Design Brief

“Flavor by design: Engineering flavored probiotics in yogurt”*

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Yogurt is rich in nutrients, supports digestive health, boosts the immune system, and offers a wide range of other health benefits. Today, yogurt is commonly flavored with fruit; however, this introduces challenges related to flavor consistency, product quality, and shelf life due to the natural variability in fruit and the complexities of the fermentation process. The aim of our project is to develop a probiotic yogurt enhanced with genetically modified bacteria that naturally produce both banana flavor and a purple hue as an indicator. Our design uses a probiotic lactic acid bacterium chassis, Lactobacillus paracasei, engineered to express these traits. To construct our system, we will begin with a shuttle vector capable of functioning in both Escherichia coli and L. paracasei. Since E. coli is highly amenable to transformation and efficient at expressing foreign proteins, it serves as our host for initial vector construction and amplification. After successful assembly and isolation, the vector will be introduced into L. paracasei, which will then be inoculated into a yogurt culture for fermentation. In conclusion, the genetic modification of Lactobacillus not only enhances the nutritional and probiotic qualities of yogurt, but also addresses significant challenges in flavor consistency, reliability, and sustainability. This approach represents an innovative step forward in the development of next-generation food products that are both functional and environmentally conscious.

Keywords: synthetic biology, shuttle vector, GMO, lactic acid bacteria (LAB), Yogurt

Yogurt is rich in nutrients, high in protein, and packed with calcium. Yogurt also contains probiotics like with Lactobacillus bulgaricus and Streptococcus thermophilus that facilitate digestive health benefits while boosting the immune system (Zavišić, et al., 2023; Tennova Healthcare North Knoxville Medical Center, n.d.). The yogurt industry often adds fruit flavored options to their products during the manufacturing process to improve taste and product appeal. However, problems are frequently encountered with the texture, flavor, and consistency of quality because of changes in pH and heating during the fermentation process. Thus, the aim of this project is to produce yogurt with additional probiotics components that are genetically modified to produce their own natural flavors.

L. paracasei is a probiotic bacterium known for its health benefits and its potential application in yogurt production (Tennova Healthcare North Knoxville Medical Center, n.d.). *L. paracasei* is not traditionally used as a primary starter culture due to its limited ability to ferment lactose efficiently. Instead, a co-culture strategy that combines *L.*

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paracasei with traditional yogurt cultures has been developed to create a yogurt-like product that enhances both its probiotic content and functional properties (Lee et al., 2017). For this reason, we chose this organism as our "chassis" to engineer into producing a protein that facilitates banana flavor. Flavor can be an extremely complex trait controlled by many genes. To simplify our design, we focused on one-gene one-flavor that led us to the ATF1 gene used by a 2006 MIT iGEM Team (Dixon & Kuldell, 2011; iGEM Parts, 2013). The ATF1 gene, originally found in yeast cells, codes for an enzyme, Alcohol AcetylTransferase (AATase) that converts isoamyl alcohol into isoamyl acetate (UniProt. n.d.; Verstrepen et al., 2003; Figure 1). Isoamyl acetate is an active flavor ester responsible for banana flavor also used in several fermentation processes such as in the fermentation of alcoholic beverages (Verstrepen et al., 2003). Isoamyl alcohol is recognized as safe for consumption (United States Food and Drug Administration [FDA], 2023) .

The tsPurple gene, also known as tinsel purple, encodes a chromoprotein found in sea anemone (Liljeruhm et al., 2018). This chromoprotein will be integrated into our system as a selective marker to facilitate the visual identification of genetically modified *L. paracasei*. This marker was chosen because it offers a safe and visually detectable alternative to traditional antibiotic resistance markers, which are commonly used in research but are not suitable for food-grade applications due to safety and regulatory concerns (Shih et al., 2015; Chen et al., 2014). In addition to its practical role in confirming successful transformation, tsPurple provides an aesthetic benefit by producing a distinctive purple color, enhancing visual appeal (Savaiano, 2014).

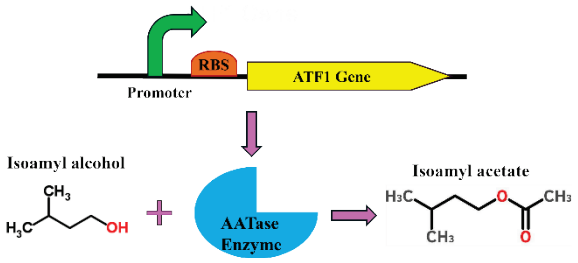

Figure 1. The ATF1 gene encodes alcohol acetyltransferase I (AATase), which is an enzyme that converts isoamyl alcohol into isoamyl acetate, creating a strong banana scent (iGEM Parts, 2013) and flavor (Verstrepen et al., 2003).

Consumer preferences for yogurt with diverse flavors and natural ingredients, as well as offering lactose intolerant individuals an alternative to traditional yogurt, provide additional support for this system (Savaiano, 2014). In conclusion, using genetically modified *Lactobacillus* to enhance flavor in yogurt production reduces the vast problems associated with growing and processing of fresh fruits, including climate change and economic issues that can cause less desirable taste, consistency, and quality, thus reducing the need for agro-food products and making it more sustainable (Deckers et al., 2020; Maryknoll Office for Global Concerns, 2013).

Systems level

Lactic acid bacteria (LAB), members of the *Lactobacillales* order, are widely utilized in the fermentation of milk into yogurt. However, a significant challenge in engineering these bacteria is their low transformation efficiency, largely due to their thick peptidoglycan layer. Additionally, the cell wall composition can vary between species, often requiring modified protocols for introducing foreign DNA via plasmids. To overcome this, we will first construct and amplify the genetically modified plasmid in *E. coli*, which is known for its high transformation efficiency and robust expression of foreign proteins. Once amplified, the plasmid will be isolated and purified, then shuttled into *L. paracasei* as the final host (Figure 2).

We will employ a shuttle vector designed to replicate in both *E. coli* and *Lactobacillus*, a method that has proven effective in previous studies for expressing recombinant proteins in LABs. For example, Suebwongsa and Panya successfully constructed a similar shuttle vector for protein expression, demonstrating its potential as a delivery system for oral vaccines. (Samperio et al., 2021; Suebwongsa et al., 2013; Suebwongsa

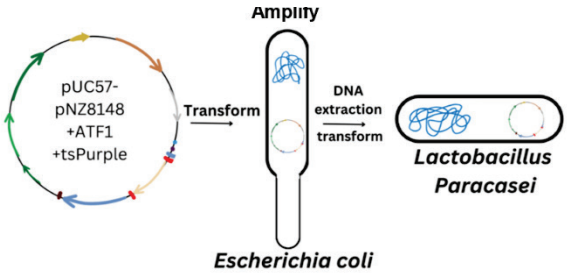

Figure 2. Overview of system-level design. Utilizing a genetically modified shuttle vector, the plasmid will be tested and amplified in E.coli before transforming it into L. paracasei.

et al., 2016).

The first step is to transform the plasmid into *E. coli* for rapid cloning and verification of gene expression. Once large quantities of plasmid DNA are extracted and confirmed, they will be introduced into *L. paracasei* (Figure 2). A successful transformation will be identified by the presence of both the banana aroma and the purple visual marker. The modified *L. paracasei* will then be cultured and used to ferment milk into yogurt, completing the development of a self-flavoring probiotic product.

This two-step transformation model enables verification of plasmid functionality in a high-efficiency host before transferring to a more challenging, food-safe host, thus ensuring a more reliable and effective approach.

Device level

The base or backbone of our construct, the plasmid, pUC57-pNZ8148, was selected because of its ability to support gene expression in both *Lactobacillus* and *E. coli*. We will insert two key genetic components into the plasmid: the ATF1 gene from plasmid pJL1, and the tsPurple gene along with an additional ribosome binding site (RBS) to ensure proper expression from plasmid pSB1C3 (Figure 3, top). To assemble the composite plasmid, we will employ the Gibson Assembly® method (NEBuilder), which allows for seamless, one-step joining of multiple DNA fragments into a single construct (Figure 3, bottom). This method is efficient and ideal for creating a fully functional shuttle vector for

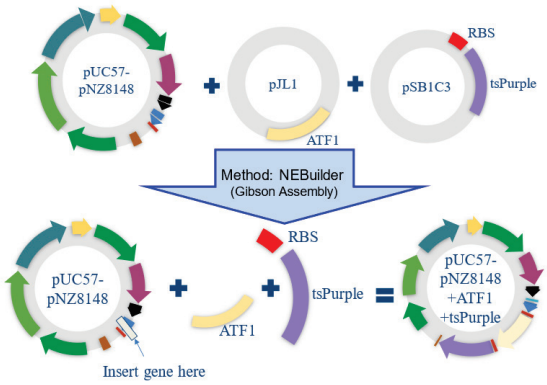

*Figure 3. **Top:** Three plasmids, the backbone and shuttle vector pUC57-pNZ8148, along with two plasmids containing our target genes, pJL1 containing the ATF1 gene and pSB1C3 containing our tsPurple gene. **Bottom:** ATF1 and tsPurple will be isolated and amplified by PCR and inserted into the shuttle vector which will be linearized by PCR. The final construct plasmid pUC57-pNZ8148+ATF1+tsPurple.*

transformation into our target chassis.

This shuttle vector design will enable both functional expression and visual confirmation of transformation across the two host organisms.

We will then transform our shuttle vector into *E. coli.* This vector includes two antibiotic resistance genes, which serve as markers for successful transformation. We will verify the proper expression of our gene in *E. coli*. Once confirmed, we will extract the plasmid and remove the two antibiotic resistance genes using PCR to ensure safety for real food applications.

Before inserting the plasmid into *Lactobacillus*, we will eliminate the antibiotic resistance genes, chloramphenicol acetyltransferase and ampicillin, as these pose significant risks to public health. Their excessive use contributes to environmental antibiotic contamination, which can lead to antibiotic-resistant infections (Centers for Disease Control and Prevention [CDC], 2024, Figure 4). Once the plasmid is modified, we will transfer it into the target host, *Lactobacillus*, employing the tsPurple chromoprotein as a visual marker to confirm successful transformation while adhering to health regulations. This plan carefully integrates precision in genetic engineering with a strong focus on safety standards.

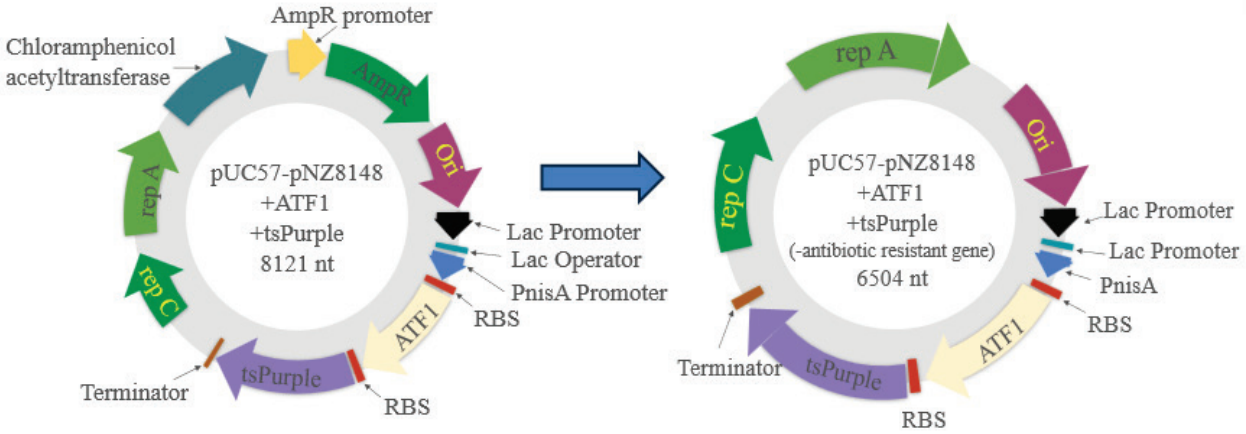

Figure 4. ***Right:*** *Plasmid map of the final construct of our shuttle vector that has been genetically engineered with target genes, ATF1 and tsPurple (reporter gene). Plasmid pUC57-pNZ8148+ATF1+tsPurple.* ***Left:*** *Vector after removal of the antibiotic-resistant genes, chloramphenicol and ampicillin after it has been tested and amplified in E. coli.*

Parts level

The plasmid pUC57-pNZ8148+ATF1+tsPurple is a shuttle vector engineered for dual-host gene expression in both *E. coli* and *Lactobacillus* species. This system incorporates two distinct promoters to drive gene expression in each host organism. For *E. coli*, the vector features the lac promoter, which is IPTG-inducible and optimized for high expression efficiency (Figure 5, Top). For *Lactobacillus*, the vector includes the PnisA promoter, which is induced by nisin, enabling controlled

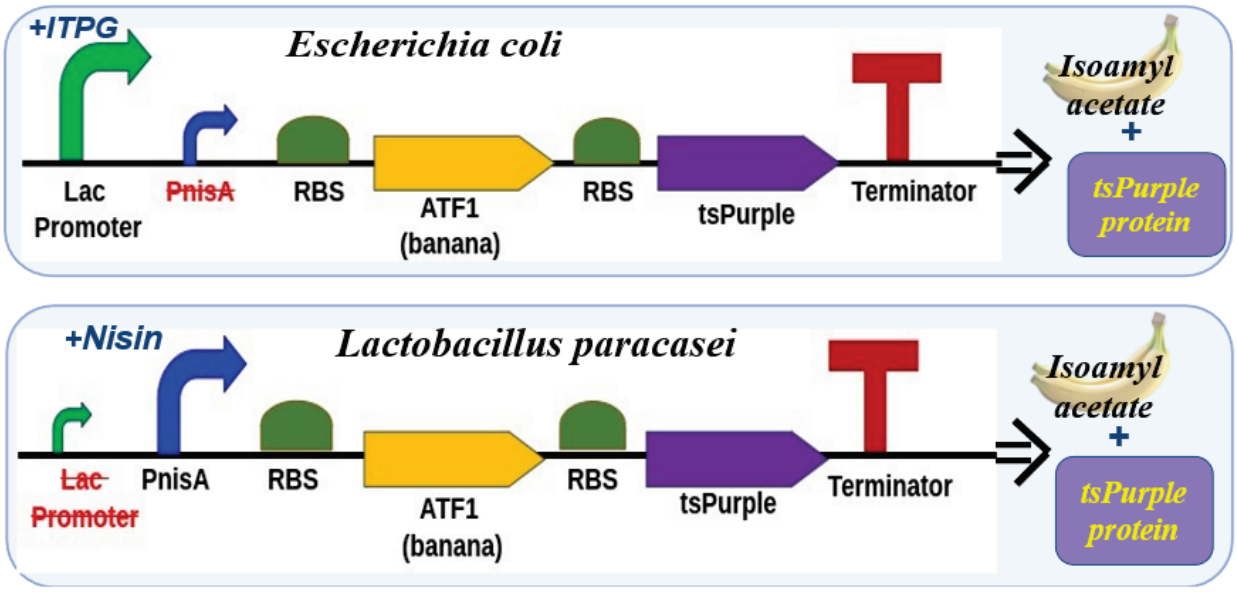

Figure 5. Top: Proposed gene expression of the shuttle vector in E. coli. ITPG will be used to induce the lac promoter leading to the transcription of the ATF1, which encodes an enzyme essential in the catalysis of the reaction. This enzyme converts isoamyl acetate to isoamyl alcohol, creating a strong banana smell, and the tsPurple reporter gene, which confirms successful transformation. Bottom: Proposed gene expression of the shuttle vector in L. paracasei. Nisin will be used to induce the PnisA promoter, leading to the transcription of the enzyme AAtase and tsPurple chromoprotein (Liljeruhm et al., 2018).

expression in probiotic strains (Mierau & Kleerebezem, 2005) (Figure 5, Bottom).

Both promoters are positioned to drive the expression of the ATF1 gene, which encodes an acetyltransferase enzyme responsible for converting isoamyl alcohol into isoamyl acetate, producing a banana-like aroma. Additionally, the tsPurple gene is included in the construct to serve as a visual marker, allowing for straightforward identification of successful transformations.

A transcription terminator sequence is also incorporated to ensure proper termination of gene expression and plasmid stability across both host systems.

Safety

E. coli is a commonly used host in synthetic biology for expressing foreign genes such as ATF1 and tsPurple. These applications must adhere to Biosafety Level 1 (BSL-1) protocols to ensure the safety of individuals and the environment. BSL-1 laboratories are designed for organisms that pose minimal potential hazard to laboratory personnel and the surrounding environment.

Proper personal protective equipment, including lab coats, gloves, and safety goggles, will be worn at all times to minimize exposure to biological materials (University of Washington Environmental Health & Safety, 2016). We will work in an aseptic environment, such as a biosafety cabinet or clean bench, to prevent contamination. Additionally, we will disinfect work surfaces before and after experiments using 70% ethanol or another approved disinfectant to maintain laboratory cleanliness and biosafety.

All waste will be disposed of properly in designated biohazard containers. Solid biological waste will be autoclaved before disposal, while liquid waste will be disinfected using a 10% bleach solution for at least 30 minutes before being safely poured down the drain with copious amounts of water (Biosafety Office of the Environmental Health & Safety Department, 2022). In addition, proper hand hygiene protocols will be followed; hands will be thoroughly washed before and after handling bacterial cultures to minimize the risk of contamination (U.S. Department of Health and Human Services, 2009).

Beyond standard biosafety protocols, the production of a food-grade product, such as banana-flavored yogurt, must comply with regulations set by the U.S. Food and Drug Administration (2024). The FDA's standard of identity for yogurt, codified in 21 CFR 131.200, defines yogurt as a product made by culturing cream, milk, partially skimmed milk, or skim milk, alone or in combination with a characterizing bacterial culture containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The final product must achieve a pH of 4.6 or lower to ensure microbiological safety and to maintain the defining qualities of yogurt (FDA, 2023a). Moreover, any additives or flavorings used must be safe and suitable for consumption. (U.S. Food & Drug Administration, 2023b).

By integrating BSL-1 biosafety protocols with FDA food-grade standards, researchers will ensure a safe, compliant, and high-quality end product.

Discussions

We considered several challenges in our proposal.

Challenge 1: Amplification and expression of foreign genes in Lactobacillus

One of the primary challenges in our proposal is the amplification and expression of foreign genes in *Lactobacillus*, which typically exhibits low transformation efficiency. We will utilize a shuttle vector that is compatible with both *E. coli* and *Lactobacillus* to address this issue. This strategy allows us to first amplify and test the plasmid in *E. coli*—a host known for its high transformation efficiency and ability to express foreign proteins—before transferring it into our final chassis, *L. paracasei*.

Challenge 2: Use of antibiotic resistance genes in food-grade systems

In traditional molecular biology, antibiotic resistance genes are commonly used in *E. coli* as selective markers to confirm successful transformation. However, these markers are not permissible in food-grade vectors due to health and environmental concerns. The presence of antibiotic resistance genes in food production raises the risk of horizontal gene transfer, contributing to the global rise in antibiotic-resistant bacteria (World Health Organization [WHO], 2011).

We will use PCR-based methods to eliminate the antibiotic resistance genes from our vector to overcome this issue, which we would then confirm through sequencing. Instead of using antibiotic resistance as a selective marker, we will incorporate the tsPurple gene, which encodes a chromoprotein that reflects purple light (Figure E–F), serving as a safe, visible indicator of successful transformation (Liljeruhm et al., 2018). Successful expression can also be confirmed through the banana aroma produced by the ATF1 gene product (acetyltransferase enzyme). These outcomes will be further validated using PCR and DNA sequencing.

Overall challenges: Regulatory and public perception

While transgenic organisms are widely used in both research and food production, their use in food systems must adhere to strict regulatory frameworks, such as the U.S. Coordinated Framework for the Regulation of Biotechnology (U.S. Food & Drug Administration, 2024). Beyond regulatory compliance, there are ongoing concerns related to biosafety, unintended consequences, and public perception. Misinformation surrounding GMOs continues to pose challenges, highlighting the need for transparency, education, and rigorous safety assessments in the development of synthetic biology-based food products.

Conclusion

The flavored yogurt market was valued at USD 50.82 billion in 2024 and is projected to grow at a compound annual growth rate (CAGR) of 8.7% from 2025 to 2030 (Grand View Research, 2025), indicating strong market potential for innovation in this sector (SkyQuest Technology, 2021; Souffriau et al., 2022). Rising consumer demand for diverse flavors, natural ingredients, and alternatives suitable for lactose-intolerant individuals further supports the feasibility of this project (Savaiano, 2021).

By genetically modifying *Lactobacillus* to enhance flavor production, our system offers a sustainable solution to many of the challenges associated with using fresh fruits in yogurt manufacturing. These challenges include the impact of climate change, economic fluctuations, and variability in fruit quality, all of which can affect taste, consistency, and shelf life. Engineered probiotics not only reduce reliance on agricultural inputs but also offer a more consistent, scalable, and eco-friendly approach to flavored yogurt production (Deckers et al., 2020).

Next steps

The immediate next phase of our project will involve testing our research design. Our first objective is to construct the shuttle vector and validate its function in *E. coli*. This includes confirming the successful expression of the ATF1 enzyme, indicated by the conversion of isoamyl alcohol into isoamyl acetate, which produces a characteristic banana aroma, and the expression of the tsPurple gene, observable as a visual purple marker.

Once successful expression is confirmed in *E. coli*, we will proceed with the amplification and purification of the construct, followed by its transformation into *L. paracasei*. The final step will involve testing our system in the context of yogurt production. We will explore two strategies:

1. Pre-culturing the modified bacteria and combining them with a yogurt starter culture.
2. Co-fermenting the system directly with milk during the yogurt fermentation process for in-situ flavor expression.

We hypothesize that co-fermentation

may be an efficient approach, as the banana flavor would be expressed from the beginning of the yogurt fermentation process, potentially resulting in a more robust aroma. However, we also plan to evaluate a pre-culturing method. Since our selected strain of *Lactobacillus* is typically used in cheese production rather than yogurt, it is important to assess its compatibility with yogurt fermentation. If *L. paracasei* can both support yogurt fermentation and express the desired flavor concurrently, co-culturing would represent a more scalable and streamlined method for future production.

Proof of concept

Successfully engineering a strain of *Lactobacillus* to produce its own flavor during yogurt fermentation would serve as a prototype for a new generation of flavor-enhanced, functional foods. This method opens the door to expressing a diverse array of natural flavors directly within probiotic cultures.

Using genetically modified organisms (GMOs) to enhance flavor in food presents a promising solution to several agricultural challenges. These include the seasonal and geographic limitations of fruit availability, the impact of climate change on crop yields, and the need to increase food production to meet the demands of a growing global population. By integrating flavor production into microbial fermentation systems, this approach offers a more sustainable, consistent, and scalable alternative to traditional flavoring methods in food manufacturing (Bel-Rhlid et al., 2018; Deckers et al., 2020; Maryknoll Office for Global Concerns, 2013).

Author contributions

This research project was conceived and developed by Ana White's Advanced Placement Biology class of 2024-25 and was a group effort. MHS was responsible for the plasmid designs and for writing the systems, device, and parts levels with the help of FB. MHS, YMO, and LOA were responsible for figures. The background information was collected and written by MMS with the help of YMO and LOA. All authors contributed to the discussion section the safety section was written by MMS and WO.

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