbe, R., Sommer, M.O.A., Crook, N.C., 2024. Programming probiotics: diet-responsive gene expression and colonization control in engineered *S. Boulardii*. *ACS Synth Biol* 13, 1851–1865. <https://doi.org/10.1021/acssynbio.4c00145>.
- Elmer, G.W., McFarland, L.V., Surawicz, C.M., Danko, L., Greenberg, R.N., 1999. Behaviour of *Saccharomyces boulardii* in recurrent *Clostridium difficile* disease patients. *Aliment. Pharmacol. Ther.* 13, 1663–1668. <https://doi.org/10.1046/J.1365-2036.1999.00666.X>.
- Erhart, E., Hollenberg, C.P., 1981. Curing of *Saccharomyces cerevisiae* 2-μm DNA by transformation. *Curr. Genet.* 3, 83–89. <https://doi.org/10.1007/BF00365710>.
- Feschotte, C., Pritham, E.J., 2007. DNA transposons and the evolution of eukaryotic genomes. *Annu. Rev. Genet.* 41, 331. <https://doi.org/10.1146/ANNUREV.GENET.40.110405.090448>.
- Finnegan, D.J., 1989. Eukaryotic transposable elements and genome evolution. *Trends Genet.* 5, 103–107. [https://doi.org/10.1016/0168-9525\(89\)90039-5](https://doi.org/10.1016/0168-9525(89)90039-5).
- Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011. National Portal of India [WWW Document]. n.d. URL <https://www.india.gov.in/food-safety-and-standards-contaminants-toxins-and-residues-regulations-2011> (accessed 11.28.24).
- Fsan, 2025. Compliance Guide to Australia New Zealand Food Standards Code Standard 1.5.2. Food Produced Using Gene Technology.
- Gao, H., Li, Y., Xu, J., Zuo, X., Yue, T., Xu, H., Sun, J., Wang, M., Ye, T., Yu, Y., Yao, Y., 2023. *Saccharomyces boulardii* protects against murine experimental colitis by reshaping the gut microbiome and its metabolic profile. *Front. Microbiol.* 14, 1204122. <https://doi.org/10.3389/FMICB.2023.1204122/BIBTEX>.
- Garvey, M., 2022. Non-mammalian eukaryotic expression systems yeast and Fungi in the production of biologics. *Journal of Fungi* 8. <https://doi.org/10.3390/JOF8111179>.
- Gately, S., 2019. Human microbiota and personalized Cancer treatments: role of commensal microbes in treatment outcomes for Cancer patients. *Cancer Treat. Res.* 178, 253–264. https://doi.org/10.1007/978-3-030-16391-4_10.
- Gelli, H.P., Hedin, K.A., Laursen, M.F., Uribe, R.V., Sommer, M.O.A., 2025. Enhancing intestinal absorption of a macromolecule through engineered probiotic yeast in the murine gastrointestinal tract. *Trends Biotechnol.* 43, 715–731. <https://doi.org/10.1016/J.TIBTECH.2024.10.019>.
- Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125, 1401–1412. <https://doi.org/10.1093/JN/125.6.1401>.
- Gietz, R.D., Schiestl, R.H., 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols* 2 (1), 31–34. <https://doi.org/10.1038/nprot.2007.13>.
- Günge, R., Rudolf, F., 2017. *Saccharomyces cerevisiae* shuttle vectors. *Yeast* 34, 205–221. <https://doi.org/10.1002/YEA.3228>.
- Goldberg, G.W., Spencer, J.M., Giganti, D.O., Camellato, B.R., Agmon, N., Ichikawa, D. M., Boeke, J.D., Noyes, M.B., 2021. Engineered dual selection for directed evolution of SpCas9 PAM specificity. *Nature Communications* 12 (1), 1–16. <https://doi.org/10.1038/s41467-020-20650-x>.
- Gomes, A.M.V., Carmo, T.S., Carvalho, L.S., Bahia, F.M., Parachin, N.S., 2018. Comparison of yeasts as hosts for recombinant protein production. *Microorganisms* 6. <https://doi.org/10.3390/MICROORGANISMS6020038>.
- Gopalan, S., Ganapathy, S., Mitra, M.N., Kumar Joshi, D., Veligandla, K.C., Rathod, R., Kotak, B.P., 2023. Unique properties of yeast probiotic *Saccharomyces boulardii* CNCM I-745: a narrative review. *Cureus* 15. <https://doi.org/10.7759/CUREUS.46314>.
- Gueldeuer, U., Heinisch, J., Koehler, G.J., Voss, D., Hegemann, J.H., 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.* 30, e23. <https://doi.org/10.1093/NAR/30.6.E23>.
- Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., Hegemann, J.H., 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24, 2519. <https://doi.org/10.1093/NAR/24.13.2519>.
- Hafner, L., Pichon, M., Burucoa, C., Nusser, S.H.A., Moura, A., Garcia-Garcera, M., Lecuit, M., 2021. *Listeria monocytogenes* faecal carriage is common and depends on the gut microbiota. *Nature Communications* 12 (1), 1–13. <https://doi.org/10.1038/s41467-021-27069-y>.
- Hamed, H., Misaghi, A., Modarressi, M.H., Salehi, T.Z., Khorasanizadeh, D., Khalaj, V., 2013. Generation of a uracil auxotroph strain of the probiotic yeast *Saccharomyces boulardii* as a host for the recombinant protein production. *Avicenna J Med Biotechnol* 5, 29.
- Han, S., Lu, Y., Xie, J., Fei, Y., Zheng, G., Wang, Z., Liu, J., Lv, L., Ling, Z., Berglund, B., Yao, M., Li, L., 2021. Probiotic gastrointestinal transit and colonization after Oral administration: a long journey. *Front. Cell. Infect. Microbiol.* 11. <https://doi.org/10.3389/FMICB.2021.609722>.
- Harrison, E., Koufopanou, V., Burt, A., MacLean, R.C., 2012. The cost of copy number in a selfish genetic element: the 2-μm plasmid of *Saccharomyces cerevisiae*. *J. Evol. Biol.* 25, 2348–2356. <https://doi.org/10.1111/J.1420-9101.2012.02610.X>.
- Hassan-Casarez, C., Ryan, V., Shuster, B.M., Oliver, J.W.K., Abbott, Z.D., 2024. Engineering a probiotic *Bacillus subtilis* for acetaldehyde removal: a hag locus integration to robustly express acetaldehyde dehydrogenase. *PLoS One* 19, e0312457. <https://doi.org/10.1371/JOURNAL.PONE.0312457>.
- He, Q., Lin, Z., Zhang, X., Qin, M., Huang, Y., Liao, X., Liu, Y., Ren, H., Sun, J., 2024. Designing a reengineered probiotic yeast to spontaneously degrade residual antibiotics in gut during antimicrobial therapy. *J. Clean. Prod.* 483, 144177. <https://doi.org/10.1016/J.JCLEPRO.2024.144177>.
- Heavey, M.K., Hazelton, A., Wang, Y., Garner, M., Anselmo, A.C., Arthur, J.C., Nguyen, J., 2024. Targeted delivery of the probiotic *Saccharomyces boulardii* to the

- extracellular matrix enhances gut residence time and recovery in murine colitis. *Nat. Commun.* 15. <https://doi.org/10.1038/s41467-024-48128-0>.
- Hedin, K.A., Rees, V.E., Zhang, H., Kruse, V., Vazquez-Urbe, R., Sommer, M.O.A., 2022. Effects of broad-spectrum antibiotics on the colonisation of probiotic yeast *Saccharomyces boulardii* in the murine gastrointestinal tract. *Scientific Reports* 12 (1), 1–9. <https://doi.org/10.1038/s41598-022-12806-0>.
- Hedin, K.A., Kruse, V., Vazquez-Urbe, R., Sommer, M.O.A., 2023a. Biocontainment strategies for in vivo applications of *Saccharomyces boulardii*. *Front. Bioeng. Biotechnol.* 11. <https://doi.org/10.3389/fbioe.2023.1136095>.
- Hedin, K.A., Zhang, H., Kruse, V., Rees, V.E., Bäckhed, F., Greiner, T.U., Vazquez-Urbe, R., Sommer, M.O.A., 2023b. Cold exposure and Oral delivery of GLP-1R agonists by an engineered probiotic yeast strain have Antiobesity effects in mice. *ACS Synth. Biol.* 12, 3433–3442. <https://doi.org/10.1021/acssynbio.3c00455>.
- Hedin, K.A., Mirhakkak, M.H., Vaaben, T.H., Sands, C., Pedersen, M., Baker, A., Vazquez-Urbe, R., Schäuble, S., Panagiotou, G., Wellejus, A., Sommer, M.O.A., 2024. *Saccharomyces boulardii* enhances anti-inflammatory effectors and AhR activation via metabolic interactions in probiotic communities. *ISME J.* 18, 212. <https://doi.org/10.1093/ISMEJO/WRAE212>.
- Hinnen, A., Hicks, J.B., Fink, G.R., 1978. Transformation of yeast. *Proc. Natl. Acad. Sci. U. S. A.* 75, 1929. <https://doi.org/10.1073/PNAS.75.4.1929>.
- Hoffmann, D.E., Fraser, C.M., Palumbo, F., Ravel, J., Rowthorn, V., Schwartz, J., 2014. Probiotics: achieving a better regulatory fit. *Food Drug Law J.* 69, 237.
- Hossain, M.N., Afrin, S., Humayun, S., Ahmed, M.M., Saha, B.K., 2020. Identification and growth characterization of a novel strain of *Saccharomyces boulardii* isolated from soya paste. *Front. Nutr.* 7. <https://doi.org/10.3389/FNUT.2020.00027>.
- How to Turn Sugar Into Fiber | The Science Behind ZBiotics [WWW Document]. URL. <https://zbiotics.com/pages/how-sugar-to-fiber-works> (accessed 11.25.24).
- Huang, C., Feng, S., Huo, F., Liu, H., 2022. Effects of four antibiotics on the diversity of the intestinal microbiota. *Microbiol Spectr* 10, e01904–e01921. <https://doi.org/10.1128/SPECTRUM.01904-21>.
- Hudson, L.E., Fasken, M.B., McDermott, C.D., McBride, S.M., Kuiper, E.G., Guiliano, D.B., Corbett, A.H., Lamb, T.J., 2014. Functional heterologous protein expression by genetically engineered probiotic yeast *Saccharomyces boulardii*. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0112660>.
- Hutkins, R.W., Krumbeck, J.A., Bindels, L.B., Cani, P.D., Fahey, G., Goh, Y.J., Hamaker, B., Martens, E.C., Mills, D.A., Rastal, R.A., Vaughan, E., Sanders, M.E., 2016. Probiotics: why definitions matter. *Curr. Opin. Biotechnol.* 37, 1. <https://doi.org/10.1016/j.COPBIO.2015.09.001>.
- Jensen, E.D., Deichmann, M., Ma, X., Vilandt, R.U., Schiesaro, G., Rojek, M.B., Lengger, B., Eliasson, L., Vento, J.M., Durmusoglu, D., Hovmand, S.P., Al'Abri, I., Zhang, J., Crook, N., Jensen, M.K., 2022. Engineered cell differentiation and sexual reproduction in probiotic and mating yeasts. *Nature Communications* 13 (1), 1–15. <https://doi.org/10.1038/s41467-022-33961-y>.
- Jessop-Fabre, M.M., Jakociunas, T., Stovicek, V., Dai, Z., Jensen, M.K., Keasling, J.D., Borodina, I., 2016. EasyClone-MarkerFree: a vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9. *Biotechnol. J.* 11, 1110–1117. <https://doi.org/10.1002/BOT.201600147>.
- Jin, Y., Yu, S., Liu, J.J., Yun, E.J., Lee, J.W., Jin, Y.S., Kim, K.H., 2021. Production of neogaroaligosaccharides by probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* engineered as a microbial cell factory. *Microb. Cell Fact.* 20. <https://doi.org/10.1186/s12934-021-01644-w>.
- Karim, A.S., Curran, K.A., Alper, H.S., 2013. Characterization of plasmid burden and copy number in *Saccharomyces cerevisiae* for optimization of metabolic engineering applications. *FEMS Yeast Res.* 13, 107–116. <https://doi.org/10.1111/1567-1364.12016>.
- Karvelis, T., Gasiunas, G., Miksys, A., Barrangou, R., Horvath, P., Siksnys, V., 2013. crRNA and tracrRNA guide Cas9-mediated DNA interference in *Streptococcus thermophilus*. *RNA Biol.* 10, 841–851. <https://doi.org/10.4161/RNA.24203>.
- Kazmierczak-Siedlecka, K., Ruskowski, J., Fic, M., Folwarski, M., Makarewicz, W., 2020. *Saccharomyces boulardii* CNCM 1-745: a non-bacterial microorganism used as probiotic agent in supporting treatment of selected diseases. *Curr. Microbiol.* 77, 1987–1996. <https://doi.org/10.1007/S00284-020-02053-9/FIGURES/1>.
- Kelesidis, T., Pothoulakis, C., 2012. Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. *Therap. Adv. Gastroenterol.* 5, 111. <https://doi.org/10.1177/1756283X11428502>.
- Khatiri, I., Tomar, R., Ganesan, K., Prasad, G.S., Subramanian, S., 2017. Complete genome sequence and comparative genomics of the probiotic yeast *Saccharomyces boulardii*. *Sci. Rep.* 7. <https://doi.org/10.1038/S41598-017-00414-2>.
- Kim, J., Cheong, Y.E., Yu, S., Jin, Y.S., Kim, K.H., 2022. Strain engineering and metabolic flux analysis of a probiotic yeast *Saccharomyces boulardii* for metabolizing l-fucose, a mammalian mucin component. *Microb. Cell Fact.* 21. <https://doi.org/10.1186/s12934-022-01926-x>.
- Kim, J., Atkinson, C., Miller, M.J., Kim, K.H., Jin, Y.-S., 2023. Microbiome engineering using probiotic yeast: *Saccharomyces boulardii* and the secreted human lysozyme lead to changes in the gut microbiome and metabolome of mice. *Microbiol Spectr* 11. <https://doi.org/10.1128/spectrum.00780-23>.
- Klein, S.M., Elmer, G.W., McFarland, L.V., Surawicz, C.M., Levy, R.H., 1993. Recovery and elimination of the biotherapeutic agent, *Saccharomyces boulardii*, in healthy human volunteers. *Pharm. Res.* 10, 1615–1619. <https://doi.org/10.1023/A:1018924820333>.
- Koblner, V., Mumper, E., Baker, S.M., 2018. Reduction in obsessive compulsive disorder and self-injurious behavior with *Saccharomyces boulardii* in a child with autism: a case report. *Integr Med* 17 (6), 38–41. <https://doi.org/10.21037/tp-23-566>.
- Kurtz, C., Calvo, M., 2024. A proposed new path for Management of Health in inherited metabolic disorders using engineered probiotic Bacteria. *Nutr. Diet. Suppl.* 16, 177–184. <https://doi.org/10.2147/NDS.S481755>.
- Lau, C.S., Chamberlain, R.S., 2016. Probiotics are effective at preventing *Clostridium difficile*-associated diarrhea: a systematic review and meta-analysis. *Int J Gen Med* 9, 27–37. <https://doi.org/10.2147/IJGM.S98280>.
- Lee, J.W., Chan, C.T.Y., Slomovic, S., Collins, J.J., 2018. Next-generation biocontainment systems for engineered organisms. *Nature Chemical Biology* 14 (6), 530–537. <https://doi.org/10.1038/s41589-018-0056-x>.
- Lee, P., Yacyszyn, B.R., Yacyszyn, M.B., 2019. Gut microbiota and obesity: an opportunity to alter obesity through faecal microbiota transplant (FMT). *Diabetes Obes. Metab.* 21, 479–490. <https://doi.org/10.1111/DOM.13561>.
- Lei n. 11.105 de 24/03/2005 - Laws - Comissão Técnica Nacional de Biossegurança &ndash CTNBio [WWW Document]. URL. https://ctnbio.mctic.gov.br/en/leis/-/asset_publisher/NT53w3Yb7zpx/content/lei-n-11-105-de-24-03-2005 (accessed 11.28.24).
- Li, M., Xie, Y., 2025. Efficacy and safety of *Saccharomyces boulardii* as an adjuvant therapy for the eradication of *helicobacter pylori*: a meta-analysis. *Front. Cell. Infect. Microbiol.* 15, 1441185. <https://doi.org/10.3389/fcimb.2025.1441185>.
- Li, R., Wan, X., Takala, T.M., Saris, P.E.J., 2021a. Heterologous expression of the *Leuconostoc bacteriocin* Leucocin C in probiotic yeast *Saccharomyces boulardii*. *Probiotics Antimicrob Proteins* 13, 229–237. <https://doi.org/10.1007/s12602-020-09676-1>.
- Li, R., Yassami, S., Kiviniemi, E.A., Qiao, W., Takala, T.M., Saris, P.E.J., 2021b. *Listeria* decontamination of chicken meat with beer brewed with bacteriocin producing *Saccharomyces boulardii*. *LWT* 152, 112323. <https://doi.org/10.1016/J.LWT.2021.112323>.
- Li, T., Yang, Y., Qi, H., Cui, W., Zhang, L., Fu, X., He, X., Liu, M., Li, P., Feng Yu, T., 2023. CRISPR/Cas9 therapeutics: progress and prospects. *Signal Transduction and Targeted Therapy* 8 (1), 1–23. <https://doi.org/10.1038/s41392-023-01309-7>.
- Ling, H., Liu, R., Sam, Q.H., Shen, H., Chai, L.Y.A., Chang, M.W., 2023. Engineering of a probiotic yeast for the production and secretion of medium-chain fatty acids antagonistic to an opportunistic pathogen *Candida albicans*. *Front. Bioeng. Biotechnol.* 11, 1090501. <https://doi.org/10.3389/fbioe.2023.1090501/BIBTEX>.
- Liu, J.J., Kong, L.I., Zhang, G.C., Jayakody, L.N., Kim, H., Xia, P.F., Kwak, S., Sung, B.H., Sohn, J.H., Walukiewicz, H.E., Rao, C.V., Jin, Y.S., 2016. Metabolic engineering of probiotic *Saccharomyces boulardii*. *Appl. Environ. Microbiol.* 82, 2280–2287. <https://doi.org/10.1128/AEM.00057-16>.
- Liu, J.J., Zhang, G.C., Kong, L.I., Yun, E.J., Zheng, J.Q., Kweon, D.H., Jin, Y.S., 2018. A mutation in PGM2 causing inefficient galactose metabolism in the probiotic yeast *Saccharomyces boulardii*. *Appl. Environ. Microbiol.* 84 (10). <https://doi.org/10.1128/AEM.02858-17>, 2858–17.
- Liu, C.H., Chang, J.H., Chang, Y.C., Mou, K.Y., 2020. Treatment of murine colitis by *Saccharomyces boulardii* secreting atrial natriuretic peptide. *J. Mol. Med.* 98, 1675–1687. <https://doi.org/10.1007/s00109-020-01987-8>.
- Lundblad, V., 1993. Yeast cloning vectors and genes. *Curr. Protoc. Mol. Biol.* 21. <https://doi.org/10.1002/0471142727.MB1304S21>, 13.4.1–13.4.10.
- Luque-Sastre, L., Arroyo, C., Fox, E.M., McMahon, B.J., Bai, L., Li, F., Fanning, S., 2018. Antimicrobial Resistance in *Listeria* Species. *Microbiol Spectr* 6. <https://doi.org/10.1128/MICROBIOLSPEC.ARBA-0031-2017>.
- Maury, J., Germann, S.M., Baallal Jacobsen, S.A., Jensen, N.B., Kildegaard, K.R., Herrgård, M.J., Schneider, K., Koza, A., Forster, J., Nielsen, J., Borodina, I., 2016. EasyCloneMulti: a set of vectors for simultaneous and multiple genomic integrations in *Saccharomyces cerevisiae*. *PLoS One* 11. <https://doi.org/10.1371/JOURNAL.PONE.0150394>.
- McFarland, L.V., 2010. Systematic review and meta-analysis of *saccharomyces boulardii* in adult patients. *World J. Gastroenterol.* 16, 2202–2222. <https://doi.org/10.3748/wjg.v16.i18.2202>.
- McFarland, L.V., 2017. Common organisms and probiotics: *Saccharomyces boulardii*. The Microbiota in Gastrointestinal Pathophysiology: Implications for Human Health, Prebiotics, Probiotics, and Dysbiosis 145–164. <https://doi.org/10.1016/B978-0-12-804024-9.00018-5>.
- McFarland, L.V., Li, T., 2025. Efficacy and safety of *Saccharomyces boulardii* CNCM 1-745 for the treatment of pediatric acute diarrhea in China: a systematic review and meta-analysis. *Front. Cell. Infect. Microbiol.* 15, 1587792. <https://doi.org/10.3389/fcimb.2025.1587792/BIBTEX>.
- Michael, S., Keubler, L.M., Smoczek, A., Meier, M., Gunzer, F., Pöhlmann, C., Krause-Buchholz, U., Hedrich, H.J., Bleich, A., 2013. Quantitative phenotyping of inflammatory bowel disease in the IL-10-deficient mouse by use of noninvasive magnetic resonance imaging. *Inflamm. Bowel Dis.* 19, 185–193. <https://doi.org/10.1002/ibd.23006>.
- Ministry of Health, Labour and Welfare: Food Produced by Recombinant DNA Techniques [WWW Document]. URL. <https://www.mhlw.go.jp/english/topics/foodsafty/dna/01.html> (accessed 11.28.24).
- Mohaqiq, M., Palou, R., Li, R., Zhang, G., Vaishnav, H., Parweez, F., Moyana, T., Carragher, D., Cameron, D.W., Ramsay, T., van der Sloot, A., Murthy, S., Coombes, B., Tyers, M., Liu, X.J., 2025. Engineered spermidine-secreting *Saccharomyces boulardii* ameliorate colitis and colon cancer in mice. *bioRxiv*. <https://doi.org/10.1101/2025.01.19.633601>, 2025.01.19.633601.
- Mojica, F.J.M., Díez-Villasenor, C., García-Martínez, J., Soria, E., 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol.* 60, 174–182. <https://doi.org/10.1007/s00239-004-0046-3>.
- More, M.I., Vandenplas, Y., 2018. *Saccharomyces boulardii* CNCM 1-745 improves intestinal enzyme function: a trophic effects review. *Clin. Med. Insights Gastroenterol.* 11. https://doi.org/10.1177/1179552217752679/ASSET/IMAGES/LARGE/10.1177_1179552217752679-FIG1.JPEG.
- Murali, S.K., Mansell, T.J., 2024. Next generation probiotics: engineering live biotherapeutics. *Biotechnol. Adv.* 72, 108336. <https://doi.org/10.1016/J.BIOTECHADV.2024.108336>.

- Natural Health Products Regulations [WWW Document]. URL: <https://laws-lois.justice.gc.ca/eng/regulations/sor-2003-196/page-1.html> (accessed 11.28.24).
- Nayebhashemi, M., Enayati, S., Zahmatkesh, M., Madanchi, H., Saberi, S., Mostafaei, E., Mirbadeh Ardakani, E., Azizi, M., Khalaji, V., 2023. Surface display of pancreatic lipase inhibitor peptides by engineered *Saccharomyces boulardii*: potential as an anti-obesity probiotic. *J. Funct. Foods* 102, 105458. <https://doi.org/10.1016/j.jff.2023.105458>.
- Neurath, M.F., 2024. Strategies for targeting cytokines in inflammatory bowel disease. *Nature Reviews Immunology* 24 (8), 559–576. <https://doi.org/10.1038/s41577-024-01008-6>.
- Nielsen, J., 2013. Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. *Bioengineered* 4, 207. <https://doi.org/10.4161/BIOE.22856>.
- Offei, B., Vandecruys, P., De Graeve, S., Foulquié-Moreno, M.R., Thevelein, J.M., 2019. Unique genetic basis of the distinct antibiotic potency of high acetic acid production in the probiotic yeast *Saccharomyces cerevisiae* var. *Boulardii*. *Genome Res* 29, 1478–1494. <https://doi.org/10.1101/GR.243147.118/-DC1>.
- Oliveira, C., Teixeira, J.A., Lima, N., Da Silva, N.A., Domingues, L., 2007. Development of stable flocculent *Saccharomyces cerevisiae* strain for continuous aspergillus *Niger* beta-galactosidase production. *J. Biosci. Bioeng.* 103, 318–324. <https://doi.org/10.1263/JBB.103.318>.
- Oliveira, C., Aguiar, T.Q., Domingues, L., 2017. Principles of genetic engineering. *Current Developments in Biotechnology and Bioengineering: Foundations of Biotechnology and Bioengineering* 81–127. <https://doi.org/10.1016/B978-0-444-63668-3.00004-4>.
- Pais, P., Almeida, Y., Yilmaz, M., Teixeira, M.C., 2020. *Saccharomyces boulardii*: what makes it tick as successful probiotic? *J. Fungi (Basel)* 6, 1–15. <https://doi.org/10.3390/JOF6020078>.
- Park, J.S., Kim, Y.W., Kim, H., Kim, S.K., Park, K., 2023. Development of a novel ATP bioluminescence assay based on engineered probiotic *Saccharomyces boulardii* expressing firefly luciferase. *J. Microbiol. Biotechnol.* 33, 1506–1512. <https://doi.org/10.4014/jmb.2305.05019>.
- Parweez, F., Palou, R., Li, R., Kadhim, L., MacMillan, H., Tyers, M., Liu, X.J., 2025. Engineered spermidine-secreting *Saccharomyces boulardii* enhances olfactory memory in *Drosophila melanogaster*. *bioRxiv*. <https://doi.org/10.1101/2025.01.30.635726>, 2025.01.30.635726.
- Pfeifer, G.P., 2020. Mechanisms of UV-induced mutations and skin cancer. *Genome Instab Dis* 1, 99. <https://doi.org/10.1007/S42764-020-00009-8>.
- Phillips, G.J., 2001. Green fluorescent protein—a bright idea for the study of bacterial protein localization. *FEMS Microbiol. Lett.* 204, 9–18. <https://doi.org/10.1111/J.1574-6968.2001.TB10854.X>.
- Pickar-Oliver, A., Gersbach, C.A., 2019. The next generation of CRISPR-Cas technologies and applications. *Nat. Rev. Mol. Cell Biol.* 20, 490–507. <https://doi.org/10.1038/s41580-019-0131-5>.
- Pöhlmann, C., Thomas, M., Förster, S., Brandt, M., Hartmann, M., Bleich, A., Gunzer, F., 2012. Improving health from the inside: use of engineered intestinal microorganisms as in situ cytokine delivery system. *Bioengineered* 4, 172. <https://doi.org/10.4161/BIOE.22646>.
- Poncelet, A., Ruelle, L., Konopnicki, D., Miendje Deyi, V.Y., Dauby, N., 2021. *Saccharomyces cerevisiae* fungemia: risk factors, outcome and links with *S. Boulardii*-containing probiotic administration. *Infect Dis Now* 51, 293–295. <https://doi.org/10.1016/J.IDNOW.2020.12.003>.
- Pronk, J.T., 2002. Auxotrophic yeast strains in fundamental and applied research. *Appl. Environ. Microbiol.* 68, 2095. <https://doi.org/10.1128/AEM.68.5.2095-2100.2002>.
- Ravussin, Y., Xiao, C., Gavrilova, O., Reitman, M.L., 2014. Effect of intermittent cold exposure on Brown fat activation, obesity, and energy homeostasis in mice. *PLoS One* 9, e85876. <https://doi.org/10.1371/JOURNAL.PONE.0085876>.
- Rebeck, O.N., Wallace, M.J., Prusa, J., Ning, J., Evbuomwan, E.M., Rengarajan, S., Habimana-Griffin, L.M., Kwak, S., Zahrah, D., Tung, J., Liao, J., Mahmud, B., Fishbein, S.R.S., Ramirez Tovar, E.S., Mehta, R., Wang, B., Gorelik, M.G., Helmink, B.A., Dantas, G., 2025. A yeast-based oral therapeutic delivers immune checkpoint inhibitors to reduce intestinal tumor burden. *Cell Chem. Biol.* 32, 98–110.e7. <https://doi.org/10.1016/J.CHEMBIOL.2024.10.013>.
- Regulation - 1829/2003 - EN - EUR-Lex [WWW Document]. URL: <https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32003R1829> (accessed 11.28.24).
- Regulations on Administration of Agriculturally Genetically Modified Organisms Safety [WWW Document]. URL: https://english.mee.gov.cn/Resources/laws/regulations/Natural_Conservation_Biosafety/201708/t20170803_419103.shtml (accessed 11.28.24).
- Ribeiro, O., Gombert, A.K., Teixeira, J.A., Domingues, L., 2007. Application of the Cre-loxP system for multiple gene disruption in the yeast *Kluyveromyces marxianus*. *J. Biotechnol.* 131, 20–26. <https://doi.org/10.1016/J.JBIOTEC.2007.05.027>.
- Rottinghaus, A.G., Ferreira, A., Fishbein, S.R.S., Dantas, G., Moon, T.S., 2022. Genetically stable CRISPR-based kill switches for engineered microbes. *Nature Communications* 13 (1), 1–17. <https://doi.org/10.1038/s41467-022-28163-5>.
- Rouches, M.V., Xu, Y., Cortes, L.B.G., Lambert, G., 2022. A plasmid system with tunable copy number. *Nature Communications* 13 (1), 1–12. <https://doi.org/10.1038/s41467-022-31422-0>.
- Roy Sarkar, S., Mitra Mazumder, P., Chatterjee, K., Sarkar, A., Adhikary, M., Mukhopadhyay, K., Banerjee, S., 2021. *Saccharomyces boulardii* ameliorates gut dysbiosis associated cognitive decline. *Physiol. Behav.* 236. <https://doi.org/10.1016/J.PHYSBEH.2021.113411>.
- Salazar-Parra, M.A., Cruz-Neri, R.U., Trujillo-Trujillo, X.A., Dominguez-Mora, J.J., Cruz-Neri, H.I., Guzmán-Díaz, J.M., Guzmán-Ruvalcaba, M.J., Vega-Gastelum, J.O., Ascencio-Díaz, K.V., Zarate-Casas, M.F., González-Ponce, F.Y., Barbosa-Camacho, F. J., Fuentes-Orozco, C., Cervantes-Guevara, G., Cervantes-Pérez, E., Cervantes-Cardona, G.A., Cortés-Flores, A.O., González-Ojeda, A., 2023. Effectiveness of *Saccharomyces Boulardii* CNCM I-745 probiotic in acute inflammatory viral diarrhoea in adults: results from a single-Centre randomized trial. *BMC Gastroenterol.* 23, 1–7. <https://doi.org/10.1186/S12876-023-02863-8/TABLES/2>.
- Sands, C., Hedin, K.A., Vazquez-Urbe, R., Sommer, M.O.A., 2024. *Saccharomyces boulardii* promoters for control of gene expression in vivo. *Microb. Cell Fact.* 23, 16. <https://doi.org/10.1186/S12934-023-02288-8>.
- Scott, B.M., Gutiérrez-Vázquez, C., Sanmarco, L.M., da Silva Pereira, J.A., Li, Z., Plascencia, A., Hewson, P., Cox, L.M., O'Brien, M., Chen, S.K., Moraes-Vieira, P.M., Chang, B.S.W., Peisajovich, S.G., Quintana, F.J., 2021. Self-tunable engineered yeast probiotics for the treatment of inflammatory bowel disease. *Nat. Med.* 27, 1212–1222. <https://doi.org/10.1038/s41591-021-01390-x>.
- Sengupta, R., Mendenhall, A., Sarkar, N., Mukherjee, C., Afshari, A., Huang, J., Lu, B., 2017. Viral Cre-loxP tools aid genome engineering in mammalian cells. *J. Biol. Eng.* 11, 1–9. <https://doi.org/10.1186/S13036-017-0087-Y/FIGURES/4>.
- Shao, Z., Zhao, H., Zhao, Huimin, 2009. DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res.* 37. <https://doi.org/10.1093/NAR/GKN991>.
- Shin, J., Lee, G., Chi, W.-J., Park, S., Jin, Y.-S., Kim, S.R., 2025. Engineering *Saccharomyces boulardii* for cell surface display of heterologous protein. *J. Biotechnol.* 397, 44–50. <https://doi.org/10.1016/J.JBIOTEC.2024.11.013>.
- Silva, R., Aguiar, T.Q., Domingues, L., 2015. Blockage of the pyrimidine biosynthetic pathway affects riboflavin production in *Ashbya gossypii*. *J. Biotechnol.* 193, 37–40. <https://doi.org/10.1016/J.JBIOTEC.2014.11.009>.
- Spatz, M., Wang, Y., Lapiere, A., Da Costa, G., Michaudel, C., Danne, C., Michel, M.L., Langella, P., Sokol, H., Richard, M.L., 2023. *Saccharomyces boulardii* CNCM I-745 supplementation during and after antibiotic treatment positively influences the bacterial gut microbiota. *Front Med (Lausanne)* 10, 1087715. <https://doi.org/10.3389/FMED.2023.1087715/BIBTEX>.
- Spigaglia, P., Mastrantonio, P., Barbanti, F., 2018. Antibiotic resistances of *Clostridium difficile*. *Adv. Exp. Med. Biol.* 1050, 137–159. https://doi.org/10.1007/978-3-319-72799-8_9.
- Sternberg, N., Hoess, R., 1983. The molecular genetics of bacteriophage P1. *Annu. Rev. Genet.* 17, 123–154. <https://doi.org/10.1146/ANNUREV.GE.17.120183.001011/CITE/REFWORKS>.
- Stovicek, V., Borja, G.M., Forster, J., Borodina, I., 2015. EasyClone 2.0: expanded toolkit of integrative vectors for stable gene expression in industrial *Saccharomyces cerevisiae* strains. *J. Ind. Microbiol. Biotechnol.* 42, 1519. <https://doi.org/10.1007/S10295-015-1684-8>.
- Svara, F., Rankin, D.J., 2011. The evolution of plasmid-carried antibiotic resistance. *BMC Evol. Biol.* 11, 1–10. <https://doi.org/10.1186/1471-2148-11-130/FIGURES/6>.
- Szajewska, H., Kołodziej, M., 2015. Systematic review with meta-analysis: *Saccharomyces boulardii* in the prevention of antibiotic-associated diarrhoea. *Aliment. Pharmacol. Ther.* 42, 793–801. <https://doi.org/10.1111/APT.13344>.
- Tan, P.Y., Mohd Johari, S.N., Teng, K.T., Loganathan, R., Lee, S.C., Ngui, R., Selvadurai, K.R., Lim, Y.A.L., 2023. High prevalence of malnutrition and vitamin a deficiency among schoolchildren of rural areas in Malaysia using a multi-school assessment approach. *Br. J. Nutr.* 129, 454–467. <https://doi.org/10.1017/S0007114522001398>.
- Tang, S., Li, J., Li, Y., Du, H., Zhu, W., Zhang, R., Wan, J., 2024a. Effects of *Saccharomyces boulardii* on microbiota composition and metabolite levels in the small intestine of constipated mice. *BMC Microbiol.* 24, 1–13. <https://doi.org/10.1186/S12866-024-03647-0>.
- Tang, C., Zhang, L., Wang, J., Zou, C., Zhang, Y., Yuan, J., 2024b. Engineering *Saccharomyces boulardii* for probiotic supplementation of l-ergothioneine. *Biotechnol. J.* 19, e202400527. <https://doi.org/10.1002/BIOT.202400527>.
- Tercio, C., Dapigny, M., Andre, F., 2019. Beneficial effects of *Saccharomyces boulardii* CNCM I-745 on clinical disorders associated with intestinal barrier disruption. *Clin. Exp. Gastroenterol.* 12, 67. <https://doi.org/10.2147/CEG.S181590>.
- The National Bioengineered Food Disclosure Standard, 2025. Overview and Selected Considerations.
- Tippelt, A., Nett, M., 2021. *Saccharomyces cerevisiae* as host for the recombinant production of polyketides and nonribosomal peptides. *Microb. Cell Fact.* 20, 1–24. <https://doi.org/10.1186/S12934-021-01650-Y/FIGURES/8>.
- Trujillo, J.M., Nuñez, W., Smith, B.A., 2021. GLP-1 receptor agonists: an updated review of head-to-head clinical studies. *Ther. Adv. Endocrinol. Metab.* 12. <https://doi.org/10.1177/2042018821997320>.
- Ulfstedt, M., Hu, G.Z., Johansson, M., Ronne, H., 2017. Testing of auxotrophic selection markers for use in the moss *Physcomitrella* provides new insights into the mechanisms of targeted recombination. *Front. Plant Sci.* 8. <https://doi.org/10.3389/FPLS.2017.01850/FULL>.
- Van Gaever, F., Vandecruys, P., Driege, Y., Kim, S.W., Thevelein, J.M., Beyaert, R., Staal, J., 2024. Multi-Step Pathway Engineering in Probiotic *Saccharomyces Boulardii* for Abscissic Acid Production in the Gut. *bioRxiv*. <https://doi.org/10.1101/2024.12.22.629964>, 2024.12.22.629964.
- Van Hul, M., Cani, P.D., 2023. The Gut Microbiota in Obesity and Weight Management: Microbes as Friends or Foe? *Nature Reviews Endocrinology* 2023 19:5 19, 258–271. <https://doi.org/10.1038/s41574-022-00794-0>.
- Vandermeulen, G., Marie, C., Scherman, D., Pr  at, V., 2011. New generation of plasmid backbones devoid of antibiotic resistance marker for gene therapy trials. *Mol. Ther.* 19, 1942–1949. <https://doi.org/10.1038/MT.2011.182>.
- Wang, T., Sun, H., Zhang, J., Liu, Q., Wang, L., Chen, P., Wang, F., Li, H., Xiao, Y., Zhao, X., 2014. The establishment of *Saccharomyces boulardii* surface display system using a single expression vector. *Fungal Genet. Biol.* 64, 1–10. <https://doi.org/10.1016/J.FGB.2013.11.006>.
- Wang, L., Sun, H., Zhang, J., Liu, Q., Wang, T., Chen, P., Li, H., Xiao, Y., Wang, F., Zhao, X., 2015. Establishment and application of target gene disruption system in

- Saccharomyces boulardii*. Biotechnol. Bioprocess Eng. 20, 26–36. <https://doi.org/10.1007/S12257-014-0197-Z/METRICS>.
- Wang, X., Zhang, H., Yu, S., Li, D., Gillings, M.R., Ren, H., Mao, D., Guo, J., Luo, Y., 2024. Inter-plasmid transfer of antibiotic resistance genes accelerates antibiotic resistance in bacterial pathogens. ISME J. 18, wrad032. <https://doi.org/10.1093/ISMEJO/WRAD032>.
- WHO Facts Sheet: Obesity and overweight [WWW Document]. URL. <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight> (accessed 11.22.24).
- Wicker, T., Sabot, F., Hua-Van, A., Benzenen, J.L., Capi, P., Chalhoub, B., Flavell, A., Leroy, P., Morgante, M., Panaud, O., Paux, E., SanMiguel, P., Schulman, A.H., 2007. A unified classification system for eukaryotic transposable elements. Nature Reviews Genetics 12 (8), 973–982. <https://doi.org/10.1038/nrg2165>.
- Wombwell, E., Bransteitter, B., Gillen, L.R., 2021. Incidence of *Saccharomyces cerevisiae* fungemia in hospitalised patients administered *Saccharomyces boulardii* probiotic. Mycoses 64, 1521–1526. <https://doi.org/10.1111/MYC.13375>.
- Xia, J., Liu, T., Wan, R., Zhang, J., Fu, Q., 2025. Global burden and trends of the *Clostridioides difficile* infection-associated diseases from 1990 to 2021: an observational trend study. Ann. Med. 57, 2451762. <https://doi.org/10.1080/07853890.2025.2451762>.
- Xiao, X., Sarma, S., Menegatti, S., Crook, N., Magness, S.T., Hall, C.K., 2022. In silico identification and experimental validation of peptide-based inhibitors targeting *Clostridium difficile* toxin a. ACS Chem. Biol. 17, 118–128. <https://doi.org/10.1021/ACSCHEMBIO.1C00743>.
- Xu, L., Bai, X., Jeong, D., Lee, D., Semidey, F., Li, C., Oh, E.J., 2025. Engineering *Saccharomyces boulardii* for enhanced surface display capacity. Microb. Cell Fact. 24, 1–13. <https://doi.org/10.1186/S12934-025-02702-3>.
- Zeng, B.X., Yao, M.D., Xiao, W.H., Luo, Y.Z., Wang, Y., Yuan, Y.J., 2021. Endogenous 2 μ plasmid editing for pathway engineering in *Saccharomyces cerevisiae*. Front. Microbiol. 12, 631462. <https://doi.org/10.3389/FMICB.2021.631462/BIBTEX>.
- Zhao, S., Jiang, E., Chen, S., Gu, Y., Shangguan, A.J., Lv, T., Luo, L., Yu, Z., 2016. PiggyBac transposon vectors: the tools of the human gene encoding. Transl Lung Cancer Res 5, 120. <https://doi.org/10.3978/J.ISSN.2218-6751.2016.01.05>.