Assessing the Effectiveness of Engineered Probiotic Yeast at Attacking Pathogenic *Clostridium Perfringens*

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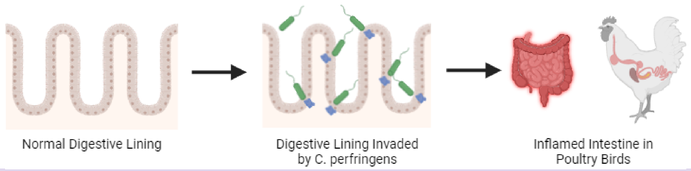
Introduction

- *Clostridium Perfringens* (*CP*) is an anaerobic, pathogenic bacteria that is primarily responsible for food-borne illnesses in humans and livestock. This is due to the production of viral toxins that destroy intestinal cells and cause gut diseases.
  - Ex. The Net B toxin is associated with the necrotic enteritis (chronic inflammation of the small intestine) disease in poultry birds.
- Antibiotics have been used as an effective method of treating this disease, however, they've been associated with increased prevalence of antibiotic resistant strains that are more pathogenic in nature.
- Alternatively, probiotics, such as the *Saccharomyces boulardii* (yeast) can be used as potential treatment options. This project focuses on modifying yeast to produce a therapeutic peptide, known as endolysin. Secreted endolysin from engineered yeast can specifically target and clear the *CP* in the gut, thus mitigating the necrotic enteritis disease.
- Modified yeast provides an effective alternative method to combating *CP* while lowering the risk of increased pathogenicity from antibiotics



Methods

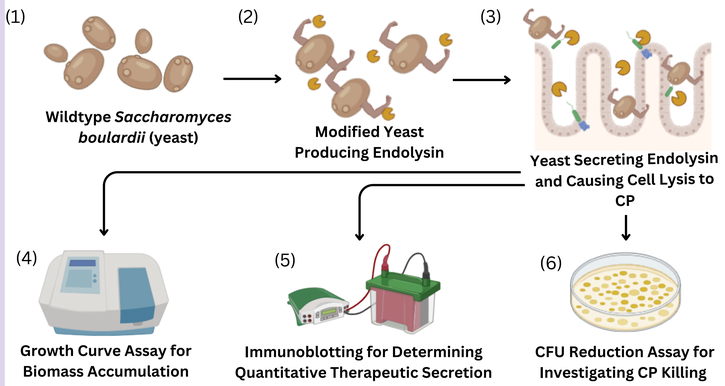


Figure 1. *Saccharomyces boulardii,* in its wildtype form (1), shows general probiotic effects against pathogens. We modified this yeast to produce heterologous proteins by null mutating certain proteases that degrade proteins (2). An endolysin gene was incorporated for constitutive production by the yeast (2). In the gut, this engineered yeast strain can effectively kill and clear *Clostridium perfringens* (3). This project focused on testing biomass accumulation and optimal enzyme production and activity *in vitro.* First, we conducted growth analysis by culturing the different strains of *S. boulardii* and constructed growth curves to conclude effective biomass accumulation (4). Supernatants from the yeast strains were immunoblotted to determine sufficient peptide production (5). To do this, a nanobody peptide of known concentration was also blotted and the densities of the nanobody bands were used to create a standard curve. This curve allowed us to determine the concentration of endolysin secreted by the different yeast strains (5). To study effective *CP* killing, we conducted a CFU reduction assay where supernatants from the engineered yeast strains where incubated with *CP* and then plated on agar plates (6). Colonies that appeared were counted and graphed as colony forming units (CFU)/mL.

Results

Engineered *S. boulardii* Strains Accumulate Sufficient Biomass via a Growth Curve

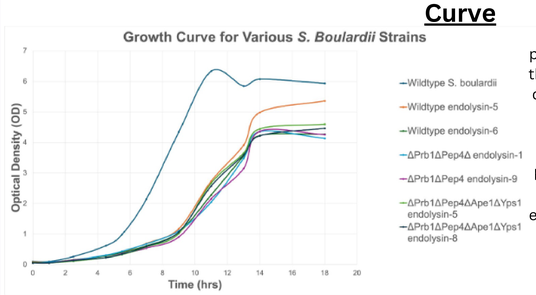
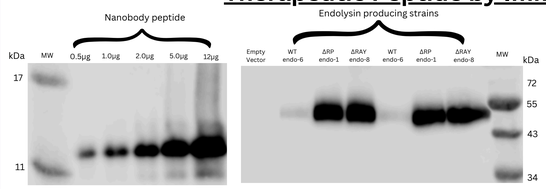


Figure 2. To improve the secretion of heterologous proteins, we previously engineered *S. boulardii* strains that contained null mutations of known proteases that degrade proteins (*PRB1, PEP4, APE1* and *YPS1).* These modifications can impede the normal growth of the cells, so a growth assay was conducted to see if the engineered strains could accumulate sufficient biomass. As expected, the graph shows that the wild-type strain grows the best. We also observed the engineered strains accumulating enough biomass over time, with the ΔPrb1ΔPep4 (ΔRP) strain growing the slowest and the ΔPrb1ΔPep4ΔApe1ΔYps1 (ΔRPAY) growing the fastest.

Engineered *S. boulardii* Displays Increased Secretion of the Endolysin Therapeutic Peptide by Immunoblotting



Strains	Concentration (µg/mL)	STDev (µg/mL)
WT endo-6	2.623957775	8.813203814
ΔPrb1ΔPep4 endo-1	22.68047616	0.7560441724
ΔPrb1ΔPep4ΔApe1 ΔYps1 endo-8	23.86077449	19.43868452

Figure 3. An immunoblotting assay was conducted to determine whether the engineered *S. boulardii* strains produce more endolysin. We constructed a standard curve to quantitatively endolysin produced by the yeast using a nanobody peptide of known concentration. The nanobody and endolysin both had the HA tag and could be probed on the same blot. Lanes 2-6 show increasing amounts of loaded nanobody. Lanes 9-14 show duplicate supernatant samples from the engineered *S. boulardii.* The density of the protein bands was determined, and a standard curve was made using the nanobody peptide. The density of the supernatant bands was then interpolated from the graph and used to calculate the concentration of endolysin. Those concentrations are summarized in the table shown.

Engineered *S. boulardii* Demonstrates Effective Killing of CP by a CFU Reduction Assay

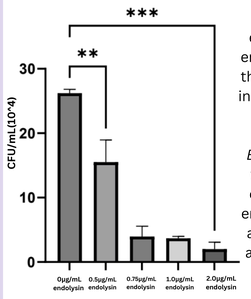


Figure 4A. To study the effectiveness of the endolysin enzyme, we first wanted to see if the endolysin gene incorporated in *S. boulardii* kills *C. perfringens.* To do this, we purified the endolysin protein from *Escherichia coli. C. perfringens* were incubated with different concentrations of the purified endolysin, plated on agar plates, and the colonies were counted and graphed as colony-forming units (CFU)/mL.

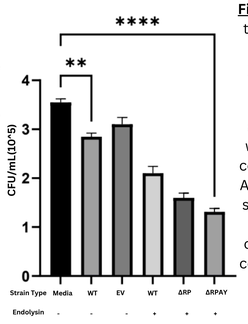


Figure 4B. We then investigated the effectiveness of endolysin secreted by the engineered yeast strains. *C. perfringens* was incubated with yeast supernatants and plated on agar plates, and the colonies were counted and graphed as colony-forming units (CFU)/mL. A one-way ANOVA test shows a significant difference between the ΔRPAY strain and the control media sample. We can conclude that the ΔRPAY strain is very effective at killing *CP* with a concentration of 21.6 µg/mL.

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BioRender and Prism for images

Conclusions

- The engineered yeast strains accumulated sufficient biomass, as shown by the growth assay
- Engineering *S. boulardii* strains by null mutating known proteases that degrade proteins (PRB1, PEP4, APE1 and YPS1) increases production of heterologous proteins i.e the endolysin therapeutic.
- The engineered *S. bouardii* strains show effective killing of CP *in vitro* - most significantly with the ΔRPAY strain
  - This also agrees with previously conducted anti-microbial assays, such as turbidity reduction assay.

Future Directions

- Establish a subclinical challenge model using live chickens to conclude the effectiveness of the engineered probiotic yeast *in vivo*.
- Create and test out a library of the endolysin gene to obtain constructs with improved killing efficiency.
- Combine other therapeutic options for treating pathogenic infections with the endolysin treatment. For example, incorporating nanobody production from yeast strains can decrease the adhesion of CP to the gut, while also killing and clearing the pathogen.

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