Decreasing the incidence of presbyopia through oxidative stress reduction with flavonoid-producing gut bacteria

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Presbyopia, or age-related farsightedness, is the most common eye disease. It is a hindrance to the day-to-day activities of more than 1.8 billion people around the globe up to the year 2015. Presbyopia is caused by a decrease in the concentration of alpha-crystallin and the accumulation of oxidative stress in the lens. This oxidative stress results in the hardening of the lens of the eye, resulting in difficulty focusing on objects nearby. Studies show that the daily intake of antioxidants—specifically, bioflavonoids—prevents the hardening of the lens. Our bodies do not produce flavonoids naturally. We gain most of our flavonoids through foods like citrus fruits, onions, and green tea. Quercetin is recognized for its antioxidant, anti-inflammatory, and anti-carcinogenic properties. Our project genetically engineers *Bifidobacterium*, a probiotic ubiquitous in the human gastrointestinal tract, to produce quercetin within our bodies. Quercetin then travels from the gut to the eyes, forming an in-situ flavonoid production and delivery system, inhibiting the accumulation of oxidative stress and preventing lens hardening. The potential pharmaceutical uses of quercetin have been extensively studied in labs and have demonstrated its positive effects. However, it's not yet widely employed in the fields of medicine. Our project would provide a permanent, cost-effective, nonsurgical treatment for presbyopia.

Keywords Presbyopia, oxidative stress, flavonoids, *Bifidobacteria*, quercetin

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Watch a video introduction by the authors at https://youtu.be/orfpB_kotvo

Background

Our eyes can perceive and process 30 to 35 frames of image memories per second due to more than 125 million light-sensing cells in each retina. These light-sensing cells make up about 70% of all sensory receptors in the human body (Brookshire and Saey, 2020; Larson, 2020). More than half of the sensory receptors of the neocortex solely process visual information. This gives the brain higher accuracy and

efficiency in processing visual information than other sensory information (Hutmacher, 2019). The eyes are an important sensory organ that helps us perceive and interpret the world. Humans rely heavily on their vision, meaning eye conditions can significantly affect one's quality of life.

A prevalent eye condition that typically appears during middle age is presbyopia. About a quarter of the world's population is affected by presbyopia (Fricke et al., 2018). Patients with presbyopia struggle to focus on nearby objects. The disease usually becomes noticeable around the age of 40 and continues to worsen for around 25 years, making day-to-day activities challenging.

Presbyopia (Figure 1) is a result of lens hardening due to the accumulation of oxidative stress (Web, 2017). Both the cornea and the lens contribute to the focusing ability of the eyes. Unlike the cornea, the lens is surrounded by a circular muscle that relaxes and constricts to focus on objects (*Presbyopia*, 2021). It consists of tightly packed fiber cells filled with highly concentrated crystalline proteins that ensure lens transparency, a state that must be maintained to avoid visual impairment (Wride, 2011). Alpha-crystallin is an insoluble chaperone protein responsible for shaping proteins. Without it, proteins will gradually build up within the lens as we age, making it harder for the lens to reshape and focus on nearby objects (Pescosolido et al., 2016).

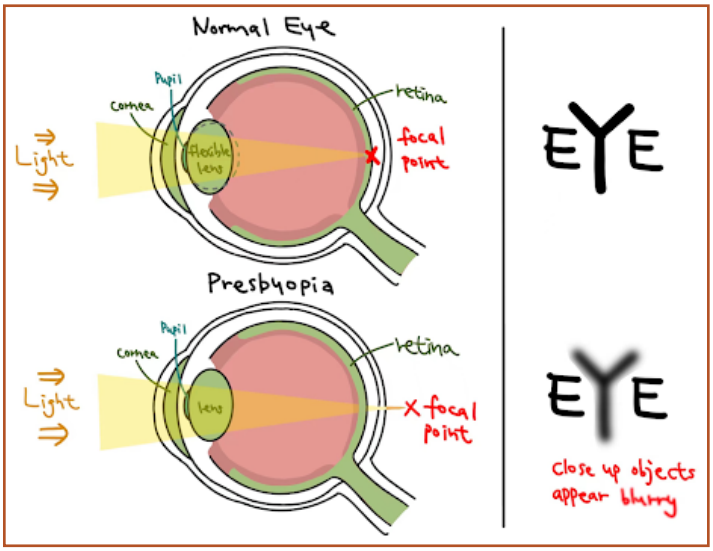

Figure 1. *Normal eye vs. presbyopia. Eyes with presbyopia have inflexible lenses that render the focal point falls behind the retina.*

Another major cause of lens hardening is oxidative stress. Unstable oxygen molecules, also known as reactive oxygen species (ROS), are generated through digestion, metabolism, and as a by-product of environmental stressors like radiation and pollution. Healthy bodies can balance ROS with antioxidants. However, when humans reach the age of forty, antioxidants in the bodies typically decrease (Black, 2021). This is why age-related eye diseases like cataracts and presbyopia happen more among older adults. Without the interference of antioxidants, ROS accumulates and induces oxidative stress in the eyes. Oxidative stress causes proteins in the lens to cross-link and aggregate, rendering them heavier and less elastic, therefore unable to adjust to focus on nearby objects (Figure 2).

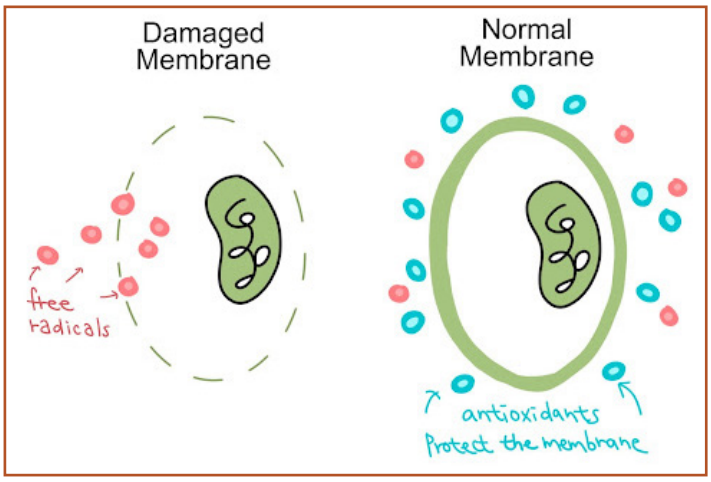

Figure 2. *Antioxidants protect cells and prevent free radicals from breaking through cell membranes, halting oxidative stress. Figure showing damaged cell membrane (left) and protected membrane (right).*

Common treatments of presbyopia include laser treatments, surgeries such as conductive keratoplasty and corneal inlays, and wearing eyeglasses or contact lenses (Mayo Clinic, 2021; Mukamal, 2022). Nonetheless, studies examining the effectiveness of these treatments stress that "the restoration of natural accommodation or an equivalent remains elusive" and that symptoms tend to come back within a short period (Wolffsohn & Davies, 2018). In addition, laser surgery is costly at $2,000 or more per eye (Kirkland, n.d.). Our team provides a more permanent solution to this vision problem: genetically engineering gut bacteria common in human bodies to produce quercetin, a dietary flavonoid abundant in nature (Anand David, Arulmoli, & Parasuraman, 2016).

Flavonoids are polyphenolic secondary metabolites that act as antioxidants in fruits and vegetables (Tungmunnithum et al., 2018). Quercetin is a well studied flavonoid in the fields of medicine and health, known for its antioxidative properties and its ability to efficiently scavenge ROS. Quercetin inactivates ROS by providing active hydrogen(Phaniendra et al., 2015). Research shows that quercetin is "effective even after the [retinal]

cells [are] exposed to oxidative stress, but before cell death [occurs]" (Majumdar & Srirangam, 2010). It is not only capable of preventing the impacts of aging but also eliminating the existing level of oxidation. By engineering Bifidobacteria to produce quercetin, we hope to prevent presbyopia.

Bifidobacterium, a bacterium ubiquitous in mammalian gastrointestinal tract microbiota, is used as a chassis. This probiotic is recognized for its ability to survive antimicrobial molecules and colonize the gut (Ruiz et al., 2012). *Bifidobacterium* would undergo several steps in the biosynthetic pathway to simulate the natural production of flavonoids in plants. There are many steps in biosynthetic pathways involved in the production of complex products. (Libretexts, 2021). The production of quercetin is one such complex biosynthetic pathway. First, TAL and 4CL convert L-tyrosine into p-coumaroyl-coenzyme A (CoA). The product is subsequently catalyzed by CHS, CHI, F3H, FLS, and CPR into producing quercetin (Stahlhut et al., 2015). We will genetically edit the eight genes needed for the production of quercetin into one plasmid using the start-stop assembly method. As *Bifidobacterium* replicates in human guts, it will consistently produce quercetin. Quercetin will be absorbed into the bloodstream via passive transport.

In the pharmaceutical industry, quercetin is well known for its positive effects as an antioxidant and the ability to control ocular blood pressure and maintain lens elasticity (Majumdar, 2010). However, it is not yet widely employed in medicine. Once the *Bifidobacterium* project is scaled up, we expect it to be a relatively inexpensive, more permanent, nonsurgical treatment for presbyopia.

Systems level

Bifidobacterium longum is a thoroughly studied strain of the genus *Bifidobacterium*. To fully simulate natural flavonoid production, four existing plasmids would be cut and annealed together. We will use electroporation to transform the four plasmids and one piece of genetic information expressing the last enzyme, *Cytochrome P450 reductase* (CPR), in sequence through the membrane into *Bifidobacterium longum*. Ideally, the restriction enzyme will cut the last plasmid used to affix the CPR gene.

To allow patients to orally consume the modified bacterium, we will utilize the freezing drying technique (*Figure 3*). According to the research by Dr. Regina Haindl and her co-workers, the bacteria will first undergo cultivation in an environment of pH 6.0 to replicate. Then it will go through a drying process of 3700 Pa constant chamber pressure after the addition of the protective medium of 75% BDM maltodextrin to preserve the viability of modified *Bifidobacterium longum*. The initial temperature is maintained at −10 °C for 12 hours, then raised to +10 °C for 6 hours to remove a large portion of the surface water, and held at +35 °C shelf temperature. This technology secures a high survival rate of 50% under +4 °C storage temperature for 70 days (Haindl et al., 2020).

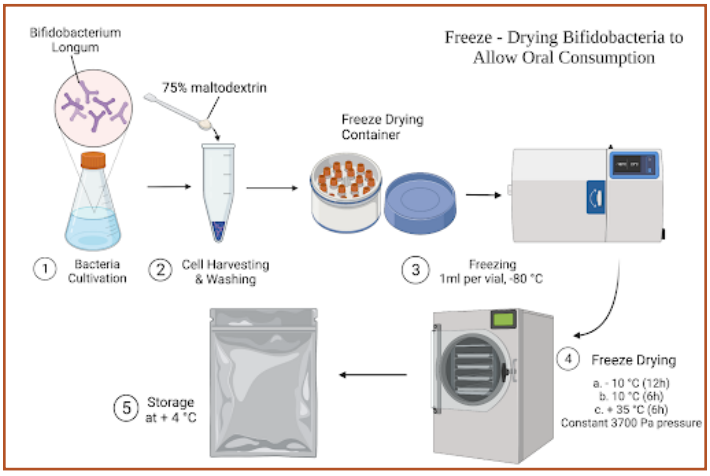

Figure 3. *Freeze-drying Bifidobacteria to allow oral consumption*

When patients orally consume the freeze-drying product, the modified bacteria will reach the stomach through the esophagus. Eventually, the bacteria will function similarly to other probiotics or common gut bacteria like *E. coli*, with one specialty of automatic generation of quercetin (Zhang et al., 2014). The quercetin molecules that *Bifidobacteria* produce within the body will cross the cell membrane and move into the bloodstream according to the concentration ingredient, resulting in medication absorption in systemic circulation (Alagga & Gupta, 2022).

Device level

We combined four plasmids, pY3, CDF (TAL, 4CL), pBR322 (CHS, CHI), and ST3 (F3H, FLS), in order to simulate real-life bioflavonoid production in *Bifidobacterium* (Figure 4). Each plasmid models one or two enzymes along the biosynthetic pathway for quercetin. Specifically, plasmid pY3 is responsible for expressing L-tyrosine. Plasmid CDF simulates the enzymatic reactions for TAL and 4CL into producing p-Coumaroyl-CoA. Plasmid pBR322 simulates the enzymatic reactions for CHS and CHI into producing Dihydrokaempferol. Lastly, we add the CPR gene into the combined plasmid to produce quercetin.

To insert eight genes into one plasmid, the start-stop assembly method is adopted for scarless combinatorial

assembly, which can have up to 15 coding sequences in one vector. This modular DNA and multi-part technology prevent scars at the highly sensitive sites and the boundaries of coding sequences from affecting the structure and function of mRNA and ribosome binding sites (Taylor et al., 2018). The genes would be inserted into one plasmid by order of the biosynthetic pathway: TAL, 4CL, CH5, CH1, F3H, FLS, and CPR.

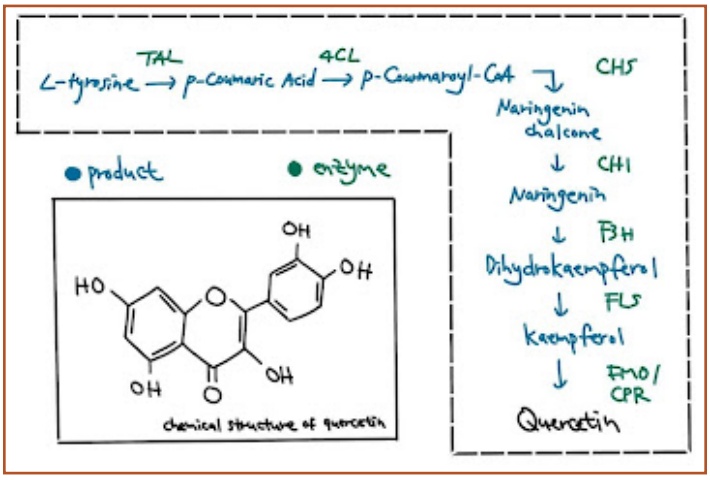

Figure 4. *Biosynthetic pathway and chemical structure of quercetin*

Parts level

The start-stop assembly method allows the combination of our eight genes of interest into one plasmid scarlessly, which decreases the possibility of assembly failure or function errors. The method has been effectively used to assemble multiple genes on one plasmid for metabolic engineering (Taylor et al., 2018). The vector backbone from each level consists of an assembly cassette, a resistance marker, one replicon, and two fusion sites: a donor fusion site and an acceptor fusion site. All the assembly cassettes include a lacZα gene that helps to identify recombinant DNA under blue/white screening. Resistance markers vary at different levels, but they all serve the same purpose of ensuring the successful transformation of our edited plasmid. A replicon determines the rate of transformation. The donor fusion site functions similarly to the restriction site in Levels 0-2 to insert the genes of interest, and the acceptor fusion site specifically aids in combining with the Level 3 vector due to its large size.

SapI and BsaI, two restriction enzymes, are used to cut off the DNA from the previous level as a whole. SapI restriction sites flank the parts stored in Level 1 inwardly to allow the insertion of Level 2 vectors with 5 targeted genes. BsaI fusion site then enables the addition of Level 2 vectors to Level 3 hierarchy that composes the last 3 targeted genes, resulting in the combination of all genes of interest in one plasmid (figure 5) (Taylor et al., 2018).

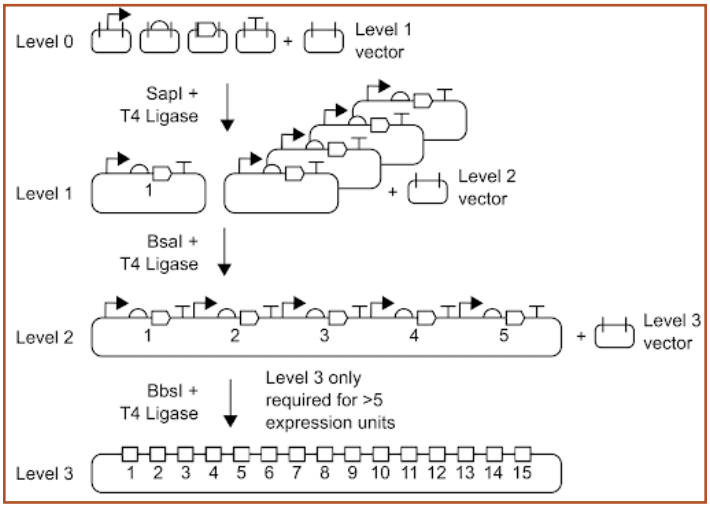

Figure 5. *Schematic illustration showing the overall framework for Start-Stop Assembly (Taylor et al., 2018)*

Safety

Our biggest concern regards implanting bacteria into human bodies. Bacteria are the major factor of ocular infections that can damage eye structures, resulting in visual impairments and blindness (Teweldemedhin et al., 2017). The safety of the presence of Bifidobacteria in the eyes is not guaranteed. This flaw turns our direction from designing a bacterial eye drop to orally consumed flavonoid-producing bacteria. The latter design prevents direct interaction between the bacteria and patients' eyes. The success of quercetin eye drops in *vivo* experiments at the McKay lab proves the safety of quercetin in human eyes (McKay and Karamichos, 2017). As a natural intestinal bacteria in the human gut, it is unlikely for *Bifidobacterium* to enter the blood circulation. However, exposure to bacteria in blood may lead to septicemia (blood poisoning). Due to its anaerobic characteristic, *Bifidobacterium* is specifically chosen to perform the task in the gut as it won't survive in the bloodstream in the presence of oxygen (Zhang et al., 2015). To further prevent the possibility of bacterial infection before our product enters the hypoxia environment of the gut, the capsule will have a shell that dissolves in the stomach and then release the active ingredient (Quality and Efficiency, 2011). Therefore, oral consumption will secure the safety and function of quercetin without the presence of bacteria in human eyes or other body parts before entering the gut.

In addition, *Bifidobacterium* is demonstrated to show long-term colonization within human bodies.

Once *Bifidobacteria* enter human guts, they can live up to as long as six years (Oki et al., 2018). Therefore, although Bifidobacterium is a probiotic that normally exists in our bodies, the potential issue of uncontrollable replications should still be studied. In *vitro* gut models run on lower costs, but they are simplified simulations of a much more complicated micro-ecosystem. In *vivo* experiments must eventually be performed on animals. Hoping to simulate real-life activities of the engineered *Bifidobacteria*, we would not conduct the experiment with germ-free animals. And due to the variability in gut microbiotas, we'd employ a larger sample size to ensure accurate information. According to guidelines in Biosafety in Microbiological and Biomedical Laboratories, The usage of Bifidobacterium is only warned against pregnant women and people with weakened immune systems, including those with AIDS, cancer, or on medications in preparations for organ transplants who are more susceptible to blood infections caused by probiotics. Additionally, although it is classified as level one biosafety contamination because of Bifidobacterium's long-term colonization, certain methods still need to be employed in order to prevent the release of engineered *Bifidobacterium* in the environment. These methods, including thymidine auxotrophy, microbial killing switch, DNAi technology which degrades targeted DNA only, need to be further evaluated (Zuo et al., 2020).

Another potential risk factor is the flavonoid that *Bifidobacterium* produces after genetic engineering. Quercetin is harmless when consumed orally: safe for most healthy adults in doses of 1 gram every day over 12 weeks. However, as *Bifidobacterium* colonizes and continues replication with the quercetin-producing function in the gut area, the possibility of overdose increases. To reduce the risk of kidney damage as a result of overdosing, the majority of plasmids that we selected contain a low-copy number replicon, which produces low DNA yields (Wood et al., 2016). Yet, due to health concerns, the mechanism still needs further research and tests.

Discussion

To realize the design, several potential obstacles need to be taken into consideration. We still need to evaluate the feasibility of genetically engineering *Bifidobacterium*. Unlike its more popular counterpart, *Escherichia coli*, *Bifidobacterium* is used as a medium less frequently in bioengineering. Electroporation is an effective method to make membranes of *E.coli* more permeable (Liu et al., 2018). Whether it is effective enough to render the membrane of *Bifidobacteria* adequately permeable is yet to be assessed.

Another potential impediment also exists in the metabolic engineering of *Bifidobacterium* for quercetin production. Compared to the size of *Bifidobacterium*, the engineered plasmid appears to be relatively big as it consists of multiple genes. The large size of the plasmid would significantly increase the difficulty of operations and affect the efficiency of transformation. As a result, the feasibility of the design may require further trials before initiating mass production.

We cannot trace *Bifidobacterium's* activities once it enters the body - once gut microbiota enters the intestine, they reproduce and colonize for the remainder of their lives. The 100 trillion gut microbiota settling in the human body form a complicated, codependent microbial system (Thursby and Juge, 2017). Although sufficient studies demonstrated that gut nutrients do manage to come into effect in the eyes, we are not sure about the specific transportation route from the intestine to the eye. Yet, we can infer from research that quercetin would travel through the vagus nerve and the bloodstream to reach the brain. Whether there would be enough concentration of quercetin that reaches the eyes to decelerate the progression of presbyopia is yet to be determined.

The relationship between the concentration of *Bifidobacterium* and its effect on the gut system should be investigated to determine the appropriate concentration of freeze-dried bacteria. We would design experiments to observe bacterial reproduction and the level of quercetin production in the gut. Another pathway will be potentially necessary if the absorption doesn't reach the ideal level of concentration. One solution would be utilizing carrier-mediated membrane transporters to actively transfer quercetin into circulation. Transporters like PEPT1 could significantly raise intestine absorption and oral bioavailability of nutrients (Katsura & Inui, 2015).

Next steps

We need to obtain our chassis, *Bifidobacterium longum* 105-A, from RIKEN-JCM in Japan for hands-on experiments. Due to its high transformation efficiency, we predict that it is the best host for the rapid culture of bacteria. In addition, our laboratory has the environment to incubate and reproduce this bacterium for future experiments (Kanesaki et al., 2014). Before the arrival of *B. longum* 105-A, we need to start looking for sources of possible promoter and terminator genes.

We must decide the specifics of combining multiple genes in the near future. In the meantime, we should continue researching plasmids that might ameliorate

the design and keep refining and testing different gene combinations to achieve the best results.

Author contributions

B.L generated the original idea. B. L., D. L., C. M., and P. W. contributed to the preliminary research on the feasibility of the project, wrote, and proofread the abstract and introduction collaboratively. B.L and D.L were responsible for the design, safety, and discussion portion of the project. B. L. and D. L. also produced the graphics and video for this article. C.M and P.W completed the writing for the next steps together. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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