

To Nurcan, Teoman, Duru and Çağlar



**ENGINEERING *Lactobacillus plantarum* AS A
RECOMBINANT HOST PLATFORM**

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**By
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Engineering *Lactobacillus plantarum* as a Recombinant Host Platform

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July 2025

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

ENGINEERING *Lactobacillus plantarum* AS A RECOMBINANT HOST PLATFORM

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Lactobacillus plantarum is a versatile lactic acid bacterium recognized for its probiotic properties and long-standing use in fermented foods. While traditional microbial chassis such as *E. coli* or *S. cerevisiae* have dominated recombinant bioproduction platforms, their limitations in food-grade and probiotic applications necessitate the development of alternative hosts. *L. plantarum*, with its GRAS status, genetic malleability, and resilience under gastrointestinal conditions, has emerged as a promising next-generation chassis, especially for the biosynthesis of health-related metabolites.

The aim of this study is to engineer *L. plantarum* WCFS1 as a recombinant host platform for the production of functional molecules, with an emphasis on 2'-fucosyllactose (2'-FL), a key human milk oligosaccharide known for its prebiotic and immunomodulatory benefits. To achieve this, advanced synthetic biology tools such as

markerless CRISPR/Cas9 editing, auxotrophic selection strategies, and modular expression systems will be employed to introduce and regulate heterologous gene pathways responsible for 2'-FL biosynthesis. The project will also focus on overcoming strain-specific challenges such as transformation efficiency, plasmid stability, and metabolic balancing within the host.

If successful, the engineered *L. plantarum* strain will serve as a live, food-compatible cell factory capable of producing 2'-FL either in fermenters or directly in situ as part of synbiotic formulations. The outcome of this work is expected to contribute to the growing field of probiotic metabolic engineering, offering a scalable, safe, and consumer-friendly platform for functional food innovation.

Keywords: *Lactobacillus plantarum*, Recombinant Host, Probiotic Metabolic Engineering, CRISPR/Cas9 genome editing, Synthetic Biology

ÖZET

REKOMBİNANT KONAK PLATFORMU OLARAK *Lactobacillus plantarum*'un MÜHENDİSLİĞİ

Derin Akman

Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

Tez Danışmanı: Urartu Özgür Şafak Şeker

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Lactobacillus plantarum, probiyotik özellikleri ve fermente gıdalardaki uzun süreli kullanımıyla bilinen çok yönlü bir laktik asit bakterisidir. *E. coli* ve *S. cerevisiae* gibi geleneksel mikrobiyal şasiler rekombinant biyoproduksiyon platformlarında uzun süredir kullanılmakta olsa da, gıda güvenliği ve probiyotik uygulamalardaki sınırlamaları, alternatif konak hücrelerin geliştirilmesini gerekli kılmaktadır. GRAS (Genel Olarak Güvenli Kabul Edilen) statüsü, genetik olarak düzenlenebilirliği ve gastrointestinal koşullara karşı dayanıklılığı sayesinde *L. plantarum*, özellikle sağlıkla ilişkili metabolitlerin biyosentezi için gelecek vadede bir şasi olarak öne çıkmaktadır.

Bu çalışmanın amacı, *L. plantarum* WCFS1 suşunu fonksiyonel moleküllerin üretimi için rekombinant bir konak platform olarak tasarlamaktır. Bu kapsamda, prebiyotik ve immünomodülatör etkileriyle öne çıkan insan sütü oligosakkaritlerinden 2'-

fukosillaktoz (2'-FL) hedeflenmektedir. Bu hedefe ulaşmak için, heterolog gen yollarının *L. plantarum* genomuna entegre edilmesi ve düzenlenmesi amacıyla markörsüz CRISPR/Cas9 düzenleme sistemleri, oksotrofik seleksiyon stratejileri ve modüler ekspresyon sistemleri gibi ileri düzey sentetik biyoloji araçları kullanılacaktır. Çalışma ayrıca, konak özelindeki transformasyon verimliliği, plazmid stabilitesi ve metabolik dengeleme gibi zorlukların aşılmasına da odaklanacaktır.

Başarılı olunması halinde, tasarlanan *L. plantarum* suşu; fermentörlerde veya sinbiyotik formülasyonlar aracılığıyla doğrudan bağırsak ortamında 2'-FL üretebilecek, canlı ve gıdayla uyumlu bir hücre fabrikası olarak işlev görecektir. Bu projenin çıktıları, probiyotik metabolizma mühendisliği alanına katkı sunarak, işlevsel gıda geliştirme süreçlerinde ölçeklenebilir, güvenli ve kullanıcı dostu bir platformun önünü açacaktır.

Anahtar Kelimeler: *Lactobacillus plantarum*, Rekombinant Konak, Probiyotik Metabolizma Mühendisliği, CRISPR/Cas9 Genom Düzenleme, Sentetik Biyoloji

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CHAPTER I: INTRODUCTION

1.1 Recombinant Microbial Platforms in Biotechnology

Recombinant DNA technology and metabolic engineering have enabled the production of high-value metabolites from a variety of microbial organisms. *Escherichia coli*, baker's yeast (*Saccharomyces cerevisiae*), and industrial Gram-positive bacteria such as *Corynebacterium glutamicum* are among the species that have been studied traditionally. These hosts have been customized to produce a variety of products, including nutraceuticals and biopharmaceuticals [1], [2]. History has come a long way in terms of synthetic biology and metabolic engineering since 1991, when the term "metabolic engineering" was first mentioned, and scientists have been trying for years to turn microbes into small cell factories that produce high-quality and valuable molecules. These efforts typically involve careful analysis of a metabolic pathway and how to engineer it, as the directed improvement of a new product or its use by another organism requires the careful handling of synthetic biology methods to modify specific biochemical processes or to create new ones[3]. A representative example of this progress is the microbial biosynthesis of human milk oligosaccharides (HMOs), such as 2'-fucosyllactose (2'-FL) which are traditionally difficult to obtain through chemical synthesis or extraction[4]. Recombinant *E. coli* strains, however, has become the primary production platform. Engineered *E. coli* strains can efficiently produce 2'-

fucosyllactose (2'-FL), a widely used HMO, reaching multi-gram per liter titers during fermentation[5]. Nevertheless, *E. coli* presents limitations for food-grade applications, as it is a Gram-negative bacterium which contains endotoxin (lipopolysaccharide) in its outer membrane, raising safety and regulatory concerns if cells were to be present in consumer products[6]. Thus, while *E. coli* remains a popular choice for microbial production due to its efficiency, the presence of endotoxins in its outer membrane requires careful purification steps, particularly when the final product is meant to be used in food, cosmetics, or health-related applications [7].

Yeast (*S. cerevisiae*) offers an attractive alternative chassis for such purposes. *S. cerevisiae* is a GRAS (Generally Recognized As Safe) organism long used in food and brewing, and it lacks endotoxins [8]. Recombinant yeast can perform complex eukaryotic metabolic functions and has been engineered to produce 2'-FL as well. Early efforts in yeast achieved only modest HMO titers (on the order of 0.5 g/L 2'-FL) [6], partly due to challenges like the need to introduce heterologous transporters and glycosyltransferases. However, subsequent metabolic enhancements have dramatically improved yields. For example, introducing a dedicated lactose transporter and optimizing the GDP-fucose synthesis pathway and fucosyltransferase expression enabled *S. cerevisiae* to produce over 25 g/L of 2'-FL in fed-batch culture [3]. This case illustrates how microbial hosts can be strategically engineered to enhance biosynthetic efficiency and meet industrial production demands for high-value biomolecules, and underscores that yeast can be a viable, endotoxin-free production host, although fermentation parameters and genetic constructs must be carefully tuned. Yeast's robustness and industrial familiarity make it suitable for large-scale processes, but *S. cerevisiae* is not of probiotic origin and generally does not colonize the human gut, which may limit its direct use in probiotic or symbiotic food products [9].

Another workhorse, *Corynebacterium glutamicum*, represents a Gram-positive platform extensively used in amino acid fermentation. *C. glutamicum* is considered safe and has no endotoxin, making it promising for food-related bioprocesses. Metabolic engineers have recently constructed *C. glutamicum* strains capable of de novo 2'-FL biosynthesis [10]. By introducing the complete GDP-L-fucose pathway and an α -1,2-fucosyltransferase, along with transport engineering, an engineered *C. glutamicum* produced about 21.5 g/L of 2'-FL in fed-batch culture [10]. This titer approaches the levels achieved in *E. coli*, demonstrating that non-pathogenic GRAS microbes can attain industrially relevant productivity. *C. glutamicum* also offers inherent advantages in fermentation robustness and has long been used at ton-scale production (e.g. for amino acids), suggesting that scale-up to manufacture HMOs is feasible [11]. Its limitation is that, like yeast, *C. glutamicum* is not of probiotic origin and generally does not colonize the human gut. Instead, it has a long history as a reliable microbial platform for industrial amino acid production, rather than serving as a live additive to functional foods [12].

Emerging GRAS hosts such as *Bacillus subtilis* have likewise been explored. *B. subtilis* is a spore-forming bacterium widely used for enzyme production and considered safe for food use. In a recent study, an engineered *B. subtilis* achieved extraordinarily high titers of 2'-FL (up to ~88 g/L) in bioreactor cultures [13]. This was accomplished by assembling a de novo 2'-FL pathway in *Bacillus* and optimizing sugar transport, cofactor regeneration, and precursor supply, combined with use of a high-copy, inducer-free expression system. Notably, the final *B. subtilis* process omitted antibiotic selection and inducer chemicals, highlighting a fully food-grade production scenario [13]. These results are remarkable as they indicate that a non-pathogenic, food-compatible microbe can rival or exceed the productivity of *E. coli*. Although *Bacillus*

subtilis can be isolated from the human gastrointestinal tract using specialized techniques, it is typically only present in low abundance and is not a stable or dominant member of the gut microbiome [14]. Therefore, its use is better suited for fermentative production rather than as a live probiotic in food applications.

Beyond HMOs, recombinant microbial platforms have also been explored for producing postbiotics, which are health-promoting compounds derived from microbial activity. Engineered bacteria, including strains originally known for their probiotic use, have shown promising results in postbiotic production. For instance, *Lactobacillus plantarum* (a lactic acid bacterium discussed in detail in Section 1.2) was recently modified to enhance γ -aminobutyric acid (GABA) production in fermented food. GABA is a health-promoting compound (credited with anti-hypertensive and calming effects) and is considered a postbiotic when delivered via fermented foods. Hussin *et al.* (2021) demonstrated that a novel self-cloned *L. plantarum* strain could significantly elevate GABA levels in a model yogurt fermentation [15]. By supplementing simple carbohydrates to the fermentation to drive the microbe's glutamate decarboxylase pathway, the GABA-enriched yogurt reached ~83.7 mg of GABA per 100 g – nearly doubling the GABA content compared to control fermentations [15]. Importantly, the GABA-rich yogurt retained high probiotic cell viability and stability during refrigerated storage, illustrating how an engineered probiotic culture can produce beneficial metabolites *in situ* without compromising the food matrix. This example highlights a key advantage of using food-grade probiotic chassis, that they can be deployed directly in fermented functional foods or as live supplements, delivering postbiotic benefits *in vivo*. In general, traditional hosts like *E. coli* or yeast cannot be used this way, since they either are not safe for consumption in an unprocessed state or lack probiotic

functions. This has motivated increasing interest in next-generation chassis that are both industrially tractable and inherently safe for nutrition and health applications [16].

These considerations have led to increased interest in GRAS and probiotic hosts that combine metabolic engineering potential with food safety. One such candidate is *Lactobacillus plantarum* WCFS1, which will be discussed in the following section.

1.2 *Lactobacillus plantarum* WCFS1 as a Next Generation Chassis

Lactobacillus plantarum (also known as *Lactiplantibacillus plantarum*) is a lactic acid bacterium widely recognized for its probiotic attributes and long history of use in fermented foods. Their cells are straight, rounded-ended rods that are typically 0.9–1.2 μm broad and 3–8 μm long. They occur single, in pairs, or in short chains [17]. They can survive under a broad range of pH values (3.4–8.8) and temperatures (12–40 $^{\circ}\text{C}$) [18]. The specific strain *L. plantarum* WCFS1 (a single-colony isolate from a human saliva-derived strain, NC_004567.2) has served as a model organism for this species, in part because it was the first *L. plantarum* strain to have its genome completely sequenced [19]. The complete genomic blueprint of WCFS1 (~3.3 Mbp, with a GC content of ~44%) is thus accessible, enabling sophisticated genetic and systems biology research. Among lactic acid bacteria (LAB), *L. plantarum* stands out for having one of the largest genomes, which encodes a wide range of functions related to stress response and carbohydrate utilization [19]. This genetic richness supports the outstanding metabolic flexibility and environmental resilience of *L. plantarum*. Strains of this species can thrive in a wide variety of environments, including plant-based fermentations such as sauerkraut and pickles, dairy products, and the human digestive tract. They readily adapt to varying conditions of pH, temperature, and oxygen availability [19]. The WCFS1 strain, in particular, has been extensively characterized,

revealing genes for tolerating acid and bile stress, polysaccharide metabolism, and other probiotic traits. Critically, *L. plantarum* is classified as a “food-grade” microorganism. It is generally regarded as safe (GRAS in the US, and QPS, or Qualified Presumption of Safety in the EU) for use in foods and supplements, given its long record of safe consumption in fermented foods. Unlike *E. coli*, *L. plantarum* produces no endotoxins; and unlike some *Bacillus* species, it does not form spores (which in presence, can complicate certain food processes). *L. plantarum* is already consumed alive in many fermented products, meaning that an engineered derivative of this species has a greater likelihood of regulatory acceptance for food or probiotic use (assuming any genetic modifications are achieved in a safe manner). Indeed, *L. plantarum* has been called a well-recognized food-grade host for recombinant protein production and biotechnology applications [16], [20], [21], [22], [23]. *Lactobacillus plantarum* has probiotic characteristics that include the ability to survive passage through the stomach, remain active for a limited time in the intestine, and interact beneficially with the gut microbiota. These features make it a suitable microbial host for delivering functional molecules within the gastrointestinal tract. Studies have shown that *L. plantarum* can enhance intestinal barrier function and modulate immune responses, and these effects may be strengthened through the recombinant production of selected biomolecules [24]. One of the advantages of the WCFS1 strain is its well-documented tolerance to stress conditions. Comparative genome analyses have identified various stress-response mechanisms in this species, including genes involved in bile salt resistance, such as those encoding bile salt hydrolases that enable survival in the gut environment [25], [26]. These features explain why *L. plantarum* can survive the acidic conditions of the stomach and the bile-rich environment of the small intestine, which stand out as the core prerequisites for a probiotic intended to deliver benefits in the host [27], [28].

Additionally, *L. plantarum* is facultatively heterofermentative and aerotolerant. It can tolerate oxygen, which it uses to degrade byproducts such as hydrogen peroxide and switch between homolactic and minor heterolactic pathways depending on environmental conditions [29], [30], [31]. This flexibility is beneficial in industrial settings where oxygen and nutrient levels can fluctuate. The strain WCFS1 also exhibits high salt tolerance and can endure refrigeration, which is useful for production and storage of starter cultures [32]. From a genomic and regulatory standpoint, *L. plantarum* WCFS1 offers a solid foundation for recombinant platform development. The availability of its complete genome sequence has enabled bioinformatic mining for metabolic pathways and potential genetic targets. It also means that metabolic engineering strategies (gene knockouts, insertions, or pathway optimizations) can be rationally designed with full knowledge of native gene context [33]. Despite many favorable attributes, there are challenges and considerations when using *L. plantarum* as a recombinant production host. One consideration is its growth profile. *L. plantarum* has a slower doubling time than *E. coli* and often requires complex, nutrient-rich media (such as MRS broth) for robust growth. This could impact volumetric productivity in fermentation processes [34], [35]. Additionally, lactic acid bacteria like *L. plantarum* secrete lactic acid as a major metabolic end-product, which can lower the pH of the culture and inhibit growth at high cell densities unless pH is controlled. Industrial processes using *Lactobacilli* typically mitigate this by base addition or employing fed-batch strategies [36]. Historically, *L. plantarum* has also been limited by its resistance to genetic modification when compared to common laboratory organisms like *E. coli*. Its strong restriction-modification mechanisms and thick peptidoglycan cell wall, which can impede DNA uptake and stability, are partially accountable for this. Furthermore, until recently, *L. plantarum* lacked a broad range of molecular tools due to its status as

a non-model organism. But in the last ten years, a lot of progress has been done. Numerous studies have shown that *L. plantarum* can be transformed and genetically altered with high efficiency when the right procedures are followed. As covered in the next section, a number of contemporary techniques, such as CRISPR-based systems, now enable more accurate and consistent changes [37], [38]. There are a few things to consider when comparing *L. plantarum* WCFS1 with traditional hosts for a product like 2'-fucosyllactose. In a controlled fermenter, *E. coli* is likely to produce more 2'-FL, but an *E. coli*-derived 2'-FL method has to guarantee that all cells and endotoxins are eliminated from the final product [16]. In contrast, a process using *L. plantarum* could conceivably include the bacteria in the final formulation (for instance, a fermented food or a dried probiotic powder that contains 2'-FL and live probiotic), adding synergistic prebiotic-probiotic effects. This concept of a synbiotic, where the probiotic produces its own prebiotic substrate (here, a HMO like 2'-FL that promotes beneficial gut microbes such as *Bifidobacterium*), is quite compelling. *L. plantarum* is an ideal candidate for such an approach given its probiotic credentials. Additionally, *L. plantarum* being Gram-positive means any proteins it secretes (or intracellular contents upon cell lysis) are free of LPS, reducing inflammation risk. Compared to yeast, *L. plantarum* might not reach the same cell densities, but it avoids issues of undesired ethanol production or different glycosylation patterns that yeasts have. And unlike *Bacillus*, *L. plantarum* does not form spores that might alter gut transit or raise concerns in certain food applications. Researchers have begun to explore *L. plantarum* as a host for delivering therapeutics and functional molecules *in vivo* [39]. Overall, *L. plantarum* WCFS1 stands out as a strong candidate for a next-generation recombinant chassis, particularly for food and health-related products, due to its safety profile, environmental robustness, and growing molecular toolkit.

1.3 Synthetic Biology Tools for Engineering *L. plantarum*

The development of sophisticated genetic tools has begun to unlock *L. plantarum*'s potential as a recombinant host. Historically, genetic editing in *Lactobacillus plantarum* (and many LAB) relied on homologous recombination via double-crossover integration, which is labor-intensive and often low-efficiency. For example, single-gene knockouts in *Lactobacillus casei* (a close relative of *L. plantarum*) until recently still depended on classical double-crossover methods, requiring multiple cloning steps and screenings [40]. In the past few years, however, powerful CRISPR/Cas-based genome editing systems have been adapted for *Lactobacillus*. Notably, Song et al. (2017) demonstrated a CRISPR-assisted, markerless editing approach in *L. casei* using a Cas9 nickase (a D10A mutant Cas9) [40]. They constructed a plasmid (pLCNICK) expressing a single-guide RNA and a Cas9^{D10A} nickase, along with a template for homologous recombination, enabling precise in-frame deletions and insertions in the chromosome [40]. By using a nickase rather than wild-type Cas9, the system creates single-strand breaks that stimulate homologous recombination without causing lethal double-strand breaks in cells that have not yet recombined. This innovation overcame some limitations of earlier LAB editing attempts and achieved editing efficiencies of 25–60% for various genes in one step, with the entire cycle (from transformation to confirmation) reduced to roughly 1–2 weeks [40]. The pLCNICK vector was also designed with a broad-host-range replicon, suggesting that the same strategy can be ported to other *Lactobacillus* species. This was a significant milestone, showing that CRISPR-mediated markerless genome engineering is feasible in lactobacilli. Following these developments, further genetic tools have been specifically developed for use in

L. plantarum. An inducible two-plasmid CRISPR/Cas9 system was recently reported for *Lactobacillus plantarum* that allows insertion of large DNA fragments into the chromosome without leaving antibiotic resistance markers [41]. This system separates the Cas9 and the editing template onto two vectors and uses an inducible promoter to control Cas9 activity, thus improving cell survival until the editing is introduced. Using this approach, functional genes (for instance, fluorescent protein reporters) have been seamlessly integrated into the *L. plantarum* WCFS1 genome [41]. Moreover, as a bottleneck in engineering LAB, some methods supplement host cells with phage-derived recombinases to enhance recombination efficiency [42]. For example, a RecE/RecT-assisted CRISPR toolbox has been developed for *L. plantarum*, wherein the λ -Red recombinase homologs promote efficient double-strand break repair by the provided template DNA, thereby increasing the frequency of desired edits. These refinements address the historically low homologous recombination rates in *L. plantarum*, which were due in part to active native nucleases and lack of natural competence [43].

A brilliant strategy to avoid antibiotic markers in engineered *L. plantarum* is to use auxotrophic selection markers. A prominent example is the alanine racemase gene (*alr*). *L. plantarum* cannot survive without D-alanine, a vital component of its peptidoglycan cell wall, and normally the bacterium produces D-Ala from L-Ala via the enzyme alanine racemase. It has been shown that *alr* is an essential gene for *L. plantarum* growth, as Δalr mutants will not grow unless exogenous D-alanine is supplied [44]. This phenotype can be exploited: one can create an *L. plantarum* host strain lacking *alr* (making it D-Ala auxotrophic) and then use plasmids or integration constructs that carry a wild-type *alr* gene as a selectable marker. Only cells that maintain the plasmid (or

have integrated *alr*) can grow on D-Ala-free medium, preventing the need for antibiotic resistance genes. After the desired genetic modification is introduced, the plasmid can be cured by adding D-alanine back to the medium, allowing isolation of clones that have lost the plasmid but retained the genomic edit. Because no antibiotic resistance remains and the only introduced gene (*alr*) restores a natural function, this method yields GMO strains that are “marker-free” and potentially acceptable for food use. A related auxotrophic marker is lactate racemase (*lar*, gene name *larA*). *L. plantarum* can utilize D-lactate in peptidoglycan cross-linking as a rescue pathway when D-alanine is limiting [45].

Beyond genome editing, *L. plantarum* has benefitted from development of modular expression systems for introducing and expressing heterologous genes. Several plasmid vector systems originally created for other lactic acid bacteria have been adapted to *L. plantarum*. One example is the pSIP system, derived from the sakacin peptide bacteriocin operon of *Lactobacillus sakei*. This system provides regulatable, high-level gene expression in *Lactobacillus* species, induced by a specific peptide pheromone [46]. It has been shown to drive strong expression of target genes in *L. plantarum* as well. Another well-known system is the NICE (nisin-controlled expression) system, developed in *Lactococcus lactis*, which uses the food-grade bacteriocin nisin as an inducer. The NICE system (with *nisA* promoter and its regulator) has been successfully implemented in *L. plantarum* too [46]. These types of inducible systems are valuable when one needs tight control of gene expression. For example, the product may be toxic or expression may be desired only at certain growth stages. In contrast, for applications requiring constitutive expression (such as a probiotic continuously producing an enzyme or metabolite), researchers have identified a set of strong constitutive

promoters in *L. plantarum*. Tauer et al. (2014) tested multiple promoter sequences (some native, some synthetic) in combination with different ribosome binding site designs and plasmid copy numbers to tune expression levels of a reporter protein [16]. They demonstrated that by choosing promoters of varying strength and adjusting the spacing of the Shine-Dalgarno sequence, one could obtain a range of expression from moderate to very high in *L. plantarum* [16]. For instance, promoters from genes encoding the transcription elongation factor (isolated from *L. plantarum* and *L. buchneri*) provided very strong expression, especially when placed on a multi-copy plasmid. They also found that an 8 nucleotide spacing between the RBS and start codon was optimal in *L. plantarum*, and deviations from this reduced expression [16]. These insights led to a set of shuttle vectors carrying different promoters, which researchers can now choose from, based on the desired expression output. Crucially, the newer expression vectors for *L. plantarum* are designed to be food-grade, meaning they avoid antibiotic resistance genes. Selection can be maintained by auxotrophic complementation (as described for Δalr , using alr^+ on the plasmid as the selectable trait) or by using resistance markers that are considered safe (e.g., erythromycin resistance is common in lab use but not acceptable for food strains; instead, one might use resistance to nisin or other food preservatives as a selection, or incorporate partitioning systems that retain plasmids without selection). Some studies have also employed an approach wherein the plasmid is only needed transiently (for delivering CRISPR machinery or integration cassette) and is then cured, yielding a plasmid-free recombinant strain. The avoidance of antibiotic markers is emphasized in literature as a key aspect of LAB synthetic biology, to reduce concerns about horizontal transfer of resistance genes in the food chain [41], [47]. In terms of DNA delivery, *Lactobacillus* species are hard to transform when compared to popular model organisms and their

transformation efficiency are highly variable even in the same species [48]. *L. plantarum* WCFS1 can be transformed by electroporation, and protocols have been optimized over time to improve efficiency. Parameters such as cell wall weakening (with glycine or lysozyme), pulse voltage, and plasmid methylation status have been tuned to overcome the species' restriction barriers [49], [50], [51]. Using such methods, researchers can introduce plasmids encoding CRISPR systems, integration cassettes, or expression constructs with reasonable success rates. Once inside, the plasmids (often based on broad-host-range origins like pWV01 or theta-replicating p256/pSH71) replicate and express in *L. plantarum* [51]. The combination of reliable transformation, precise genome editing, and flexible expression control is currently available for *L. plantarum* WCFS1, which positions this strain as a truly engineerable probiotic platform.

1.4 Functional Molecules: Human Milk Oligosaccharides (HMOs)

It is well established that the gut microbiota in early life plays a fundamental role in the development of the immune system and long-term metabolic health [52]. Numerous studies have identified a critical window during the early postnatal period (prior to weaning) during which environmental factors can induce alterations in microbial structure and composition. Such disturbances have been shown to trigger pathological processes later in life, which may contribute to immune imbalances [53], and increasing susceptibility to inflammatory bowel diseases (IBD) [54], [55].

In early infancy, the composition of the microbiota is primarily shaped by environmental exposures, such as mode of delivery [56], antibiotic treatment [55], and feeding practices, particularly whether the infant is breastfed or formula-fed [57]. Human breast milk is well known to promote microbiome and immune system

maturation and provides all the essential nutrients for infant growth, together with numerous bioactive molecules (including antibodies, antimicrobial proteins, and cytokines) [58].

Human milk oligosaccharides (HMOs) are one of the most important bioactive constituents of breast milk that affect the gut microbiota and the health of newborns. Colostrum and mature milk have about 20 g/L and 5–10 g/L of HMOs, respectively. This makes HMOs one of the most prevalent and essential components of milk [59], [60]. HMOs are somewhat selective substrates that help beneficial gut microbes grow, especially *Bifidobacterium longum subsp. infantis*, which is exceptionally common in breastfed babies and helps make a healthy microbiome in the early stages of life [61]. Oligosaccharides found in cow's milk or baby formula, on the other hand, are usually present in much lower amounts and are less complex in structure, so they don't have the same effect on the microbiome as HMOs [62]. In recent years, there has been more and more interest in the health benefits of *Bifidobacterium* species in early life, especially how they help the immune system develop. The genomes of *Bifidobacterium* spp. possess an abundance of different genes that are responsible for breaking down HMOs. This means that the relationship between the availability of HMOs and the colonization of *Bifidobacterium* is a key factor in keeping the immune system stable in early life [63].

Structurally, HMOs are composed of five monosaccharide units: (a) glucose, (b) galactose, (c) N-acetylglucosamine, (d) fucose, and (e) N-acetylneuraminic acid (also known as sialic acid). These units can be linked through various glycosidic bonds to generate over 200 distinct oligosaccharide structures. Based on their core composition, HMOs are broadly classified into three categories: fucosylated, sialylated, and neutral

oligosaccharides. Among the most abundant HMOs identified are 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-N-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL) [64].

1.5 2'-Fucosyllactose (2'-FL): Biological Role and Industrial

Interest

2'-fucosyllactose (2'-FL) is the most common fucosylated structure among the different human milk oligosaccharides (HMOs). It is also one of the most studied in terms of its health benefits [65]. Animal studies have shown that 2'-FL may modulate the gut-brain axis, which is an important pathway for the development of the central nervous system. For example, mice that were given extra 2'-FL in their diets had better cognitive function, learning ability, and memory performance [66]. Also, experimental data show that 2'-FL may help protect against necrotizing enterocolitis, a serious condition that occurs frequently in premature babies [67]. It has also been shown to lower the risk of early colonization by enteric pathogens, especially in models of *Campylobacter*-associated diarrhea [68]. All these studies emphasize important biological functions of 2'-FL for newborn health.

After infancy, 2'-FL has been evaluated as a promising prebiotic that is safe for adults and has selective growth-promoting effects on *Bifidobacterium* species [69]. For a long time, researchers have linked the low levels of inflammation observed in breastfed babies to the presence of complex HMOs [70]. More recently, orally administering 2'-FL was found to make experimental ulcerative colitis models less severe by increasing the expression of mucin (MUC2) in the colon [71]. The same study also stressed how important the gut microbiota is in causing these physiological changes. These results

suggest that the health benefits of 2'-FL arise from a combination of factors, including the enrichment of beneficial taxa like *Bifidobacterium spp.*, and the production of short-chain fatty acids via microbial fermentation, which in turn help maintain intestinal homeostasis. Based on its: (i) high natural abundance in human milk, (ii) relevance to neonatal health, (iii) efficacy as a well-tolerated prebiotic in adults, (iv) demonstrated safety profile, and (v) strong supporting literature, 2'-FL was selected in this thesis project as a target compound for synthetic biology-based production.

Despite global recommendations by the World Health Organization encouraging exclusive breastfeeding for the first six months of life [72], only 42% of infants worldwide meet this target according to the UNICEF 2019 report [73]. These data highlight a significant gap in achieving optimal breastfeeding coverage during the early months of life. Given the unique health effects of HMOs in early life, and the widespread shortfall in exclusive breastfeeding, interest in the industrial-scale production of 2'-FL for use in infant nutrition has grown significantly. Two randomized controlled clinical trials published in 2022 showed that infant formulas with 2'-FL were safe, well-tolerated, and could cause a "bifidogenic" effect, which is similar to the microbial composition found in breastfed babies [74]. A few countries have recently given the green light to adding 2'-FL to baby formulas, and other countries are currently looking into adding it as a new food ingredient. It is important to note that the European Food Safety Authority (EFSA; EU 2017/2470) and the U.S. Food and Drug Administration (FDA; GRN 650) have both approved 2'-FL made from *Escherichia coli* BL21.

In infants who cannot be breastfed, the use of 2'-FL as a dietary supplement during the critical early-life window may have substantial health benefits. In parallel, its documented ability to increase *Bifidobacterium* levels also makes it a valuable prebiotic

candidate for adults. Given its growing global adoption, establishing technological capacity for the production of 2'-FL and supporting its application as a supplement is of strategic importance for countries aiming to improve nutritional interventions. The lack of natural sources that produce high concentrations of 2'-FL besides human milk is one of the main obstacles in its production. Moreover, the technical complexity and prohibitive cost of its chemical synthesis persists [75]. Consequently, metabolic engineering-designed microbial cell factories have become popular as an alternative method for producing complex prebiotics like HMOs [76]. The majority of 2'-FL production efforts to date have utilized genetically modified *E. coli* strains in batch or fed-batch fermentation settings [76]. Because of its endogenous pathway for GDP-fucose biosynthesis, a crucial precursor in the production of 2'-FL, *E. coli* is especially well-suited for this task. Due to this, *E. coli* has been the host organism in a significant portion of the literature [77], [78], [79]. Various engineering strategies have been implemented to improve yield and support industrial-scale production, especially under batch fermentation regimes [65]. In addition to *E. coli*, other microbial platforms such as *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *Corynebacterium glutamicum* have also been explored for 2'-FL biosynthesis under controlled fermentation conditions [76], [80].

1.6 Aim and Scope of the Study

This thesis aims to develop a novel recombinant strain of *Lactobacillus plantarum* WCFS1 for the biosynthesis of 2'-fucosyllactose (2'-FL) through metabolic engineering. As discussed in the previous sections, *L. plantarum* WCFS1 is a well-characterized probiotic bacterium with GRAS status, making it a suitable candidate for

food and therapeutic applications. While conventional probiotic strains contribute to host health, their capacity to restore gut microbiota or deliver specific bioactive compounds remains limited. Recent advances in synthetic biology have enabled the functional enhancement of probiotic microbes, allowing them to produce health-promoting molecules within the host [81]. To date, no studies have reported the successful engineering of *L. plantarum* for de novo synthesis of human milk oligosaccharides, such as 2'-FL. This thesis proposes an original approach by both constructing a metabolically engineered probiotic capable of synthesizing 2'-FL, and by integrating this strain into functional food applications. Unlike previous studies that focus solely on large-scale industrial production, this work aims to evaluate the performance of a modified probiotic strain within living systems and, for the first time in the literature, as part of a functional food product in the form of yogurt. The recombinant *L. plantarum* strain will be engineered using a CRISPR-Cas9 system tailored for food applications. This system will enable genome editing through knockout and knock-in strategies, without the use of antibiotic selection markers. Instead, auxotrophy-based and food-compatible complementation systems will be applied, allowing for precise and modular genomic integration of synthetic cassettes. To enable 2'-FL production, three essential metabolic modules will be introduced: first, the pathway responsible for synthesizing GDP-L-fucose as the critical intermediate; second, high-level expression of a lactose permease gene to import lactose into the cell; and third, the integration of a fucosyltransferase gene that catalyzes the fucosylation of lactose using GDP-L-fucose as the donor molecule.

The synthesis of GDP-L-fucose is the central step in engineering the strain for 2'-FL production. This metabolite can be generated via either the *de novo* or salvage pathway. However, the salvage pathway depends on the external addition of fucose, which raises

production costs and reduces long-term feasibility. For this reason, the *de novo* route, adapted from the colanic acid biosynthesis pathway of *E. coli* is preferred. This pathway involves the sequential conversion of fructose-6-phosphate, derived from glycolysis, into GDP-L-fucose through the action of five key enzymes: ManA, ManB, ManC, Gmd, and WcaG. Once synthesized, GDP-L-fucose serves as a donor substrate for the fucosylation of lactose, catalyzed by the fucosyltransferase enzyme encoded by the *fucT* gene [75].

Although this study primarily focuses on the metabolic engineering of *L. plantarum* for 2'-FL production, future applications may include integrating the engineered strain into fermented food products such as yogurt. In this context, milk could serve both as a lactose source and as a fermentation matrix, enabling cost-effective production and delivery. The development of live or heat-treated functional yogurt containing 2'-FL and postbiotic components remains a potential extension of this work, with relevance for both probiotic and lactose-sensitive populations.

The development of a genetically engineered probiotic strain that can synthesize 2'-FL and its application in functional food systems represents a novel contribution to the field. This work aims to combine synthetic biology and probiotic biotechnology to create a safe, effective, and practical nutritional solution that has not yet been realized in literature or the market.

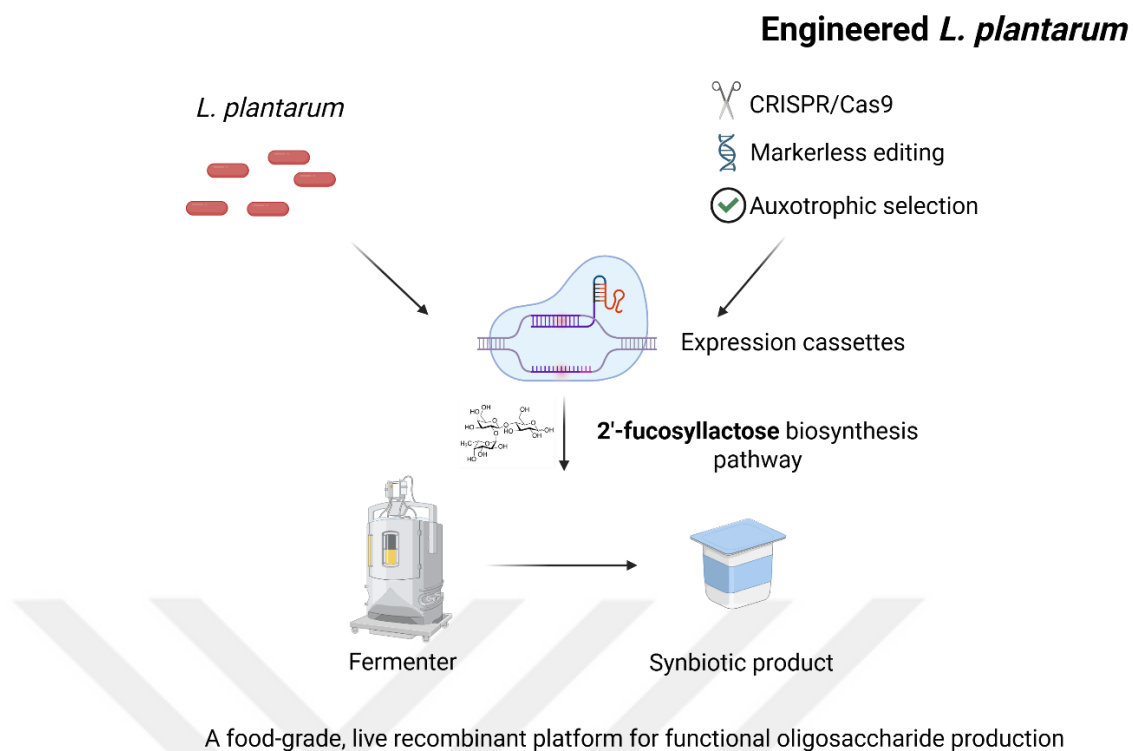


Figure 1. Schematic overview of the engineering strategy for *Lactobacillus plantarum* WCFS1 as a food-grade platform for 2'-fucosyllactose (2'-FL) production.

CHAPTER II: MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

Escherichia coli DH5 α strain was used for cloning studies due to its high insert stability and DNA yield. This strain carries the PRO cassette, which encodes genes for the constitutive production of TetR and LacI repressors, as well as spectinomycin resistance genes.

Lactobacillus plantarum WCFS1 (wild-type) strain was originally provided by Bioprogrammable Materials group led by Prof. Shrikrishnan Sankaran at the INM-Leibniz Institute for New Materials (Germany) and was subsequently kindly provided to our laboratory by Assoc. Prof. Dr. Ceren Özkul Koçak (Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Hacettepe University, Turkey).

Cell stocks were maintained in 25% (v/v) glycerol in Lysogeny Broth (LB) solution (recipe given in Appendix E, Table E.1) for *E. coli* cells and in 25% glycerol in De Man- Rogosa- Sharpe (MRS) Broth (Merck, Darmstadt, Germany; Cat. No. 1106610500) solution for *L. plantarum* cells at -80°C.

E. coli DH5 α was cultured at 37°C shaking incubator at 200 rpm, in Lysogeny Broth (LB) for liquid cultures; on LB agar plates (see Appendix E, Table E.1) at 37°C standing incubator for Petri dish cultures. *L. plantarum* WCFS1 was grown in De Man-Rogosa-Sharpe (MRS) broth or MRS agar (Merck, Darmstadt, Germany; Cat. No. 1106600500) under anaerobic or microaerophilic conditions at 30–37°C standing incubator.

Anaerobic or microaerophilic conditions were established either by placing the cultures in a sealed glass container along with a lit candle until oxygen was depleted, or by using AnaeroPack™-MicroAero or AnaeroPack™-Anaero gas-generating sachets (Mitsubishi Gas Chemical, Tokyo, Japan). 5% CO₂ incubator as culturing condition was also investigated, but decided not to proceed with. To decide the best culturing condition, a single colony was selected and inoculated into MRS liquid medium. After 24 h, cells were centrifuged at 3000-4000 g for 10 minutes and dissolved in 1mL 1X PBS. Minimum of 3 replicates of each sample were distributed into a microplate reader. Absorbance was measured at 600 nm using a SpectraMax M5 Microplate Reader (Molecular Devices).

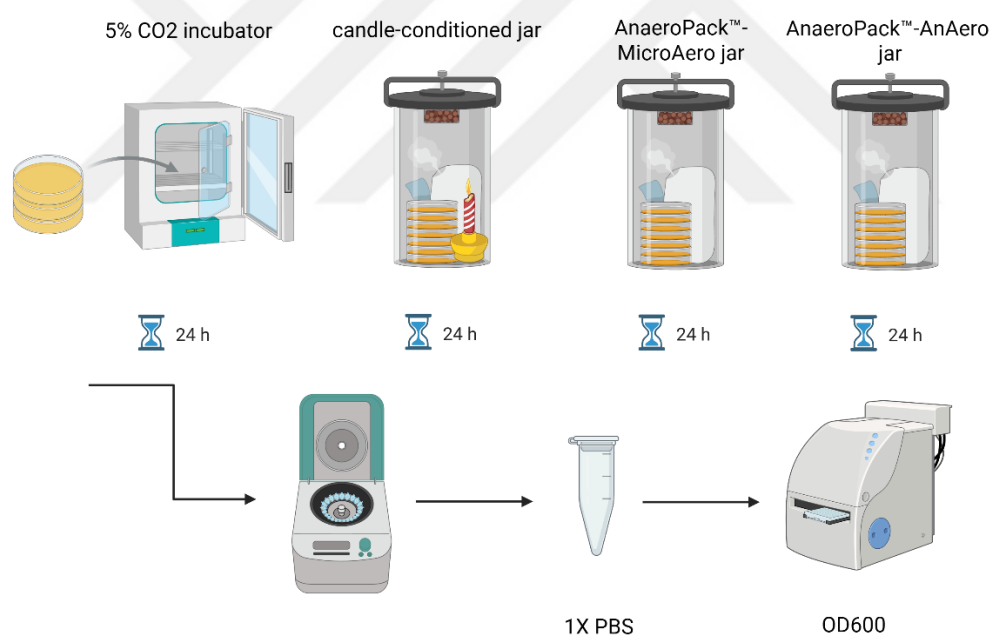


Figure 2. Assessment of optimal culturing conditions for *Lactobacillus plantarum* WCFS1. A single colony was cultured in MRS broth under different atmospheric conditions (candle jar, AnaeroPack™-Anaero, AnaeroPack™-MicroAero) for 24

hours. After centrifugation ($3000\text{--}4000 \times g$, 10 minutes) and resuspension in PBS, OD_{600} was measured using a microplate reader. Illustration is created using BioRender.com.

2.2 Plasmid Transformation Procedures

Plasmid introduction (for cloning experiments) into *E. coli* was carried out using chemical transformation. To prepare chemically competent *E. coli* DH5 α cells, cultures were first grown overnight from -80°C glycerol stocks in LB medium containing the appropriate antibiotics. These cultures were then diluted 1:100 into fresh antibiotic-supplemented LB and incubated until reaching an optical density (OD_{600}) between 0.2 and 0.5 (measured using Thermo Scientific™ GENESYS™ 10 UV-Vis Spectrophotometer). At this point, the cells were placed on ice for 10 minutes to cool. Following this, centrifugation was performed at $1000 \times g$ for 10 minutes at 4°C , and the supernatant was discarded. The resulting pellet was gently resuspended in Transformation and Storage (TSS) Buffer (see Appendix E, Table E.5) at a 1:10 dilution ratio. Aliquots of 100 μL were then prepared and stored at -80°C for future transformation use. Chemically competent cells were thawed on ice prior to transformation. Subsequently, 100 ng of plasmid DNA was added to each 100 μL aliquot, and the mixture was incubated on ice for 20 minutes to facilitate initial DNA binding. A heat shock was then applied at 42°C for 45 seconds to promote DNA uptake, followed by immediate chilling on ice for an additional 5 minutes to stabilize the cell membrane. Next, 250 μL of LB medium was added to each sample, and the cultures were incubated at 37°C with shaking at 200 rpm for 45 minutes to allow recovery and expression of antibiotic resistance. The cells were then pelleted by centrifugation at

8000 rpm for 5 minutes. After removing the supernatant, leaving behind approximately 100 μ L, the pellet was gently resuspended in the remaining volume. The entire suspension was plated onto LB agar plates containing the appropriate antibiotics, and plates were incubated overnight at 37°C.

Plasmid introduction into *L. plantarum* was carried out using electroporation. To prepare wild-type electrocompetent *L. plantarum* WCFS1 cells, cultures were first grown overnight from -80°C glycerol stocks in MRS medium containing the appropriate antibiotics under anaerobic or microaerophilic conditions. These cultures were then diluted 1:50 into fresh antibiotic and 1% glycine supplemented MRS broth and incubated until reaching an optical density (OD₆₀₀) of 0.8. Cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4 °C. The supernatant was discarded, and the pellet was gently resuspended in 5 mL of ice-cold 10 mM MgCl₂. This wash step was repeated twice under the same conditions. Subsequently, the cells were washed once with 5 mL of an ice-cold Suc-Gly solution (see Appendix E, Table E.8), followed by another centrifugation at 5000 rpm for 10 minutes at 4 °C. After removing the supernatant, the pellet was resuspended in 1 mL of the same Suc-Gly solution, centrifuged again, and finally resuspended in 500 μ L of Suc-Gly. Aliquots of 60 μ L were transferred into sterile, pre-chilled tubes and kept on ice until electroporation. All solutions were kept ice-cold throughout the procedure, and all centrifugation and handling steps were performed on ice or at 4 °C. For transformation, plasmid DNA was added to 60 μ L of competent cells and incubated on ice for 10 minutes. The mixture was transferred into pre-chilled 1 mm electroporation cuvettes and pulsed at 1.8 kV,

200 Ω resistance, and 25 μ F capacitance, resulting in a single pulse of approximately 5 ms. Immediately after the pulse, 1 mL of MRS recovery medium (See Appendix E, Table E.9) was rapidly added to the cuvette. The suspension was then transferred to a fresh microcentrifuge tube and chilled on ice for 3–5 minutes. Cells were recovered by incubation at 37 °C under anaerobic or microaerophilic conditions for 4 hours. Following recovery, cells were plated on MRS agar containing the appropriate antibiotics and incubated at 37 °C for 18–24 hours under the same atmospheric conditions.

2.3 pLH01 Transformation into *L. plantarum* WCFS1 and Plasmid

Curing

To optimize effective transformation conditions into wild-type *L. plantarum* WCFS1, pLH01 plasmid (Addgene, Plasmid #117261) containing the recombinase enzymes required for gene deletion was used [43]. 300 ng of pLH01 was transformed into electrocompetent *L. plantarum* cells (see 2.2) using either 1mm or 2mm cuvette. Transformed cells were either inoculated in normal MRS medium or MRS recovery medium and grown for either 4 h or 24 h, then spread onto 10 μ g mL⁻¹ chloramphenicol containing MRS agar plate. After electroporation, colony PCR was performed (using homemade PFU DNA polymerase) to determine the presence of the plasmid and the accuracy of the strain. See Appendix E, Table E.10 and E.11 for colony PCR protocol. During colony PCR, a 300 bp region in the pLH01 plasmid was amplified (using prLABP1 and prLABP2, see Appendix B). To detect the presence of the 16S rRNA gene of *L. plantarum* bacterium, a 1500 bp region was amplified (using primers prLABP3 and prLABP4, see Appendix B). Colony PCR products were observed in agarose gel containing SYBR™ Safe DNA gel stain.

Following transformation, serial passaging was performed to eliminate the recombinant plasmid from *L. plantarum* strains containing pLH01. For this purpose, the bacteria were passaged daily at a 1:10,000 dilution in antibiotic-free MRS medium. At the end of the third day, the bacterial cultures were collected, serially diluted, and plated onto MRS agar. After incubation, colony growth was observed, and colonies were counted.

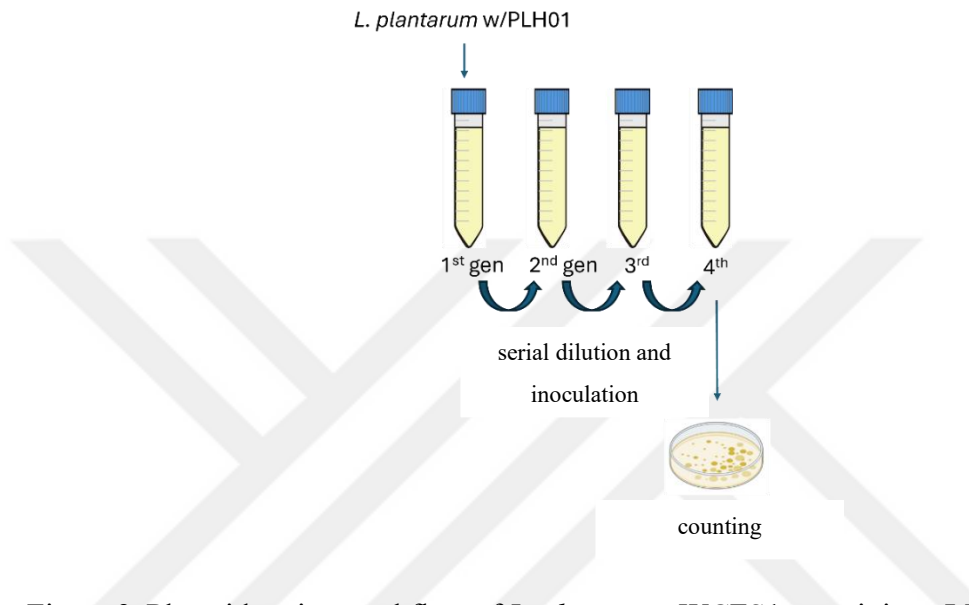


Figure 3. Plasmid curing workflow of *L. plantarum* WCFS1 containing pLH01 plasmid.

Illustration is created using BioRender.com.

2.4 sgRNA Design and Cloning into CRISPR Plasmid

To inactivate selected genes in *L. plantarum* WCFS1, multiple single-guide RNAs (sgRNAs) were designed targeting key genomic loci. The target genes included *alr*, *argH*, *upp*, *trpA*, *larA*, and *trpB*, which were chosen to create defined auxotrophies and facilitate selection (e.g., *alr* encodes alanine racemase, whose knockout yields D-alanine auxotrophy useful for integration selection). Candidate 20-nt targeting sequences for each gene were identified using the CHOPCHOP online tool (v3) [82]. This tool was used to select sgRNAs with high on-target efficacy and minimal off-target

hits in the *L. plantarum* genome. Several sgRNA candidates per gene were screened for suitable GC content (approximately 40–60%) and lack of off-targets; the final chosen guide for each target had 0 predicted off-target sites and was typically on the coding strand to maximize transcription efficiency (insert reference here). Table A.1 summarizes the sgRNA sequences designed for each target gene. Each sgRNA includes the requisite protospacer adjacent motif (PAM, 5'-NGG-3') at the 3' end of the target sequence to enable *Streptococcus pyogenes* Cas9 binding and cutting.

The CRISPR/Cas9 plasmid pHSP02 (Addgene, Plasmid #117259) was used as the backbone for sgRNA cloning. This vector, originally developed by Huang et al. (2019) for genome editing in *L. plantarum* [43] carries a constitutively expressed Cas9 gene and a guide RNA scaffold under a strong promoter. In pHSP02, the sgRNA expression is driven by the P11 promoter (a synthetic promoter from the pCas vector system), which ensures high levels of guide RNA transcription. The plasmid also contains a default guide sequence cassette flanked by unique restriction sites (XbaI and ApaI) for cloning new target sequences (pHSP02 is derived from a pCas shuttle backbone [43]). To insert the designed sgRNAs, the cloning strategy recommended for this vector was followed [43]. Initially, the pHSP02 DNA was linearized by restriction digestion, removing the existing guide sequence. The plasmid was double-digested with XbaI (NEB) and ApaI (NEB) to excise the 20 bp protospacer region (digestion reaction protocol given in Appendix E, Table E.3). The expected fragments (approx. 12 kb vector backbone and a ~2kb fragment containing the old guide cassette and homology arms) were resolved on an agarose gel containing SYBR™ Safe DNA gel stain. However, since in practice this digest did not yield a clearly purified linear backbone (the 12 kb fragment was not recovered as expected), possibly due to incomplete digestion or difficulty isolating the large fragment.

Given the challenges with restriction cloning, an alternative PCR-based cloning approach was employed for inserting the sgRNAs. The pHSP02 vector was linearized by PCR with Q5 polymerase, using primers (prHMO5 and prHMO6) that amplify the entire plasmid minus the original guide region and homology arms for recombination. These primers were designed with ends near the XbaI/ApaI sites, effectively removing the old spacer. Separately, the new sgRNA sequence was introduced by PCR amplification of the guide scaffold. For sgRNA-1 of *alr* gene, primers carrying the 20-nt target sequence as an overhang (primers prHMO7 and prHMO8) were used to amplify the plasmid's guide region and put the new protospacer in place. The two PCR products (the linearized backbone and the small insert containing the new guide) of appropriate length were loaded on an agarose gel containing SYBR™ Safe DNA gel stain and run. The expected bands on the agarose gel were cut out for DNA extraction using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel), following the manufacturer's instructions. After measuring the concentration with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer, the resulting 160 bp 'gRNA scaffold + sgRNA sequence', and 12kb backbone (not containing the original sgRNA sequence, gRNA scaffold and homology arms) were assembled either using NEBuilder® HiFi DNA Assembly following the manufacturer's instructions, or using in-house Gibson Assembly (reaction mix protocol given in Appendix E, Table E.4)

Following the assembly reaction, the mixture was transformed into chemically competent *E. coli* DH5α cells. Transformation steps are mentioned in 2.2. Colonies were picked from the overnight incubated LB-Agar plate for Next Generation Sequencing (NGS). Verification data for colonies containing the *alr*-gRNA-1 part in pHSP02 backbone were analyzed using Geneious Prime® (2021.1.1) software. Verified colonies were inoculated in 10 mL of LB medium containing 25

$\mu\text{g mL}^{-1}$ kanamycin and incubated in a shaking incubator at 37°C and 200 rpm overnight. Cells stocks were prepared and stored at -80°C the following day.

This cloning procedure was tested initially with the first sgRNA targeting the *alr* gene (*alr*-sgRNA-1). The same cloning strategy was followed for *argH*-sgRNA-1 and -sgRNA-2 with primers prHMO7 - prHMO9 and prHMO7 – prHMO10, respectively, for amplifying the unique sgRNA region. Similarly, *upp*-sgRNA-1 and -sgRNA-2 were amplified using primer pairs prHMO7–prHMO11 and prHMO7–prHMO12, respectively.

Cloning of the remaining sgRNAs (for *trpA*, *larA*, *trpB*) into pHSP02 is in progress and will follow the same protocol. All sgRNA plasmids were designed to be maintained with kanamycin selection (25 $\mu\text{g/mL}$) for *Escherichia coli* DH5 α and erythromycin selection (10 g/mL) for *Lactobacillus plantarum* WCFS1. Each constructed pHSP02-sgRNA plasmid will later be introduced into *L. plantarum* for genome editing once verified.

2.5 Homology Arm Designs

The constructed plasmids in Section 2.5 were devoid of homology arms, unlike originally developed by Huang et al. (2019) [43]. Homology arms were planned to be amplified by PCR reaction and given to *L. plantarum* cells externally, linearly during knockout transformations. For this purpose, the genomic DNA of wild-type *L. plantarum* WCFS1 was extracted using Monarch[®] Spin gDNA Extraction Kit following the manufacturer's instructions. 500 bp upstream and 500 bp downstream of each knockout target gene was amplified by PCR using Q5 polymerase using this

genomic DNA template. Upstream and downstream fragments were designed to be compatible with each other, with 30 bp of overlapping regions in order to be assembled with Gibson Assembly. After the Gibson Assembly, they were amplified using the primers from the beginning of the upstream part as forward and from the end of the downstream part (generating approximately 1kb products). For *alr* gene's homology arm PCR reactions, prHMO13, prHMO14, prHMO15 and prHMO16 primers were used. For *argH* gene's homology arm PCR reactions, prHMO17, prHMO18, prHMO19 and prHMO20 primers were used. For *upp* gene's homology arm PCR reactions, prHMO21, prHMO22, prHMO23 and prHMO24 primers were used. The same strategies will be employed for *trpA*, *larA*, and *trpB* genes.

At the end, 1000 bp of homology arms of each gene containing both upstream and downstream regions are ready with no intervening sequence, as required for markerless gene knockout. These homology arms enable precise allelic replacement via homologous recombination (with the help of pLH01 plasmid) following CRISPR-Cas9-mediated double-strand break induction [43].

1000 bp assembled homology arm pieces were also designed to be implemented into the pHSP02 plasmid, as suggested by Huang et al. [43]. For *alr* homology arm, the linear 1 kb piece was amplified by PCR using Q5 polymerase (with primers prHMO13 and prHMO25) in such a way that both of its ends contained *Apal* restriction site. This design allowed for subsequent ligation into a pHSP02 derivative that had been previously cleared of unrelated homology arms and contained a single *Apal* site downstream of the sgRNA scaffold region.

Following PCR, the *alr* homology arm product was gel-purified using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel), and its concentration was measured with

a NanoDrop™ 2000 spectrophotometer. Then, the extracted piece and the pHSP02 backbone containing *alr*-targeting sgRNA were linearized with ApaI-HF (NEB) at 37 °C for 2 hours (digestion reaction protocol provided in Appendix E, Table E.3). The digested plasmid and homology arm piece were also purified by agarose gel electrophoresis and extracted as described above.

Both the insert and the linearized backbone were ligated using T4 DNA ligase (NEB) under standard conditions (ligation protocol provided in Appendix E, Table E.7). The resulting ligation mix was transformed into chemically competent *E. coli* DH5α cells via heat shock transformation. Transformed colonies were selected on LB agar plates containing 25 µg/mL kanamycin and screened by colony PCR for correct insert size. Positive clones were confirmed by Sanger sequencing and stored at –80 °C in 25% glycerol stocks for further use in transformation of *L. plantarum*.

2.6 Induction of Recombinase System and Knockout Strategy

pLH01 plasmid-containing (as previously described) *L. plantarum* WCFS1 cells were inoculated in MRS media containing 10 µg/mL chloramphenicol and grown overnight at 37°C under microaerophilic conditions. Culture was then diluted 1:50 into fresh antibiotic and 1% glycine supplemented MRS broth and incubated until reaching an optical density (OD₆₀₀) of 0.3. At this point, the synthetic inducer peptide “MAGNSSNFIHKIKQIFTHR” was added into the culture at a final concentration of 100 ng/mL to induce the expression of *recE* and *recT* recombinase genes (RecE/T) encoded by pLH01 [43]. The peptide was ordered from GenScript Co. Ltd. (Nanjing,

China). Induced cells were harvested upon reaching $OD_{600} \approx 0.4\text{--}0.5$ and processed into electrocompetent form as previously described (see Section 2.2). These competent cells were then used for transformation with the editing-plasmid pHSP02 along with linear double-stranded homology arm fragments (see Section 2.5). Approximately 800-1000 ng of pHSP02 CRISPR plasmid was given together with 500-700 ng of linear homology arm pieces. When the homology arms were placed into the CRISPR plasmid, 1 μ g of DNA was given. Following transformation, cells were allowed to recover in MRS recovery medium for 2–3 hours at 37°C under microaerophilic conditions before being plated on MRS agar supplemented with 10 μ g/mL erythromycin and 10 μ g/mL chloramphenicol.

Planned screening for genome edits was to be conducted by colony PCR using primers flanking the homologous regions of the target gene (prHMO26 and prHMO27). Positive colonies would have been further verified by Sanger sequencing to confirm chromosomal modifications. Following successful knockout verification, integration of complementary gene expression cassettes was intended. These knock-in constructs, such as the *manA–manB–manC* operon along with *alr* gene, were designed to restore function and enable biosynthetic capabilities (e.g., GDP-L-fucose synthesis) under control of synthetic promoters. Plasmid curing was designed to proceed via subculturing in MRS medium supplemented with 10 μ g/mL chloramphenicol for three consecutive generations, followed by streaking on MRS agar with and without erythromycin to distinguish cured clones. (Note: Colony formation was not observed during this step; see next chapters for further discussion.)

This recombineering-assisted CRISPR-Cas9 strategy was adapted from the protocol reported by Huang et al. [43] and optimized for *L. plantarum* WCFS1 in the context of this study.

2.7 Design of Synthetic Expression Cassettes

To enable de novo 2'-fucosyllactose (2'-FL) biosynthesis in *L. plantarum*, synthetic operons were designed to heterologously express the necessary pathway genes. The overall strategy was to introduce the GDP-L-fucose biosynthesis pathway from *E. coli* along with a fucosyltransferase (from *Helicobacter pylori*), allowing the host to convert intracellular lactose and GDP-fucose into 2'-FL. In total, up to seven genes were targeted for expression: five genes (*manA*, *manB*, *manC*, *gmd*, *wcaG*) encoding the GDP-fucose synthesis pathway enzymes, one gene (*fucT*, an α -1,2-fucosyltransferase) to produce 2'-FL from GDP-fucose and lactose, and potentially a copy of the lactose transporter gene (*WJL_0209*, from *Lactiplantibacillus plantarum* *WJL*) to enhance lactose uptake. This gene is also known as lactose and galactose permease GPH translocator family, with GenBank accession number KPN43136.1. For practical assembly, these genes were grouped into synthetic operons. For example, one operon design included *manA*, *manB*, *manC* together with a selection marker (*alr*, alanine racemase), while a second operon was planned to carry *gmd* and *wcaG*, and a third operon for *fucT* (and possibly *WJL_0209* if needed). Each operon was constructed as a single transcriptional unit under a strong promoter, with intercistronic ribosome binding sites to allow polycistronic expression in *L. plantarum*. This section describes the design of these expression cassettes, focusing on the first operon (*manA-manB-manC-alr* as a representative example).

Gene Sources and Optimization: The genes *manA*, *manB*, *manC*, *gmd*, and *wcaG* were sourced from *E. coli* BL21 (DE3), which catalyze the formation of GDP-mannose and its conversion to GDP-L-fucose in the *de novo* pathway [83]. The *fucT* gene (also known as *futC*) was sourced from *Helicobacter pylori* (which encodes an α 1,2-fucosyltransferase enzyme that can synthesize 2'-FL) [84]. Codon Adaptation Index (CAI) values were calculated using the BiologicsCorp CAI Calculator [85], with a codon usage table specific to *L. plantarum* WCFS1 [86]. BL21 variants of the genes exhibited higher CAI scores (CAI >0.7) compared to those from other *E. coli* strains such as K-12, making them more suitable candidates for heterologous expression. While no additional codon optimization was performed, this selection strategy aimed to ensure efficient translation without requiring gene synthesis or sequence alteration at this stage. The *alr* gene included in the operon was the native *L. plantarum* WCFS1 alanine racemase gene sequence.

Regulatory Elements (Promoter and UTR): A strong constitutive promoter, **P11**, was chosen to drive the synthetic operons. P11 is a synthetic sequence derived from an rRNA promoter of *L. plantarum* WCFS1 and is also utilized in the pHSP02 CRISPR plasmid for sgRNA expression. It has been demonstrated to exhibit one of the strongest constitutive activities in *L. plantarum* and related lactic acid bacteria [16]. Downstream of P11, a customized 5' untranslated region (5'-UTR) was designed to maximize translation. This UTR design incorporated a *RiboJ* self-cleaving ribozyme element and a ribosome binding site (RBS) sequence. The *RiboJ* sequence (a self-cleaving RNA element) was placed immediately after the promoter to insulate the transcripts from promoter-proximal sequence effects; it ensures that each mRNA transcript begins with a defined sequence after cleavage, thereby improving the consistency of gene expression levels. Following *RiboJ*, a strong Shine-Dalgarno RBS (5'-AAGGAGG-3')

was included to recruit *L. plantarum* ribosomes. An 8-nucleotide spacer (5'-AAATTTAC-3') was added between the RBS and the start codon of the first gene, as recommended by prior optimization studies in *Lactobacillus* [16]. Each subsequent gene in the operon was also preceded by its own RBS and short spacer to ensure each cistron can be efficiently translated. A schematic overview of the operon construct is shown in Figure 4. The *E. coli rrnB T1* terminator, known for its strong hairpin-forming structure that promotes RNA polymerase dissociation, was inserted downstream of the operon to ensure proper transcriptional termination and prevent undesired read-through into genomic DNA [87].

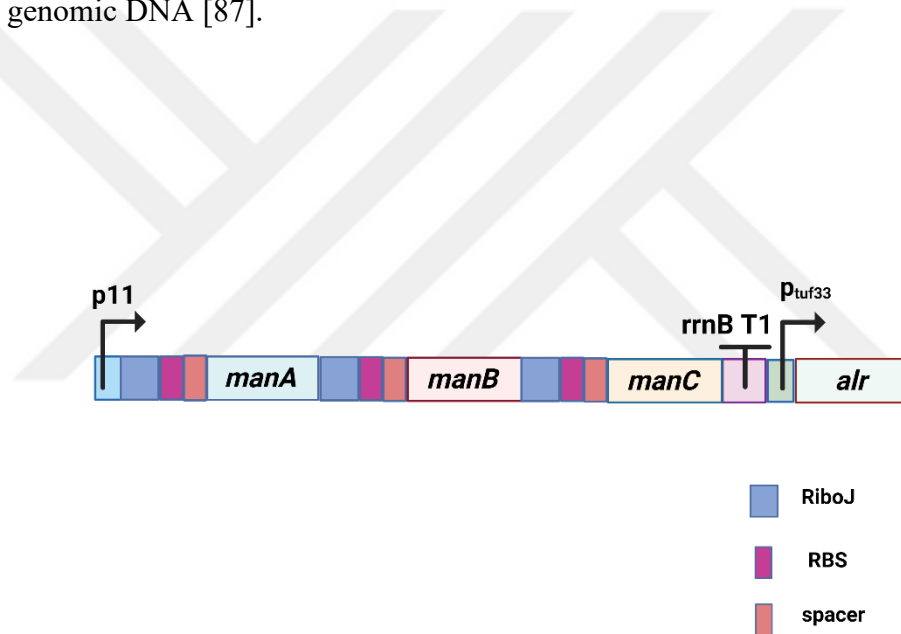


Figure 4. Schematic of the synthetic *manA-manB-manC-alr* expression cassette. Illustration is created using BioRender.com.

Assembly Strategy (Overlap PCR and Gibson Assembly): The synthetic operon cassette was assembled in vitro using a combination of overlap-extension PCR and Gibson Assembly. First, each gene was amplified with overlapping ends such that adjacent fragments shared ~20–30 bp of homology. For instance, the 3' end of *manA* fragment was designed to overlap with the 5' end of *manB* fragment, and so on. Using

overlap extension PCR [88], pairs of fragments were fused: e.g., *manA* and *manB* were joined in a PCR reaction to create a fused *manAB* product, then *manC* was merged with *manAB*, and so forth. After a series of such PCR fusions, a full-length 5.8 kb product containing *manA-manB-manC-alr* was obtained (the exact assembly was optimized by joining two fragments at a time to maintain high PCR efficiency). Each intermediate and the final operon PCR product were checked on agarose gels for correct size and extracted using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel). For overlapping PCR, the common PCR mixture (Appendix E, Table E.2) was prepared without adding primers. Instead of a template, PCR parts were added as 100ng/kb. For example, if the part is 300bp, 30ng is added. The first 8 cycle was run without the primers, at overlap T_m . Then the primers were added, and the usual PCR reaction (with 28 cycles) was followed. Alternatively, in cases that overlap PCR failed due to non-optimal overlap T_m , Gibson Assembly method was pursued. For the amplification of *manA*, prHMO32 and prHMO33; for the amplification of *manB*, prHMO36 and prHMO37; for the amplification of *manC*, prHMO38 and prHMO39 was utilized as primers. RiboJ was constructed using primers prHMO30, prHMO31, prHMO34, prHMO35 and prHMO40 depending on its location in the operon. *rrnB T1* was amplified using prHMO41 and prHMO42. Since p11 promoter is already present in the pHSP02 backbone, Cas9 was extracted from the plasmid (prHMO28 and prHMO29). New pHSP02 backbone was linearized using prHMO5 and prHMO6 via PCR using Q5 polymerase, and the expression cassette is placed right after the p11 promoter using Gibson Assembly method.

Following the *rrnB T1* terminator of the synthetic operon, a separate complementation cassette was designed to contain the *alr* gene under the control of the strong constitutive *Ptuf33* promoter. This promoter, previously characterized for its robust expression in

L. plantarum [16], was selected to ensure efficient transcription of *alr* in potential knock-in configurations. Since the *Ptuf33* sequence was not available in plasmid format, it was synthesized by hybridizing two 60 bp oligonucleotides (prHMO43 and prHMO44) designed as reverse complements. See Appendix E, Table E.12 for hybridization procedure. Resulting duplex along with its ribosome binding site (as previously reported [16]), was amplified in a total of five PCR reactions using overhang-containing primers. The *alr* gene, in its native sequence from *L. plantarum* WCFS1, was then PCR-amplified (prHMO54 and prHMO55) and fused downstream of *Ptuf33* via Gibson Assembly.

Importantly, the *manA-manB-manC* operon was first assembled and inserted into a Cas9-free pHSP02 backbone in the absence of homology arms. In parallel, the *Ptuf33-alr* fragment was constructed separately, with the intention of inserting it downstream of the operon in a modular fashion. This modularity was achieved by designing *ApaI*-compatible junctions flanking the *Ptuf33-alr* fragment, allowing it to be seamlessly inserted after the *rrnB T1* terminator via cut-ligate cloning. This strategy provides flexibility, as the complementation cassette can be selectively included or omitted depending on the results of the upstream *alr* knockout or auxotrophic selection. Although the initial constructs were prepared without homology arms to reduce metabolic burden and facilitate cloning, future knock-in experiments will involve flanking the entire expression cassette (including the *manA-manB-manC* operon and the *Ptuf33-alr* unit) with appropriate homology arms. These will target the genomic locus corresponding to the *alr* gene, guided by a new sgRNA designed to introduce a double-stranded break within the terminator region of the deleted native *alr*. The presence of these homology arms will enable RecE/T-assisted recombination (as

mediated by the pLH01 helper plasmid), integrating the synthetic operon into the host genome.

The remaining two synthetic operons (containing the *gmd-wcaG* and *fucT-WJL_0209* gene pairs) will be designed following the same modular principles described above. Specifically, these operons will be constructed independently and then assembled with appropriate complementation modules downstream, based on the outcomes of auxotrophic selection experiments. Among the candidate genes considered for generating auxotrophic strains (e.g., *argH*, *upp*, *trpA*, *larA*, and *larB*), only a subset will ultimately be selected for complementation. The final choice will depend on which gene deletions yield the most reliable selection phenotype in *L. plantarum* WCFS1. Once identified, the corresponding complementation cassettes will be cloned downstream of the synthetic operons, using the same cut-ligate strategy to preserve modularity.

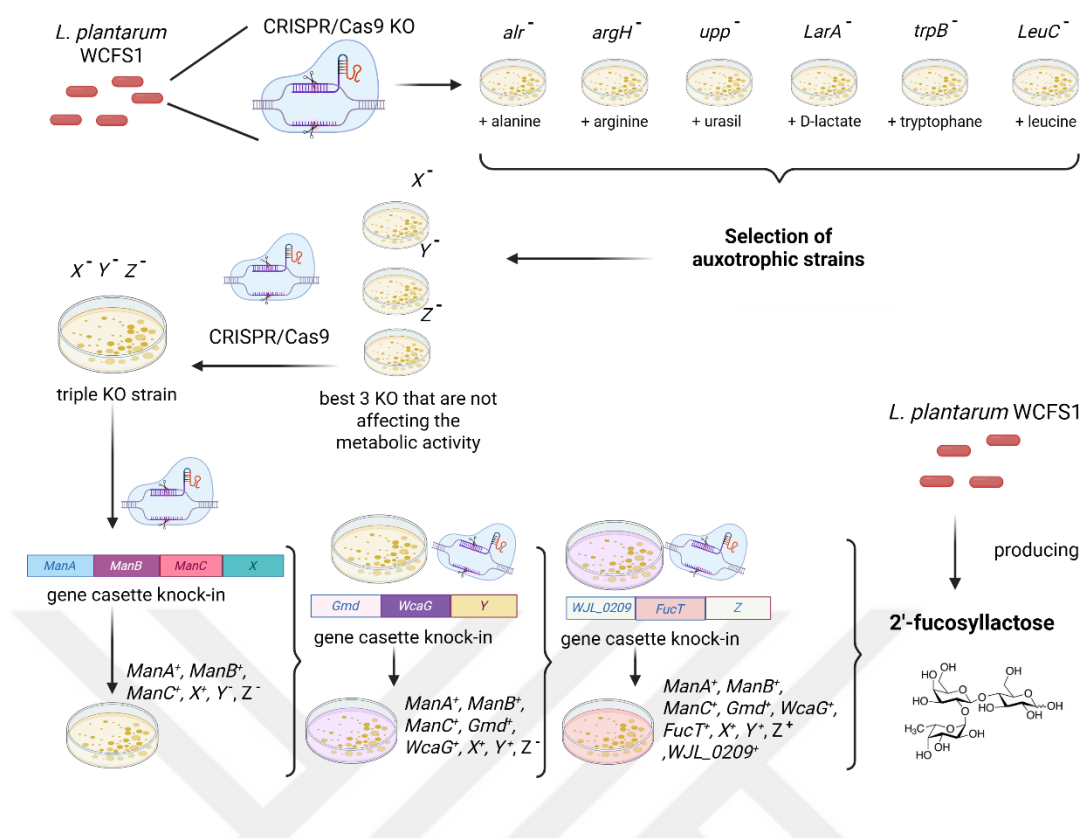


Figure 5. Schematic overview of the metabolic engineering workflow for 2'-FL production in *L. plantarum* WCFS1. Illustration is created using BioRender.com.

As an overview plan, process begins with gene knockouts using a CRISPR-Cas9 system (pHSP02) and RecE/T-assisted homologous recombination (pLH01). Following auxotrophic gene knockouts (*alr*, *argH*, *upp*, *trpA*, *larA*, *trpB*), the most effective markers will be selected based on growth performance and burden. One selected marker will be included downstream of each operon for complementation. Synthetic operons enabling 2'-FL biosynthesis are introduced: *manA-manB-manC* for GDP-mannose production, *gmd-wcaG* for GDP-L-fucose synthesis, and *WJL_0209-fucT* for lactose transport and fucosylation. The final strain is expected to produce 2'-FL from lactose via the de novo pathway.

CHAPTER III: RESULTS AND DISCUSSION

3.1 Growth Condition and Transformation Optimizations for *L. plantarum* WCFS1

After obtaining the strain *L. plantarum* WCFS1, optimal culturing conditions were to be determined. As was previously described, cells were cultured at 37°C statically for 24 hours in four different atmospheric conditions: in 5% CO₂ incubator, in a jar with candle-depleted oxygen, in a jar with AnaeroPack™-MicroAero (Mitsubishi Gas Chemical, Tokyo, Japan) and in a jar with AnaeroPack™-AnAero (Mitsubishi Gas Chemical, Tokyo, Japan).

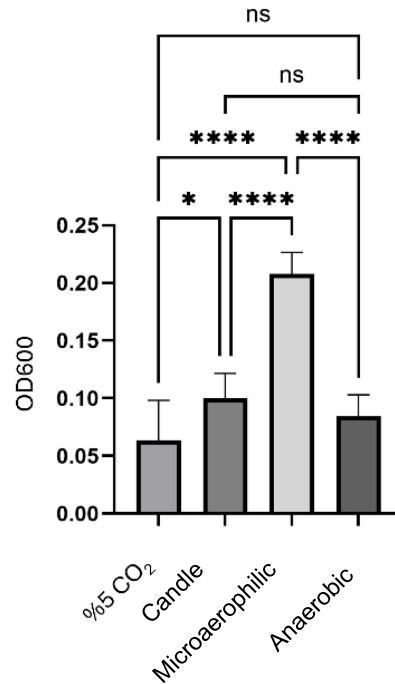


Figure 6. Growth of *Lactobacillus plantarum* WCFS1 under different oxygenation conditions, measured by OD600. Cultures were incubated under 5% CO₂, candle jar (microaerobic), gas-generating microaerobic, and strict anaerobic conditions. The highest OD600 values were observed in gas-generated microaerobic conditions. Data are presented as mean $\pm$ SD (n = 3). Statistical significance was evaluated using one-way ANOVA followed by Tukey's multiple comparisons test; *p < 0.05, ****p < 0.0001, ns = not significant. The graph was generated using GraphPad Prism software.

As described in Figure 6, cultures grown under microaerobic conditions using AnaeroPack™-MicroAero exhibited significantly higher OD600 values compared to those incubated under 5% CO₂ and strict anaerobic conditions. These results suggest that mild oxygen limitation promotes the growth of *L. plantarum* WCFS1 more effectively than fully anaerobic or standard CO₂-enriched environments.

After preparation for electroporation and overnight storage at -80°C , *L. plantarum* WCFS1 cells were electroporated with 300 ng of pLH01 plasmid using parameters of

1.8 kV, 200 Ω , and 25 μ F. 300 ng was selected as the optimal plasmid DNA amount for electroporation after comparing colony formation efficiency across different DNA concentrations (300 ng, 600 ng, and 900 ng). As illustrated in Figure 7, 300 ng yielded a sufficient number of colonies without increasing background growth or risking cytotoxicity due to DNA overload. Higher amounts did not provide significantly improved transformation efficiency; thus 300 ng was adopted as the standard for subsequent experiments.

Following electroporation, transformed cells were incubated in antibiotic-free recovery media under four conditions: 4 hours in standard MRS broth, 24 hours in standard MRS broth, or 4 hours and 24 hours in MRS supplemented with 80 mM MgCl_2 , prior to plating on MRS agar containing 10 $\mu\text{g mL}^{-1}$ chloramphenicol. The highest colony numbers were observed when cells were recovered for 4 or 24 hours in MRS supplemented with 80 mM MgCl_2 . Based on these results, a 4-hour recovery in the recovery medium was adopted for further transformation experiments. 1mm and 2mm cuvettes were also tried but no significant differences were found, thus the data is not shown.

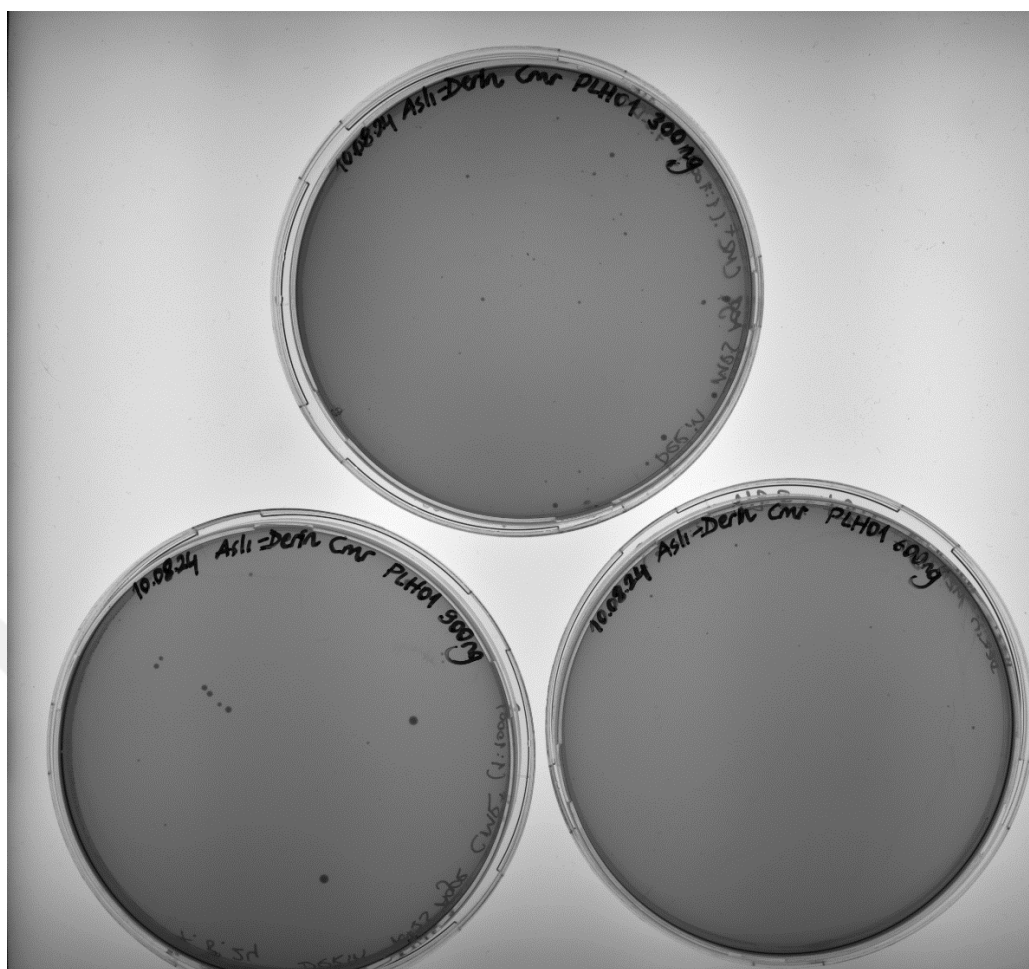


Figure 7. Colony formation after *L. plantarum* WCFS1 electroporation with varying concentrations of pLH01 plasmid DNA. Transformation was performed using 300 ng (top), 900 ng (bottom left), and 600 ng (bottom right) of plasmid DNA under identical electroporation.

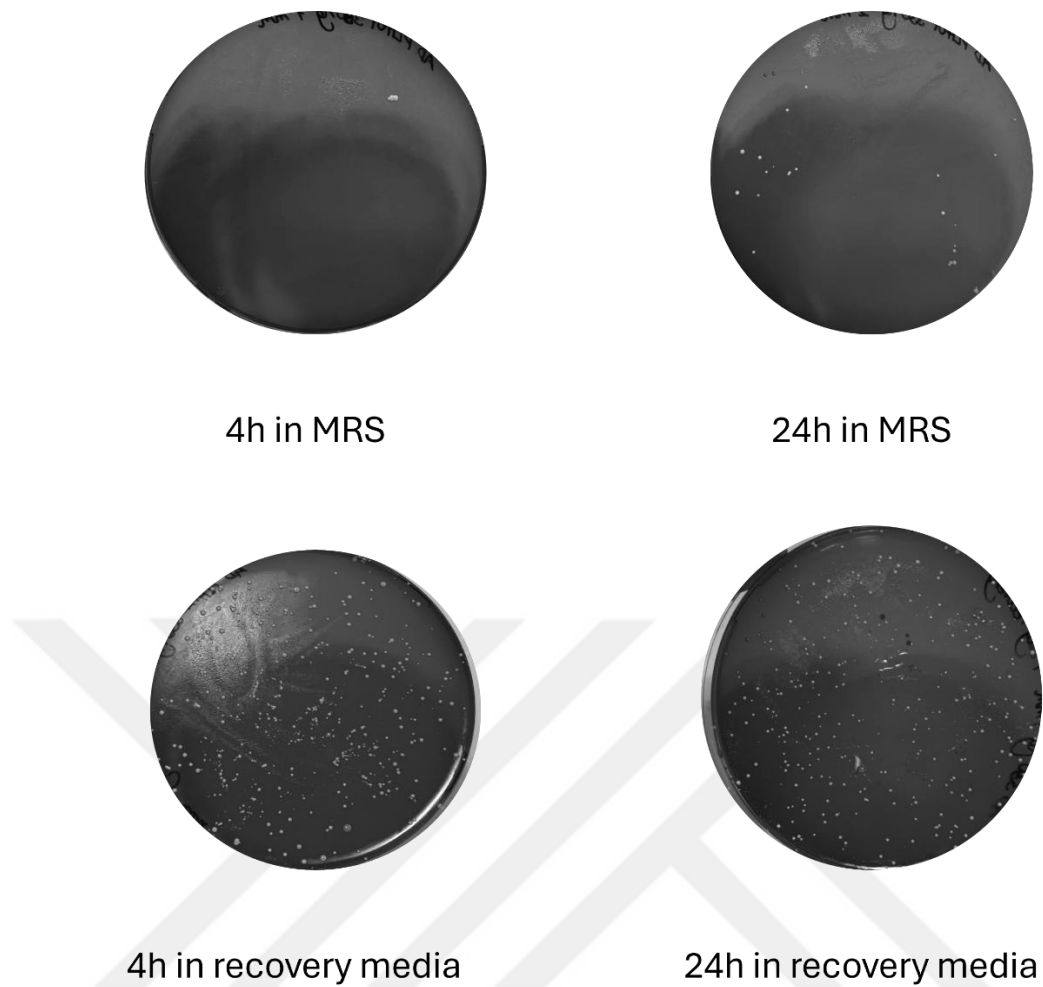


Figure 8. Effect of recovery conditions on transformation efficiency of *L. plantarum* WCFS1 after electroporation with pLH01 plasmid. Colony formation increased with MgCl_2 -supplemented recovery.

Following electroporation, colony PCR was performed to confirm both the presence of the plasmid and the identity of the strain. A 1.5 kb fragment of the 16S rRNA gene was amplified to verify the presence of *L. plantarum*, while a 300 bp region specific to the pLH01 plasmid was amplified to confirm successful plasmid uptake. Based on the colony PCR results, the transformed colonies were verified to contain the pLH01 plasmid and to belong to *L. plantarum* WCFS1.

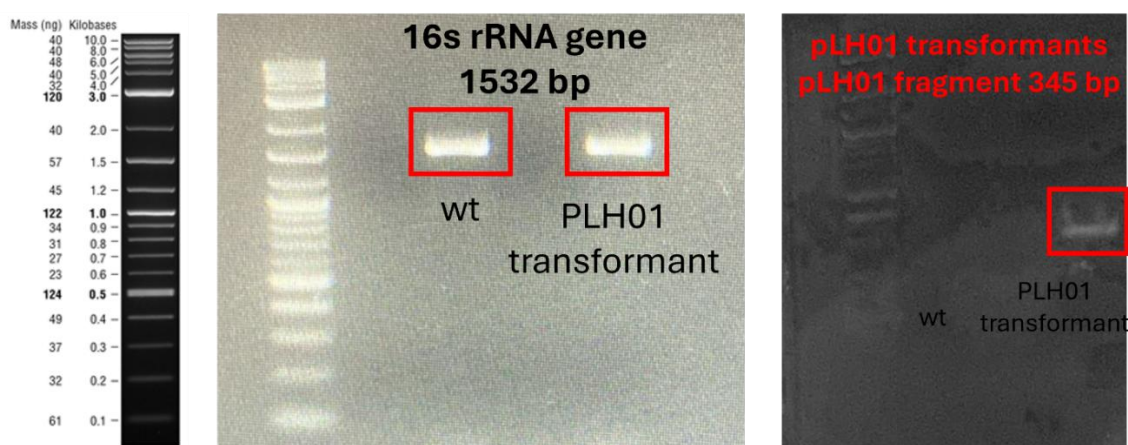


Figure 9. Agarose gel analysis confirming the presence of the plasmid. The left gel shows PCR amplification of the 16S rRNA gene from both wild-type and transformed strains. The right gel shows amplification of a 345 bp region from the pLH01 plasmid in wild-type and transformed strains.

To make sure if the optimized transformation protocol works on other plasmids too, pTRK892 (Addgene #71803) and pMSP3535 (Addgene #46886) plasmids were transformed into electrocompetent *L. plantarum* WCFS1 cells. (Figure 10).

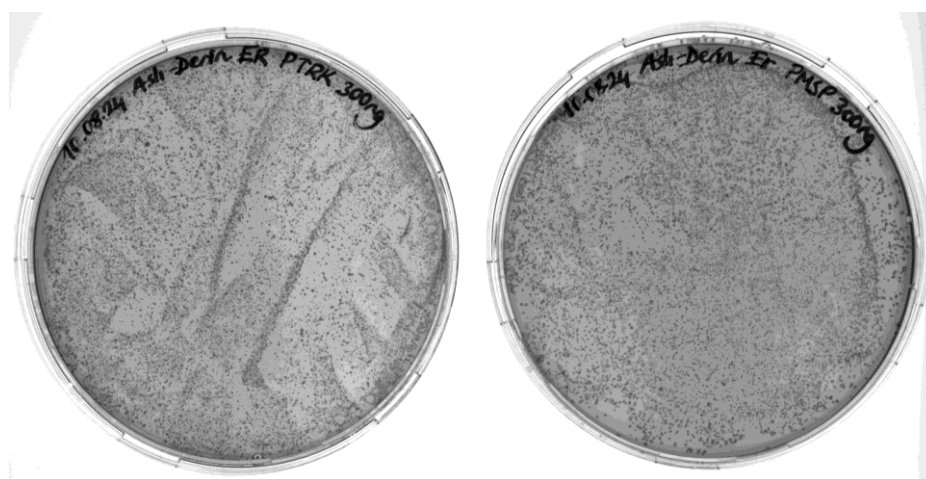


Figure 10. Transformation of *L. plantarum* WCFS1 with pTRK892 (left) and pMSP3535 (right) plasmids using the optimized electroporation protocol. Both

transformations were performed with 300 ng of plasmid DNA, demonstrating the applicability of the protocol to different plasmids.

3.2 Plasmid Curing

Following transformation, serial passaging was performed to promote plasmid loss from *L. plantarum* strains harboring pLH01. For this, cultures were diluted 1:10,000 daily into antibiotic-free medium. At the end of the third day, cells were harvested, serially diluted, and plated on non-selective solid media. After incubation, colonies were counted. The emergence of colonies in the absence of antibiotic selection by day three indicated successful plasmid curing and the presence of plasmid-free *L. plantarum* cells.

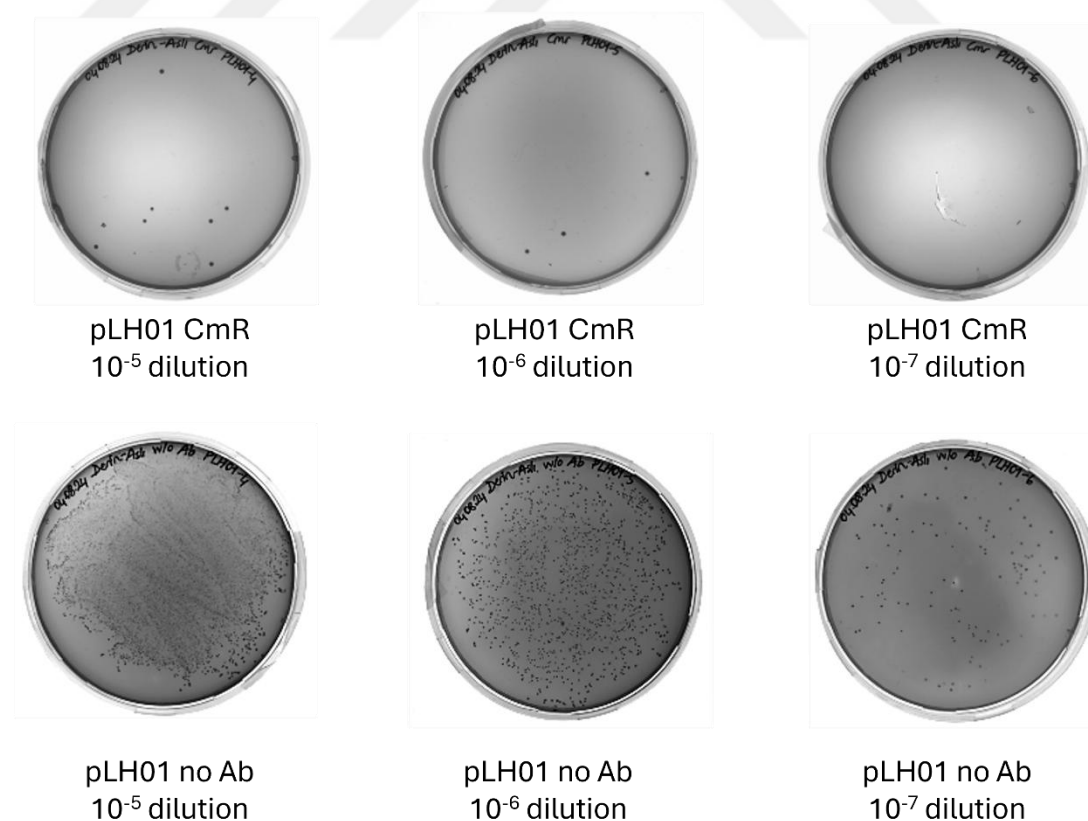


Figure 11. Plasmid Curing of *L. plantarum* WCFS1 After Serial Passaging.

3.3 sgRNA Clonings

In order to insert newly designed gRNA sequences into the gRNA/CRISPR plasmid pHSP02, a cloning strategy was developed. The plasmid carries a constitutively expressed Cas9 region and a gRNA-coding region transcribed under the P11 promoter. The constant region of the gRNA scaffold was PCR-amplified using primers containing the specific gRNA sequences. To remove the existing gRNA insert and linearize the plasmid backbone, a double digestion with *ApaI* and *XbaI* restriction enzymes was planned. This digestion was expected to yield two fragments of approximately 12 kb and 2.1 kb in size.

However, as shown in the agarose gel image (Figure 12), the restriction digestion did not yield the expected band pattern. The absence of the anticipated fragment sizes indicates that the digestion was unsuccessful. Due to this failed restriction outcome, an alternative strategy was pursued.

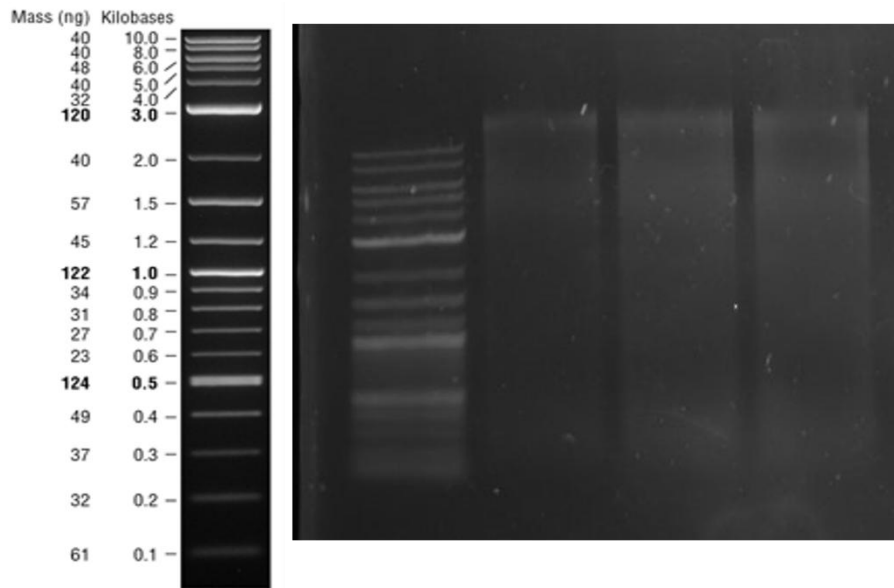


Figure 12. Agarose gel electrophoresis of the *Apal*–*XbaI* double digestion of pHSP02 plasmid. The expected bands (12 kb and 2.1 kb) were not observed, indicating unsuccessful digestion.

The plasmid backbone was instead linearized by PCR amplification using primers flanking the gRNA expression region. This linearized vector was then used in a Gibson Assembly reaction to fuse it with the newly designed gRNA fragment.

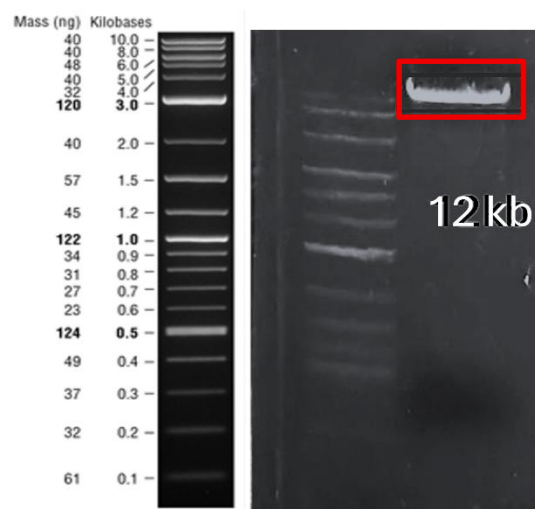


Figure 13. Amplification of pHSP02 vector with prHMO5 and prHMO6 primers.

Based on agarose gel electrophoresis, the vector was successfully obtained at the expected size. To insert the gRNA sequence targeting the *alr* gene, PCR amplification was performed using primers prHMO7 and prHMO8, which added the specific gRNA region to the constant gRNA scaffold on the pHSP02 vector. The resulting PCR product was analyzed by agarose gel electrophoresis (Figure 14).

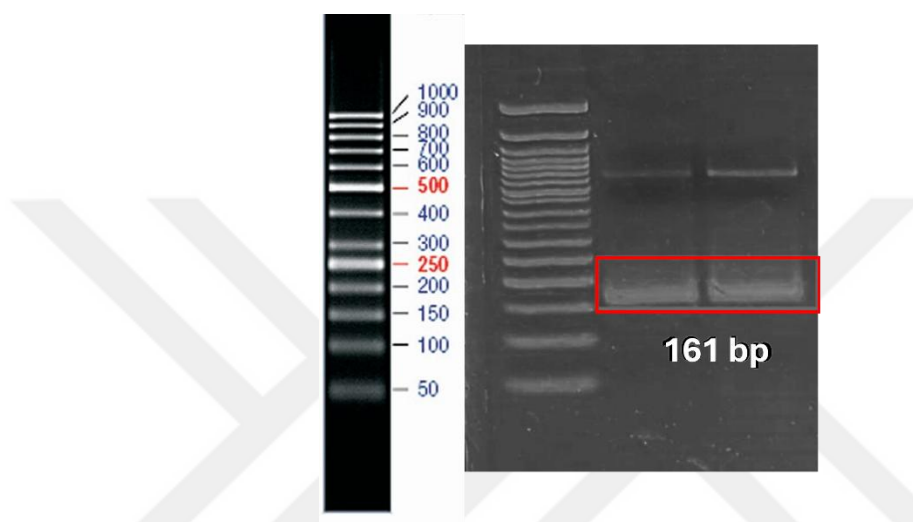


Figure 14. Amplification of gRNA1 targeting the *alr* gene. The PCR product was analyzed on an agarose gel using the Thermo Scientific GeneRuler 50 bp DNA Ladder as a molecular weight marker.

Based on the agarose gel analysis, the desired DNA fragment was successfully amplified. During the amplification of this gRNA, Gibson overhangs were added to the gRNA-encoding region. These overhangs were designed to facilitate the assembly of the linearized vector and the gRNA DNA fragment via Gibson Assembly. The Gibson Assembly reaction was set up as described in Appendix E, Table E.4 and incubated at 50 °C for 1 hour. Upon completion of the reaction, the assembled DNA construct was transformed into chemically competent *E. coli* DH5 α cells. The cells were plated onto agar plates supplemented with the appropriate antibiotic and incubated overnight.

In the initial optimization, the linearized vector and the gRNA-coding DNA fragment were added to the reaction at an equimolar ratio. However, no colonies were observed the next day, indicating that the assembly may not have occurred under these conditions. Subsequently, the reactions were repeated using 1:3 and 1:9 vector-to-insert molar ratios, yet no colony formation was observed. In later experiments, successful assembly was achieved using higher vector-to-insert molar ratios of 1:10 and 1:15. The same steps were also followed for *alr*-gRNA-2 clonings.

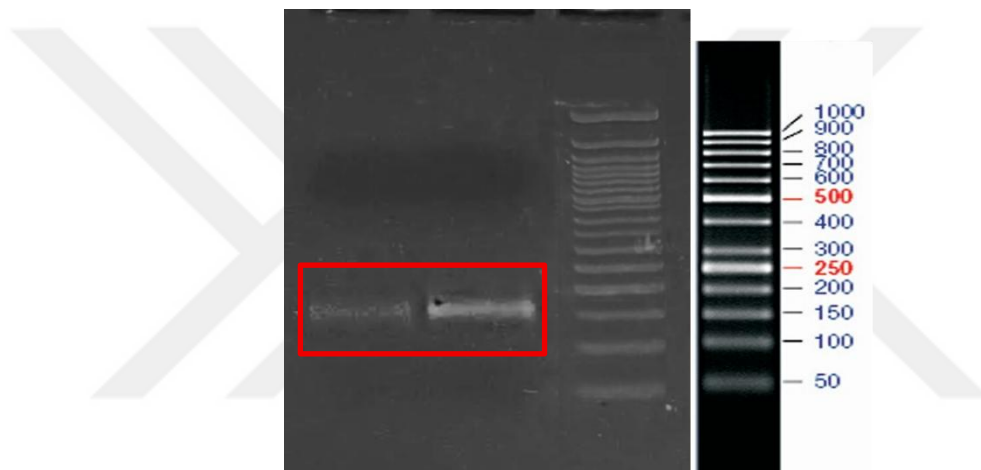


Figure 15. Amplification of gRNA2 targeting the *alr* gene. The PCR product was analyzed on an agarose gel using the Thermo Scientific GeneRuler 50 bp DNA Ladder as a molecular weight marker.

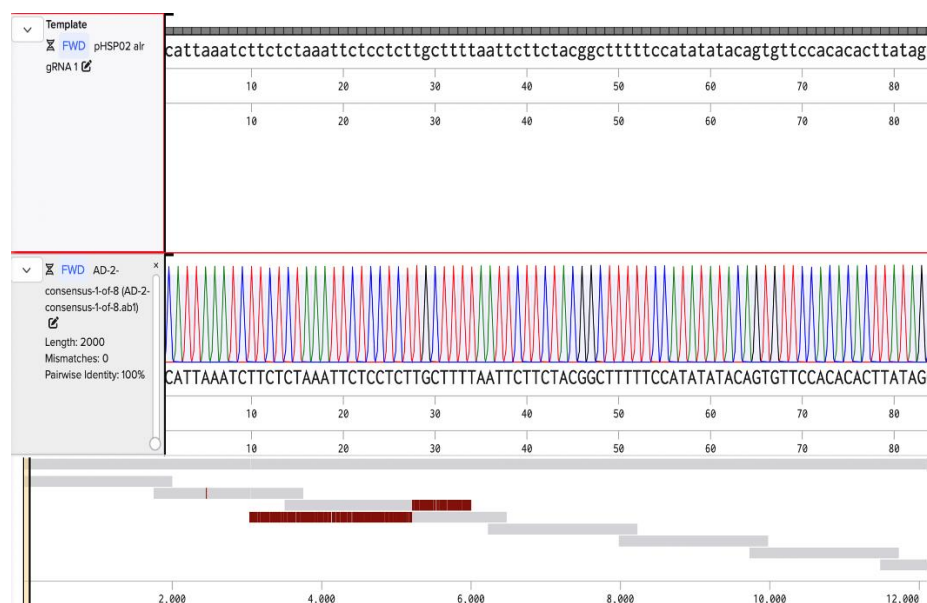


Figure 16. NGS-based confirmation of *alr*-gRNA1 cloning into the pHSP02 backbone. The inserted region was verified by sequencing and aligned using Benchling.

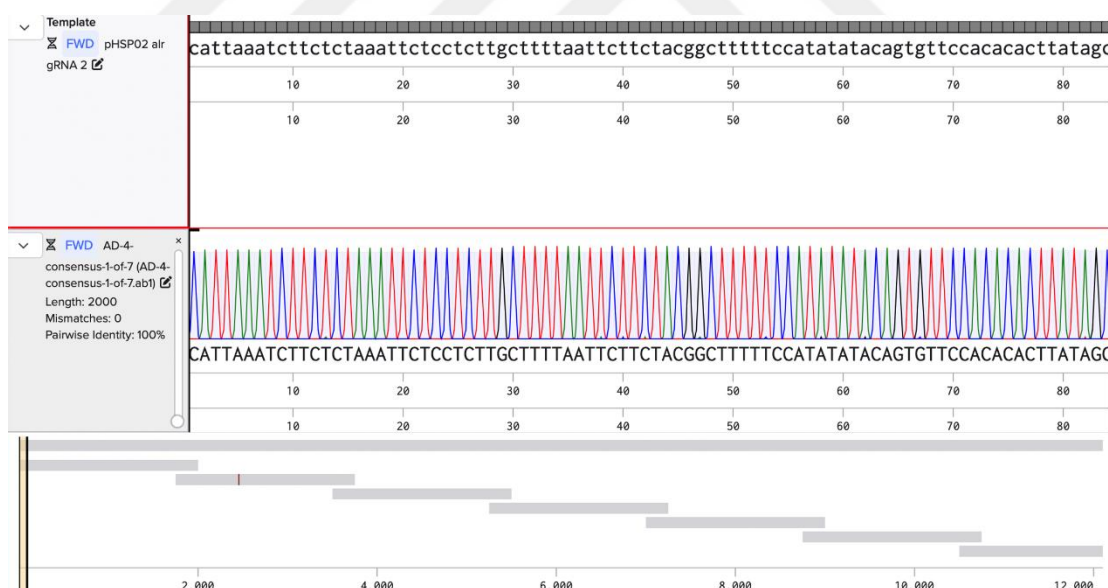


Figure 17. NGS-based confirmation of *alr*-gRNA2 cloning into the pHSP02 backbone. The inserted region was verified by sequencing and aligned using Benchling.

Following the successful optimization of the Gibson Assembly conditions for the *alr*-targeting gRNA, the same cloning strategy was systematically applied to introduce additional gRNA sequences targeting *argH* (gRNA1 and gRNA2) and *upp* (gRNA1 and gRNA2) genes. In each case, the specific gRNA sequence was appended to the constant scaffold region via primer-mediated PCR amplification, and the resulting fragments were assembled with the linearized pHSP02 backbone using the previously optimized high insert-to-vector molar ratios. This unified strategy enabled the seamless construction of a series of CRISPR/Cas9 plasmids, each containing a distinct gRNA sequence designed to target essential loci in *L. plantarum* WCFS1.

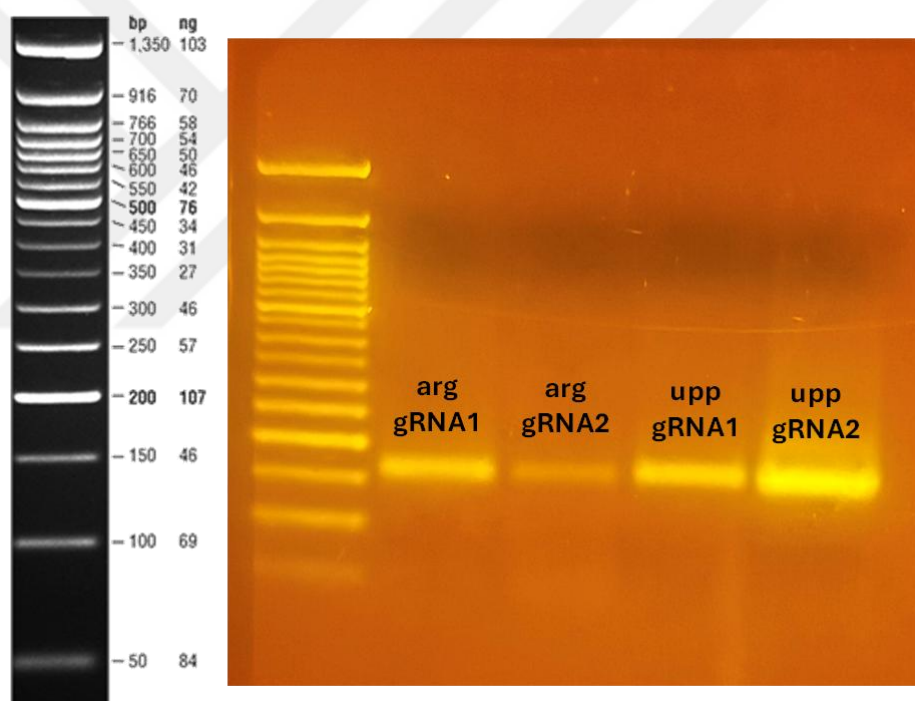


Figure 18. Amplification of gRNA1 and gRNA2 targeting the *argH* and *upp* genes. The PCR product was analyzed on an agarose gel using the Thermo Scientific GeneRuler 50 bp DNA Ladder as a molecular weight marker.

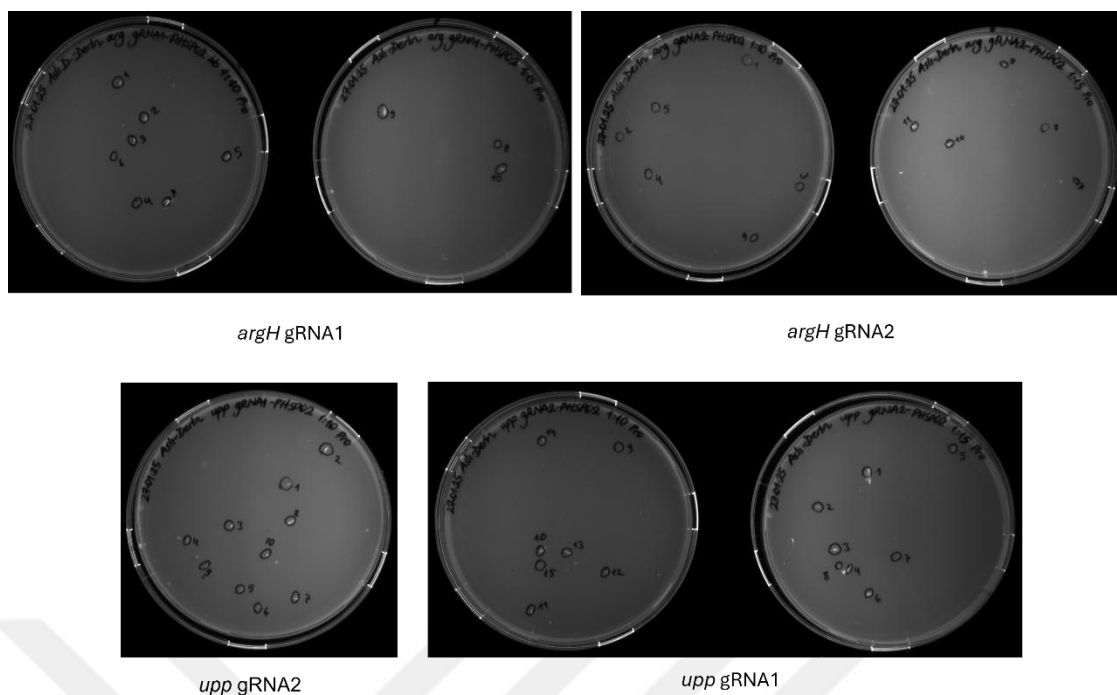


Figure 19. Electrocompetent *E. coli* DH5 α cells were transformed with Gibson Assembly products of gRNA1 and gRNA2 targeting the *argH* and *upp* genes into the pHSP02 plasmid backbone. Colonies were observed on selective LB agar plates supplemented with the appropriate antibiotic, indicating successful cloning events.

See Appendix D for Next Generation Sequencing (NGS) results.

3.4 Homology Arms Construction

Homology arms flanking the target genes were amplified by PCR using *L. plantarum* WCFS1 genomic DNA as a template. Products corresponding to ~500 bp were observed for both upstream and downstream regions of *alr*, *upp*, and *argH*, confirming successful amplification. Thermo Scientific GeneRuler 1 kb Plus DNA Ladder was used as the molecular weight marker.

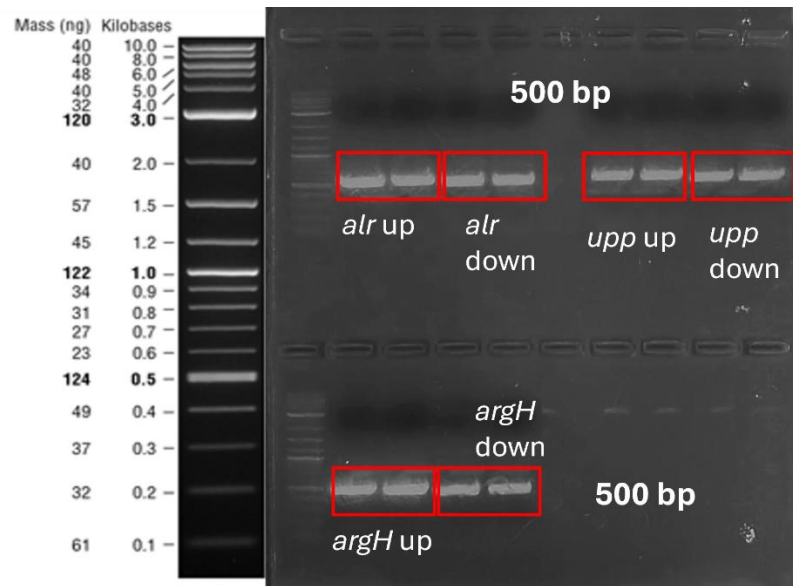


Figure 20. PCR amplification of 500 bp upstream and downstream homology arms for *alr*, *upp*, and *argH* genes.

These PCR products were subsequently assembled into a single cassette using Gibson Assembly. The integrity of the assembled fragments was confirmed by PCR amplification using primers targeting the 5' and 3' ends of the construct. Successful amplification of the expected full-length product indicated correct assembly of the upstream and downstream homology arms (see Figure 20 and Figure 21).

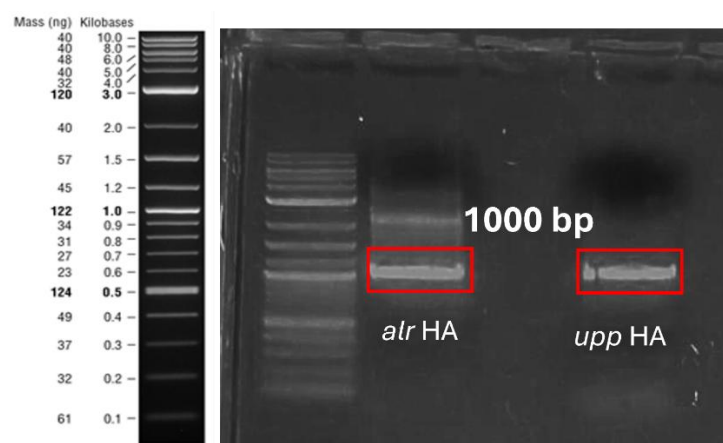


Figure 21. PCR amplification of the assembled 1000 bp *alr* and *upp* homology arms using primers flanking the 5' and 3' ends. Agarose gel electrophoresis shows single

bands at the expected size, confirming successful assembly of the upstream and downstream regions.

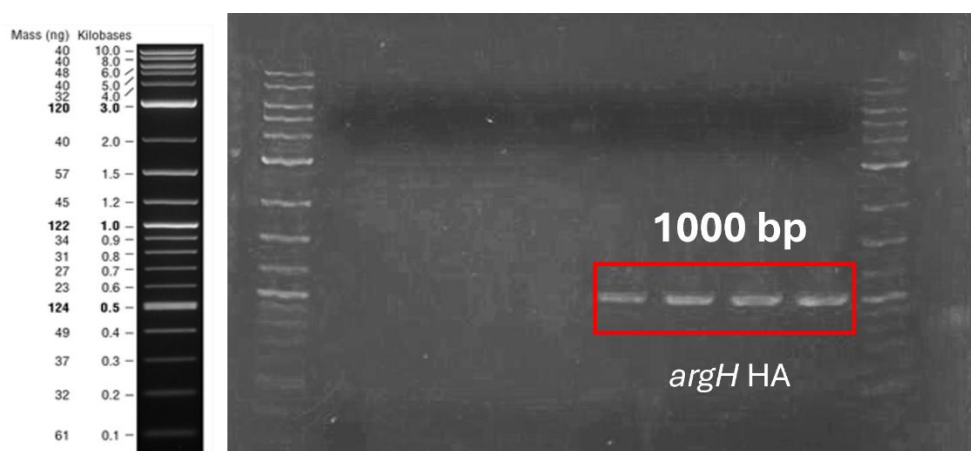


Figure 22. Gradient PCR amplification of the *argH* homology arm.

A 1000 bp band corresponding to the assembled *argH* HA fragment was successfully obtained following gradient PCR optimization.

The *alr* homology arm was successfully cloned into the pHSP02 vector containing the corresponding sgRNA. The 1 kb insert, amplified with primers introducing *Apa*I sites at both ends, was ligated into an *Apa*I-linearized pHSP02 backbone. Following ligation, transformation into *E. coli* DH5 α yielded colonies that were screened by colony PCR and confirmed by Sanger sequencing to contain the correctly assembled construct. These confirmed plasmids were subsequently stored as glycerol stocks for future transformation into *L. plantarum*.



Figure 23. Benchling-based Sanger sequencing analysis confirming integration of *alr* homology arms into pHSP02-gRNA2 construct.

Successful insertion of both upstream and downstream *alr* homology arms into the pHSP02 vector carrying the *alr*-targeting gRNA2 was confirmed by Sanger sequencing. Although only the upstream region is fully visible in the representative screenshot, the alignment coverage bars indicate that the entire ~1 kb homology region was correctly assembled. Minor signal noise at the read edges is typical for Sanger sequencing and does not affect confidence in the correct integration. Sequence analysis was performed using Benchling.

3.5 Knockout Experiments

Initial knockout attempts focused on the *alr* gene, which encodes alanine racemase, due to the well-characterized auxotrophy that results from its deletion. Previous studies have shown that *alr* deletion leads to strict D-alanine auxotrophy, with significant

viability loss (>99%) and morphological abnormalities during extended growth without exogenous D-alanine supplementation [89], [90].

To achieve this, electrocompetent cells pre-induced with the recombinase plasmid pLH01 were transformed with *alr*-targeting pHSP02 constructs, in which sgRNA expression was driven by the P11 promoter. Two strategies were pursued in parallel: one using pHSP02 plasmids containing pre-cloned homology arms flanking *alr*, and another using linear PCR-generated homology arms co-electroporated with a sgRNA-only version of pHSP02. Corresponding DNA concentrations and transformation conditions are detailed in the Materials and Methods section. Control groups consisted of electrocompetent *L. plantarum* WCFS1 cells already carrying the pLH01 plasmid, which were electroporated without any additional DNA. These cells successfully grew on chloramphenicol-containing plates, confirming their viability and retention of pLH01. As expected, no colonies appeared on plates containing both chloramphenicol and erythromycin, since no erythromycin resistance plasmid had been introduced. In contrast, experimental groups that were electroporated with pHSP02 plasmids (either containing integrated homology arms or co-transformed with external linear arm fragment) were plated on media supplemented with chloramphenicol, erythromycin, and D-alanine to support growth of potential *alr* knockout strains. No colony formation was observed in any of these groups, suggesting failure in second plasmid uptake or subsequent genome editing.

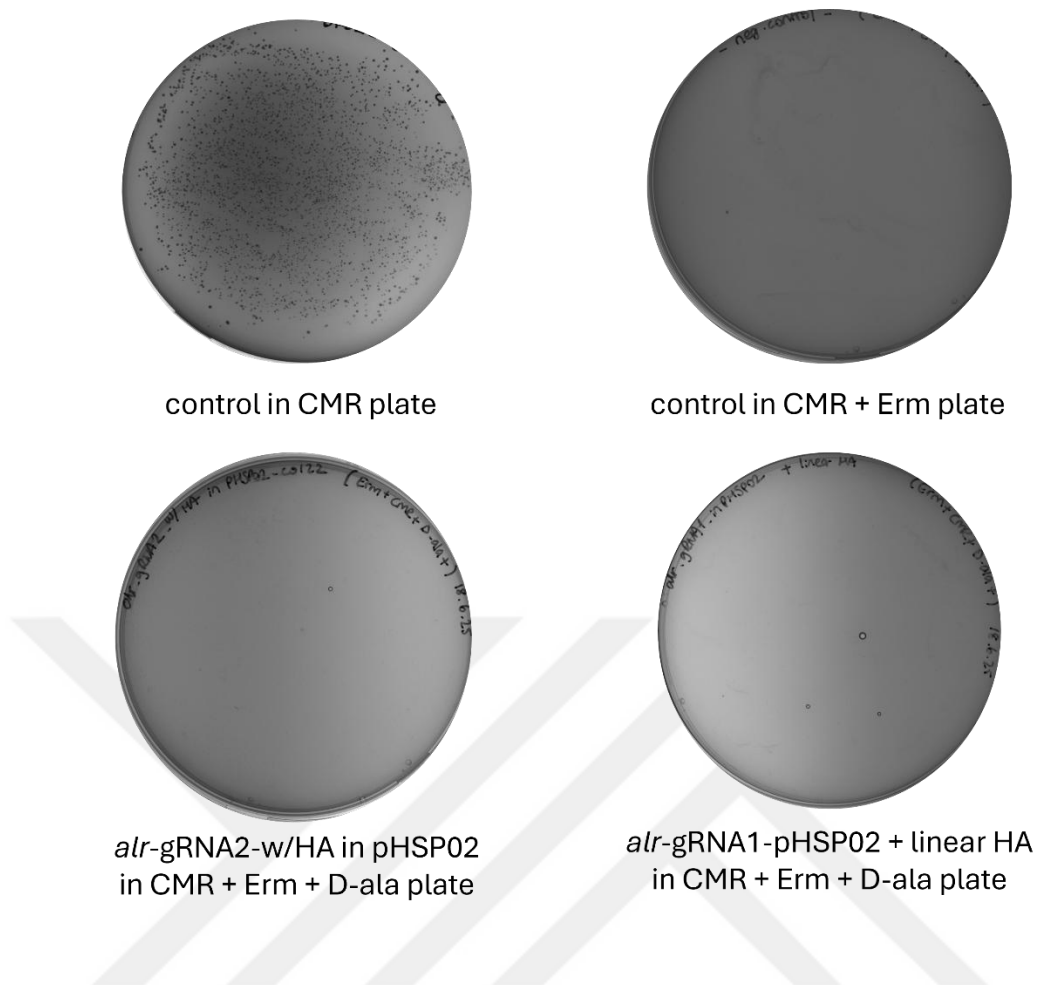


Figure 24. Electroporation-based genome editing attempts in *L. plantarum* WCFS1 using pHSP02 plasmids targeting *alr*. Top left: Control plate (CMR only) showing successful growth of pLH01-containing cells electroporated without a second DNA. Top right: Same control group plated on CMR+ERM shows no growth, confirming absence of erythromycin resistance. Bottom panels: Experimental groups electroporated with pHSP02 constructs encoding *alr*-targeting sgRNA and either internal or co-transformed external homology arms, plated on CMR+ERM+D-alanine. No colony formation was observed in either condition. Plates contained chloramphenicol (10 μ g/mL), erythromycin (10 μ g/mL), and D-alanine (2,25mM end concentration).

As part of our initial attempts to knock out *alr*, we designed and tested two different sgRNA sequences targeting distinct regions of the gene. Despite repeated efforts using these constructs, no colony formation was observed on selective media. To rule out the possibility that our design strategy was the root cause of failure (problems with gRNA designs or homology arm length and so on), we next attempted to replicate the original method described by Huang et al. [43]. In this approach, we used the unmodified version of pHSP02, which was ordered by Addgene, as stated previously. This original version of the plasmid is for deleting the Lp_0537 gene (which encodes the pyruvate decarboxylase, or pdc homolog) in *Lactobacillus plantarum* WCFS1 [43]. Knockout colonies may be selected on MRS agar containing chloramphenicol and erythromycin plates. Again, no colonies were observed on the selection plates. This result was remarkable to understand that the core problem was not the specific designs of the knockout strategy.

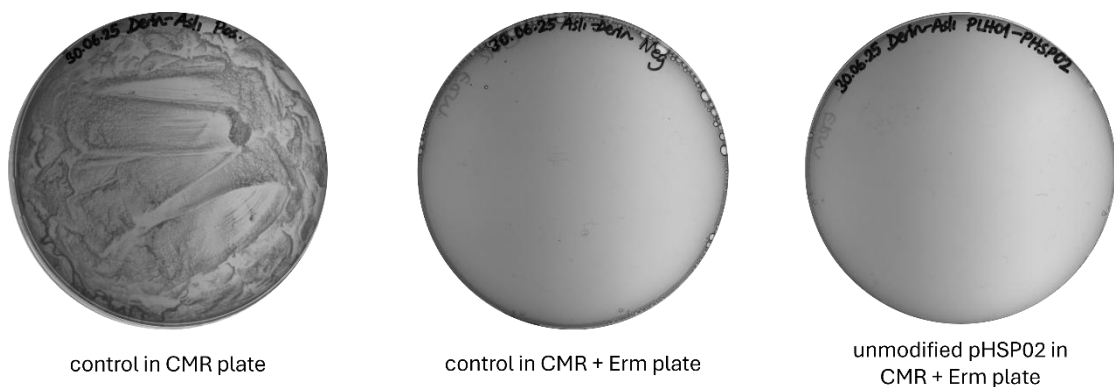


Figure 25. Replication of the original pHSP02-based knockout targeting Lp_0537, as described by Huang et al. [43]. No colony growth was observed on MRS plates containing erythromycin and chloramphenicol.

To better understand the reason behind the repeated lack of colony formation, we wanted to clarify whether the issue was due to ineffective genome cutting by Cas9 or from unsuccessful homologous recombination. One possibility was that Cas9 was indeed making the intended double-stranded breaks, but the recombination machinery induced by pLH01 was not functioning properly, preventing repair and thus leading to cell death. To make sure the issue was not caused by poor recombinase induction due to faulty peptide synthesis or peptide distortion due to freeze-thaw, we repeated the experiments using pLH01-induced cells prepared with two versions of the MAGNSSNFIHKIKQIFTHR peptide: one sourced from GenScript (Peptide 1) and the other synthesized by Peptideam Kimya A.Ş. (Peptide 2). Despite this variation, no colonies appeared on any of the erythromycin- and chloramphenicol-containing plates. These results suggested that the main obstacle was not related to recombinase induction or Cas9 activity, but rather to an inability of the pLH01-carrying cells to successfully take up and retain a second plasmid. To isolate this variable, we set up a control experiment using the pTRK892 plasmid (Addgene #71803), which does not carry Cas9 and does not trigger any genome editing. This plasmid only confers erythromycin resistance and has been previously used successfully in *L. plantarum* [91]. By introducing this plasmid alone into electrocompetent cells already carrying pLH01, we aimed to see whether a second plasmid could be taken up and maintained under our experimental conditions. The transformed cells were plated on MRS agar containing erythromycin and chloramphenicol, but once again, no colonies were observed. This outcome suggested that the main obstacle was not the editing strategy itself but a more general issue with introducing a second plasmid into the pLH01-containing cells.

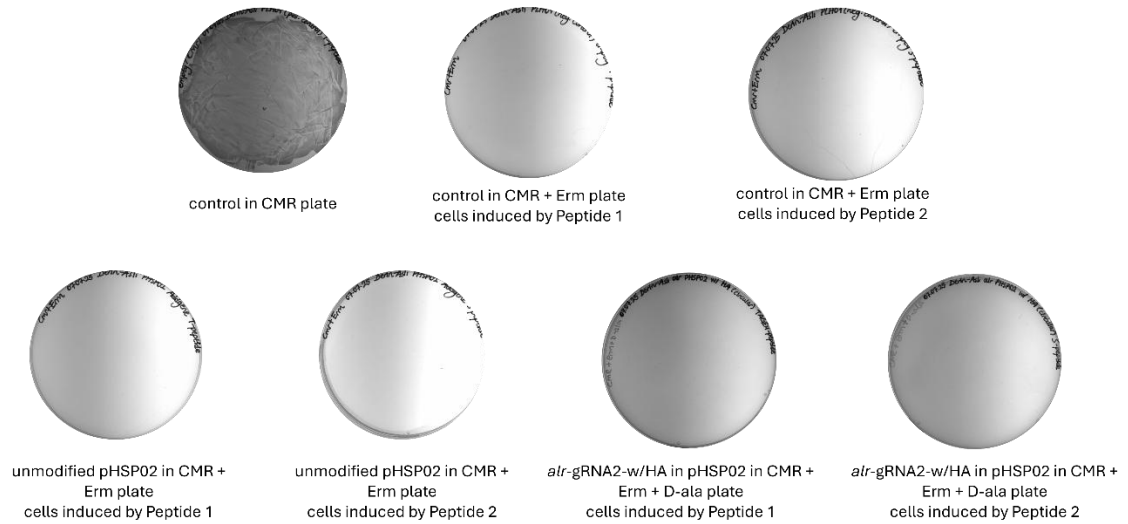


Figure 26. Comparison of knockout attempts using the original unmodified pHSP02 plasmid (targeting *Lp_0537*) and a custom-designed pHSP02 plasmid expressing gRNA2 against *alr*, which also contains plasmid-encoded homology arms. For each plasmid, two separate batches of recombinase-induced electrocompetent *L. plantarum* WCFS1 cells were prepared using synthetic peptide MAGNSSNFIHKIKQIFTHR sourced from either GenScript (Peptide 1) or Peptideam Kimya A.Ş. (Peptide 2). Transformed cells were plated on MRS agar supplemented with chloramphenicol (10 µg/mL), erythromycin (10 µg/mL), and D-alanine (2,25 mM end concentration). No colonies were observed in any condition, suggesting that neither the gRNA design nor the peptide origin rescued the transformation outcome.

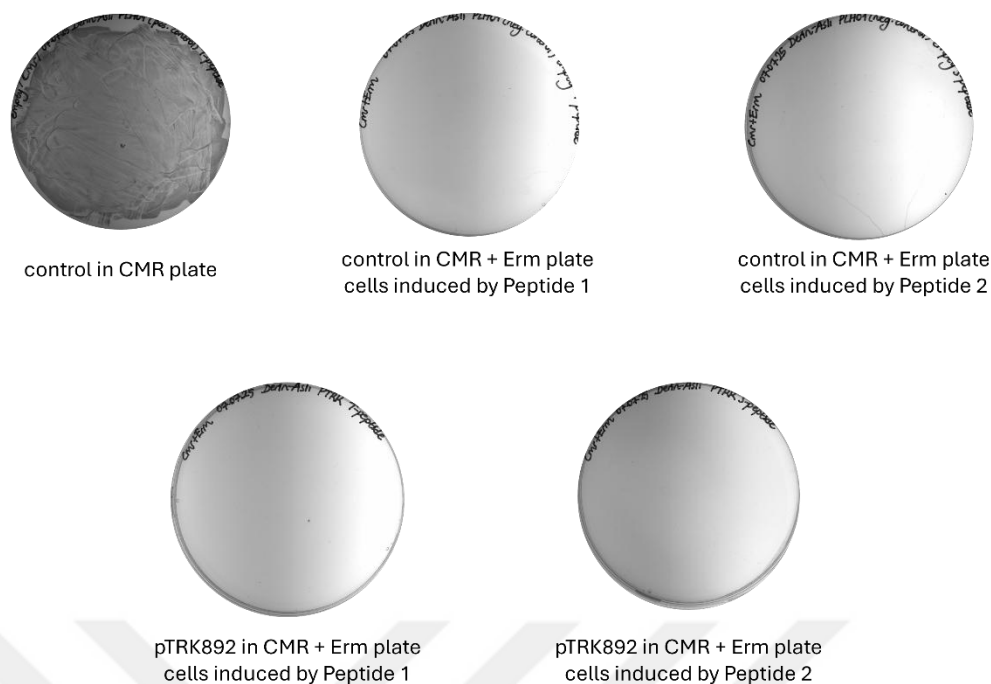


Figure 27. Evaluation of second plasmid uptake in pLH01-containing *L. plantarum* cells induced with two different peptides. To evaluate whether *L. plantarum* WCFS1 cells already carrying pLH01 could successfully take up and express a second plasmid, electrocompetent cells were prepared using two different sources of the RecE/T-inducing peptide (GenScript-Peptide 1 and Peptideam-Peptide 2) as indicated. As controls, cells were either not transformed (top row) or transformed with pTRK892 (bottom row), a plasmid that does not contain Cas9 or any genome editing components but confers erythromycin resistance. All groups were plated on MRS agar containing chloramphenicol and erythromycin. No colonies were observed in any of the pTRK892 transformation plates, suggesting that pLH01-containing cells could not maintain a second plasmid under dual selection, regardless of the peptide source used during induction.

3.6 Expression Cassette Designs

The expression cassette was initially designed to include the *manA*, *manB*, and *manC* genes, forming the central segment of the GDP-mannose biosynthesis pathway. This construct aimed to enhance the intracellular availability of mannose precursors forming the central segment of the GDP-mannose biosynthesis pathway for use in 2'-FL production in engineered *L. plantarum*.

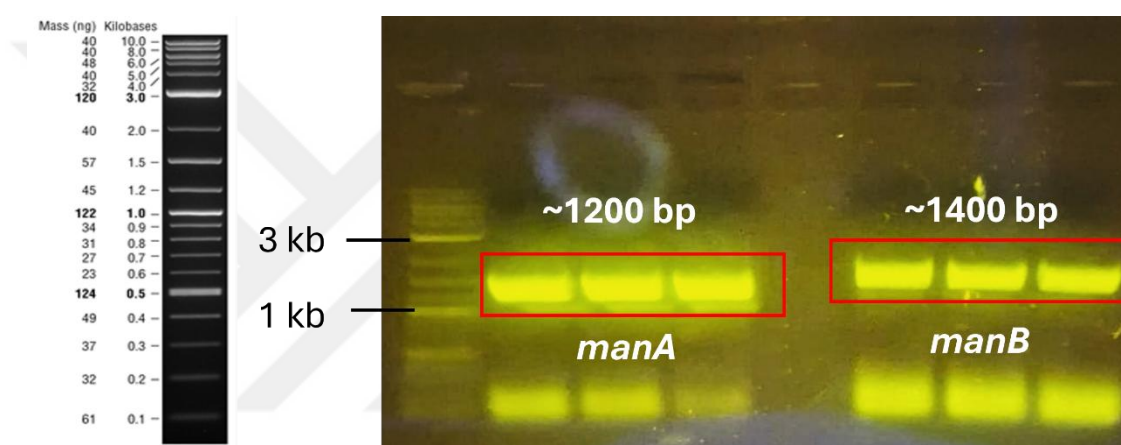


Figure 28. PCR amplification of *manA* and *manB* genes. Agarose gel electrophoresis results showing successful amplification of the *manA* and *manB* genes from *E. coli* BL21 (DE3) genomic DNA.

As shown in Figure 28 and Figure 29, expected band sizes were approximately 1200 bp for *manA*, 1400 bp for *manB*, and 1450 bp for *manC* which were clearly observed in the corresponding lanes.

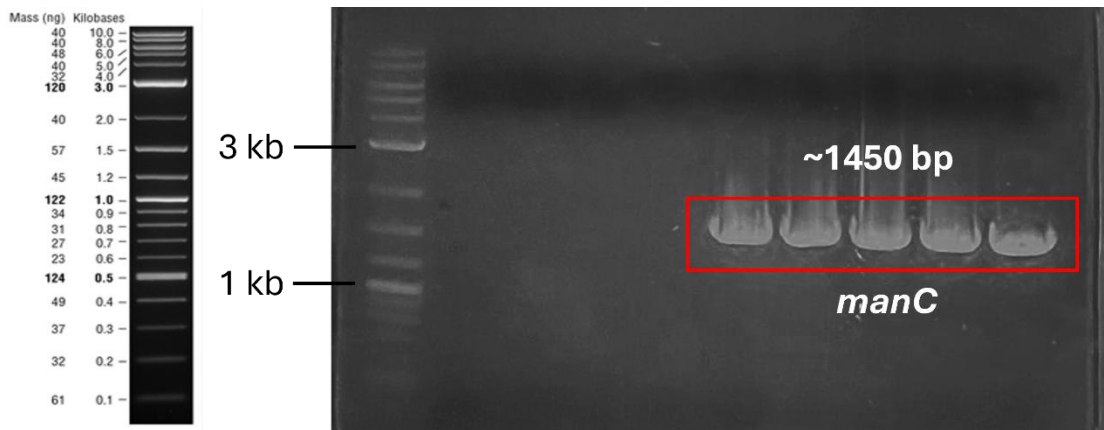


Figure 29. PCR amplification of *manC* gene. Agarose gel electrophoresis showing successful amplification of the *manC* gene (~1450 bp) from *E. coli* BL21 (DE3) genomic DNA. The absence of bands in the first few lanes reflects suboptimal PCR conditions tested during gradient optimization.

To ensure modular and reliable expression of each gene in the synthetic operon, we incorporated the self-cleaving ribozyme RiboJ upstream of *manA*, *manB*, and *manC*. This element helps insulate gene expression by reducing transcriptional context effects, leading to more consistent downstream translation.

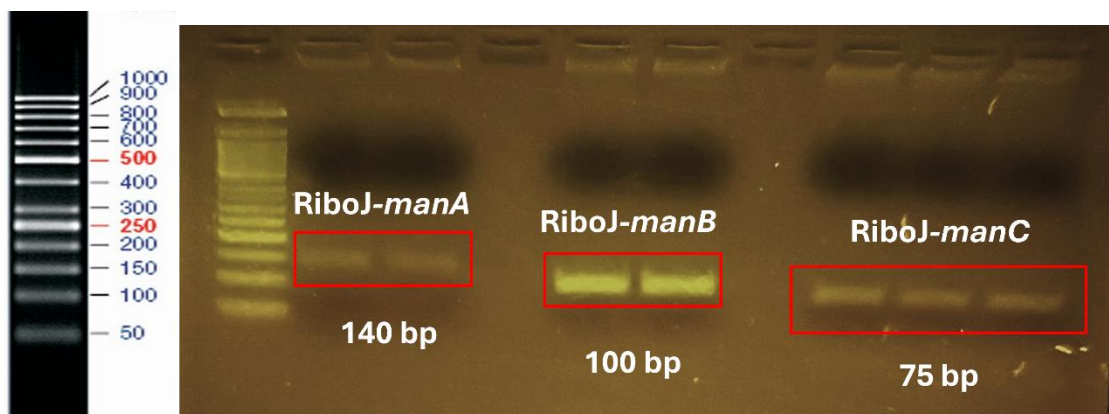


Figure 30. PCR amplification of RiboJ fragments designed to be fused upstream of *manA*, *manB*, and *manC* genes. Amplicons of approximately 140 bp (RiboJ-*manA*), 100 bp (RiboJ-*manB*), and 75 bp (RiboJ-*manC*) were successfully obtained, confirming the

generation of gene-specific RiboJ fusion elements for subsequent assembly into the multi-gene expression cassette.

In Figure 30, the gel shows successful amplification of each RiboJ-gene fragment: *RiboJ-manA* (~140 bp), *RiboJ-manB* (~100 bp), and *RiboJ-manC* (~75 bp). These RiboJ fragments were later assembled with their respective gene fragments (see Figure 31).

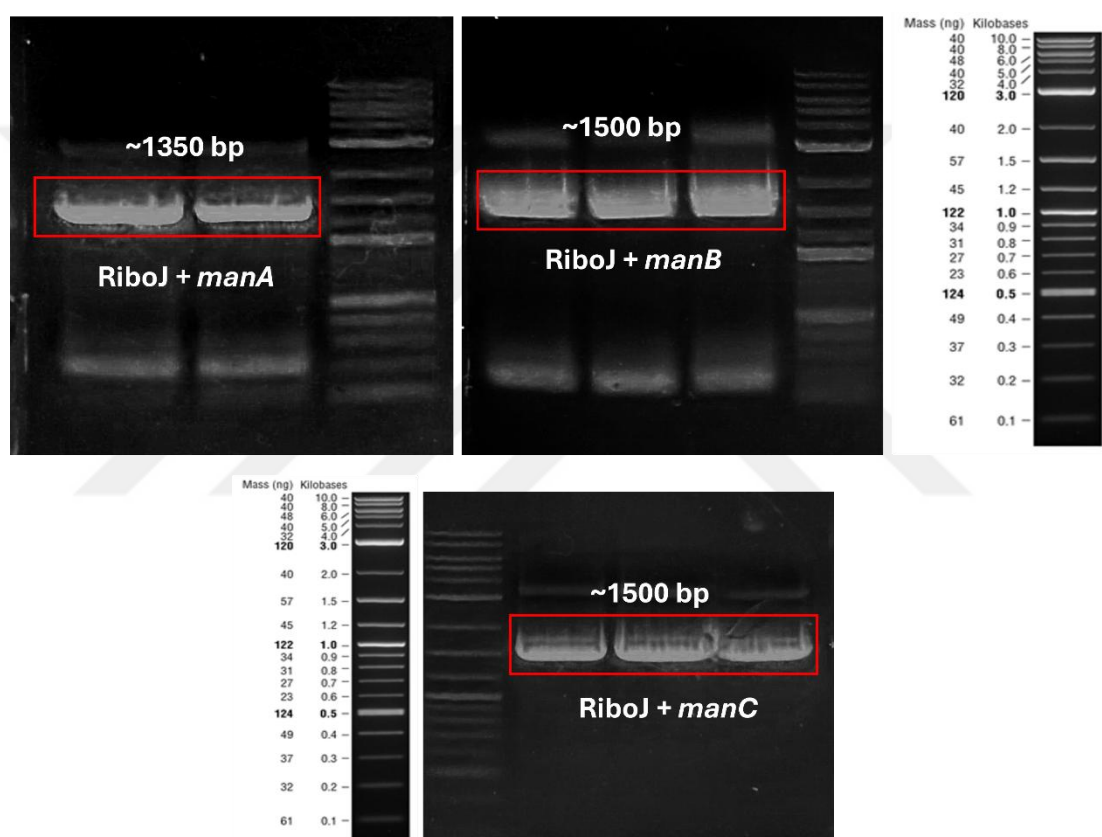


Figure 31. PCR amplification of RiboJ-fused *manA*, *manB*, and *manC* gene fragments. Expected band sizes were obtained for each fusion construct: ~1350 bp for RiboJ+*manA*, ~1500 bp for RiboJ+*manB*, and ~1500 bp for RiboJ+*manC*, confirming successful overlap extension of regulatory and coding sequences prior to operon assembly.

Terminator sequence was also generated via PCR amplification to be used as a part of expression and complementation cassettes.

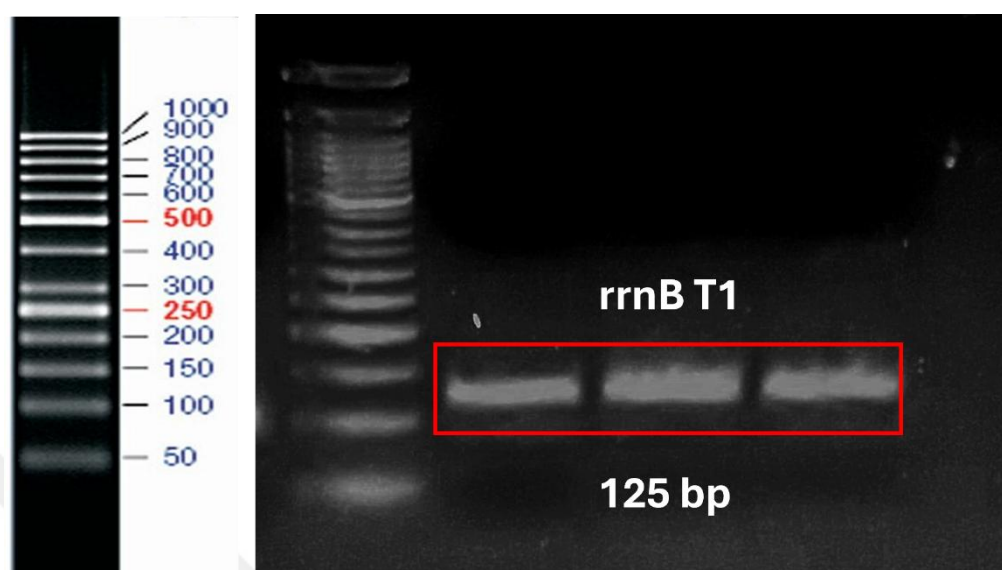


Figure 32. Verification of the *rrnB* T1 terminator sequence PCR amplification. Expected band size of 125 bp was obtained.

As specified before, to enable selection and genomic integration without relying on antibiotic markers, the *alr* gene was also incorporated into the cassette. This gene, which restores growth in D-alanine–deficient media, will serve as a functional selection marker based on the auxotrophic phenotype of the engineered strain. Importantly, the *alr* gene was positioned such that it could be removed in future iterations without disrupting the main expression module. *Ptuf33* promoter was also synthetically generated without relying on a DNA template, as detailed in the Materials and Methods section. *Ptuf33* promoter and *alr* gene fragment (amplified from *L. plantarum* WCFS1 genome) were assembled.

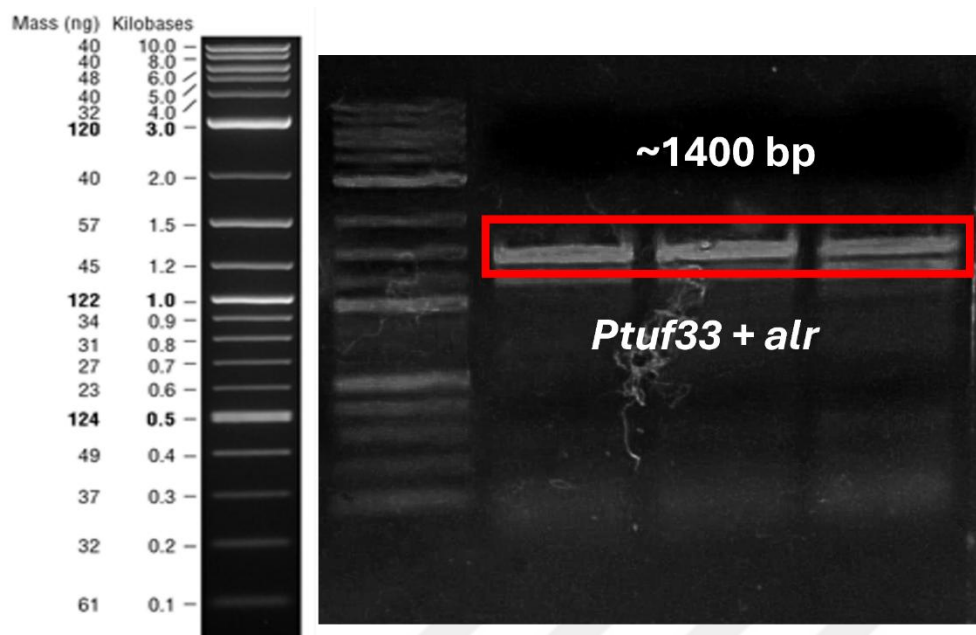


Figure 33. Verification of the *Ptuf33* promoter assembled with *alr* gene fragment. The expected band size of 1400 bp was obtained.

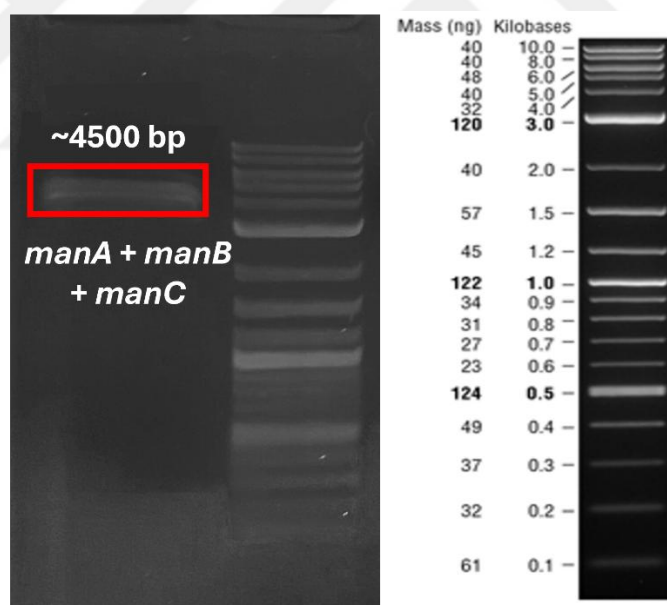


Figure 34. Agarose gel electrophoresis image showing the final assembly of the expression cassette containing *manA*, *manB*, and *manC*, along with their respective RiboJ insulators, ribosome binding sites (RBS), spacer sequences, and a downstream transcription terminator. The band at approximately 4500 bp confirms the successful amplification of the full-length operon construct designed for multigene expression.

Currently, efforts to finalize the expression cassette are still ongoing. The previously assembled *Ptuf33-alr* complementation cassette will be fused with the tricistronic expression module shown in Figure 34. This complete construct is planned to be cloned downstream of the constitutive P11 promoter in a Cas9-deleted version of the pHSP02 backbone. The resulting plasmid will be sent for sequence verification via next-generation sequencing. Once this construct is successfully validated, similar cloning strategies will be initiated for additional operons designed for 2'-FL biosynthesis, such as those carrying *gmd* and *fucT*, as was discussed in detail in the previous chapter. These steps are intended to establish a flexible and modular framework for the expression of various biosynthetic gene clusters in *Lactobacillus plantarum*.

CHAPTER IV: CONCLUSION

In this thesis, a comprehensive strategy was developed to engineer *Lactobacillus plantarum* WCFS1 as a recombinant host platform capable of producing 2'-fucosyllactose (2'-FL), a health-promoting human milk oligosaccharide. By integrating advanced tools of synthetic biology such as markerless CRISPR/Cas9 genome editing, this work aimed to expand the functional capacity of a food-grade probiotic organism into a metabolically active recombinant host for biosynthetic applications.

Throughout the study, essential groundwork was laid by optimizing transformation conditions, establishing electroporation protocols for *L. plantarum*, and successfully implementing a plasmid curing workflow. sgRNA sequences targeting key genes such as *alr*, *argH*, and *upp* were designed, cloned, and sequence-verified. Homology arm construction and integration strategies were also completed, including the external delivery of linear DNA to support CRISPR-mediated double-strand break repair. Although genome editing trials did not yet yield viable colonies, the molecular infrastructure (e.g. plasmids, homology arms, and expression cassettes) was successfully constructed and validated.

At a broader point of view, this work demonstrates the feasibility and value of engineering non-model probiotic organisms using rational, modular, and food-compatible approaches. While traditional chassis such as *E. coli* offer high yields, they remain unsuitable for *in situ* applications due to safety concerns. *L. plantarum*, on the other hand, offers the unique advantage of functioning as both a recombinant

production host and a live therapeutic agent. Establishing this strain as a biosynthetic probiotic opens the door for applications where beneficial compounds are produced directly in the gut or in fermented food matrices.

Though challenges remain, particularly in achieving high transformation efficiency and maintaining metabolic balance during pathway integration, this thesis on engineering *Lactobacillus plantarum* as a recombinant host platform establishes a strong foundation for future efforts in recombinant probiotic engineering. The progress achieved here represents a meaningful first step toward developing a dual-function *L. plantarum* strain that not only survives and colonizes the gastrointestinal environment but also actively synthesizes functional biomolecules with proven health benefits.

CHAPTER V: FUTURE PROSPECTS

This study established the key markerless genetic tools required for engineering *Lactobacillus plantarum* WCFS1 but did not yet achieve complete genome editing or functional expression of the 2'-fucosyllactose biosynthesis pathway. The main limitations encountered were low transformation efficiency, difficulties in second plasmid uptake and retention, and possible cellular stress from multiple simultaneous DNA inputs. Future work should focus on resolving these specific issues.

Optimizing electroporation conditions remains a critical step. While the optimized conditions are effective for initial transformation of wild-type cells, they appear to be insufficient for secondary transformations in previously transformed strains. Tuning of pulse parameters, buffer composition, and cell preparation protocols can be changed to improve the second DNA uptake. In addition, simplifying the editing workflow, for example by reducing the number of constructs introduced at once or embedding homology arms directly into the editing plasmid, could enhance efficiency and reproducibility.

Once genome edits can be reliably introduced, verifying the expression and activity of the synthetic genes will be essential. This involves confirming the production of pathway intermediates and the final product (2'-FL) using analytical methods such as HPLC or LC-MS. Testing the engineered strain in realistic environments, such as dairy-based fermentation systems, will also be necessary to evaluate its performance under production-like conditions.

While the essential genetic tools have been put in place, building a fully functional *Lactobacillus plantarum* strain that produces 2'-FL is still an ongoing effort. The next steps will involve careful troubleshooting, confirming gene expression and activity, and testing how the strain performs under realistic conditions. With continued work and gradual improvements, this approach could lead to a practical microbial system suitable for both nutritional and therapeutic use.



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APPENDIX A

DNA SEQUENCES OF CONSTRUCTS

Table A. 1: Gene sequences and regulatory elements used in this study

Gene Name	Sequence
<i>alr</i>	ATGGTTGTAATTGGGGAGCACCGCCACACACAAGTCACA GTCGACTTGCAGGCAATTAAGACAAATATTAGTAATGAA ATGGCGCAAAAGGATGAGTTGACCGAGTTATGGGCAGTC GTTAAAGCGAATGGTTATGGACATGGAATTATCCAAGTT GCTCAGGCCGCCAAAGAAGCCGGGGCGACCGGCTTTTGT GTTGCAATCCTGGATGAGGCCTTAGCGTTGCGGGCCGCT GGCTTTGCGGAACCCATCCTAGTACTTGGAATTACGGAA CCGGAATACGCCCCACTGGTAGCTGAAAAGGATATTTCA CTAGCTGTTGGAACGCAAGATTGGCTGACTACGGCCGCA GCAATTTTAGCGGCTAATCAAGTGACGACACCACTTCAC GTTTCATCTTGCAATAGATAACGGGTATGGGACGAATCGGG TTTCAGACGCCCCGAAGAATTGGCAACGGCGGTTACGACT TTGCGTCAACCGCAGTCACCATTGACTTTGAAGGGATT TTACGCATTTTGCAACGGCTGACCAGGCAGATGATACGT ATTTTACTCATCAATTAAATAATTGGAAACACTTGATTGC AGTGGTGGATGAGCTACCACGCTATGTCCACGTGTCCAA TTCGGCCACCAGTCTCTGGCATCAAGCTTGCAATGGCAA CATGGTGCGCTTTGGGGTTGCACTCTATGGTCTAAATCCT TCTGGTCGCGAACTCAGCGCACCATACCCCTTGCAACCC GCGTTGTCGCTAACGGCACGCTTGACGTTTGTTAAACGC TTGGCTCGGGGCAAATCGGTGAGCTATGGTGCCACGTAT ACGGCCGCACAGGATGAATGGATTGGCACGGTGCCGATT GGGTATGCGGACGGCTATGAACGCCGATTACAAGGCTTC CATGTACTTGTTGATGGTGAGTTTTGCGAAATCGTCGGA CGGGTCTGCATGGACCAGCTGATGGTTCGTCTGCCACAT GAAGTACCGGTTGGAGCTAAGGTAACCTTTGGTTGGCACG GACGGTGCTCGTACCATTTCTGTTGCAAGATATTGCTGACT ATTGTGGGACAATTCATTATGAGATTGCTTGTGGGTTAG CACCACGAGTGCCGAGAGTTTATATAGATTAA
<i>argH</i>	ATGAGTACCCAGAAGCTATGGGGTGGCCGGTTTACCGAA ACGGGTGCTAAGTATGTTGATGACTTTGGGGCATCAATT AGCTTTGACCAGTTGCTTGCCGCAGAAGATATTGCTGGT TCACTGGCACACGTTAAAATGTTGAAGAAAACCTGGTATT TTGCCCCGCCGCTGATGTTGATCAAATTGTTGCTGGTTTGG AAACCTTGGCTGAGCGTCAAAAACAAGGTGAGTTGGAAT TTTCAACGGTCAACGAAGACATTCATATGAATATTGAGT CATTATTGACCGAAGAGATTGGGCCGGTCGCTGGGAAGT

	TGCACACGGCCCCGGTCGCGGAATGACCAAGTTGCCACGG ATTTCCACTTGTACTTGAAGCATCAATTACCACTAATATT GGACCGGCTACATGAACTGGAAACAGTATTGGTCGATAA AGCGAGTGAAAACGTGGAGACGGTCATGCCGGGGTACA CGCATTTGCAACACGCCCAACCCATCTCGTACGCGCACT ACCTGTTAGCTTATTACCAAATGTTCAAGCGGGACATGG AACGCTTCGAGTTTAACCTGAAGCATACCGATATTTTCGC CATTGGGAGCCGCTGCACTTGCTGGAACGACTTTTCCAA TCGACCGGGAATATAGTGCGAAACTATTAGGATTCAAGTG AAGTCTATCATAATAGTTTGGATGCCGTTTCTGACCGGG ACTTCGTGCTTGAGTTTCTCAGCAATGCTTCGATCCTGAT GATGCATCTCTCACGGTTTTGTGAAGAAATTGTCAGCTG GAGCAGTTATGAATTCAAGTACATCAGTTTGAGCGATCA GTTTTCAACGGGTAGTTTCGATTATGCCGCAAAGAAGAA CCCTGACATGGCCGAATTGATTCGGGGCAAGTCCGGCCG GGTTTACGGCAACTTGATGGGATTGCTAACTGTCATGAA AGGGATTCCGCTCGCTTACAATAAGGATCTGCAAGAAGA CAAAGAAGGGGCGTTTGATACGGTCACGACCGTGTTAAC GAGTCTCCACGTCTTCACCGGCATGTTGGCAACGCTGAC CGTTAATGAAGATCGGATGGCTGATGCCACGACTAACGA TTTCTCAAATGCGACCGAACTAGCCGATTACCTTGCAAA CAAAGGAATCCCATTTTCGGGAGGCCCATGCCATTGTGGG GAAGCTCGTCTTGATGGCTTGAAAAAACAGCGGCCGTT GCAGGATATTCCACTCAAAGAATATCAGTCAATCTCACC GCTGATCCAAGAAGATGTCTACCATGATTTGAACGCAAA AGTTGCGGTGGAACGGCGGCATTTCGCTAGGTGGTACTGG TTTTGATCAGATTAGACAAGAATTGCAACGGGCACAAAC GCAATTGTCAGCCTATCAATCTGAAACCACTGATTAA
<i>upp</i>	ATGGGTAAGTTTGAGGTTTTGGATCATCCGCTCATTCAAC ATAAATTGACGATCATTCGGGAAAAGAAGTGTGGTACTA AGGTCTTCCGGGAAATGGTTAACGAAATTTCAACGTTGA TGGCGTACGAAGTATCACGCGATATGCCGTTGAAGGACA TTGAGATTGAAACACCGATTGCCAAGTCAACGCAAAAAA CGTTGGCAGGTAAGAAAGTCGCCATTGTACCAATCTTAC GGGCTGGCTTAGGGATGGTTGACGGTTTCTTAAACATGA TTCCGGCAGCTAAAGTTGGGCACGTTGGGATGTATCGGG ATGAAAAGACCTTGAAGCCGGTCGAATATTTCTGTTAAAT TACCAAGTGATATTTCAACAACGGCAATTATTTGTCGTTGA TCCAATGTTAGCCACTGGTGGTTCAGCCATCATGGCGAT GGATATGTTGAAAAAGCGCGGCGCAAGTAATATTAAGTT TATGTGTTTAGTTGCGGCGCCTGAGGGTGTTAAGGCACT TCGGGACGCACATCCAGACATCGACGTTTACACGGCAGC ACTTGATGACCATTAAATGAAGATGGCTACATCGTTCC TGGTTTGGGCGATGCGGGCGACCGGTTATTTGGTACGAA GTAA
<i>manA</i>	ATGCAAAAACCTCATTAACTCAGTGCAAAACTATGCCTGG GGCAGCAAAACGGCGTTGACTGAACTTTATGGTATGGAA AATCCGTCCAGCCAGCCGATGGCCGAGCTGTGGATGGGC GCACATCCGAAAAGCAGTTCACGAGTGCAGAATGCCGCC GGAGACATCGTTTCACTGCGTGATGTGATTGAGAGTGAT

	AAATCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCTTT GGCGAACTGCCTTTCCTGTTCAAAGTATTATGCGCAGCA CAGCCACTCTCCATTCAGGTTTCATCCAAACAAACACAAT TCTGAAATCGGTTTTTGCCAAAGAAAATGCCGCAGGTATC CCGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAAC CACAAGCCGGAGCTGGTTTTTTGCGCTGACGCCTTTCCTTG CGATGAACGCGTTTCGTGAATTTTCCGAGATTGTCTCCCT ACTCCAGCCGGTCGCAGGTGCACATCCGGCGATTGCTCA CTTTTTACAACAGCCTGATGCCGAACGTTTAAGCGAACT GTTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATC CCACGCGCTGGCGATTTTAAAATCGGCCCTCGATAGCCA GCAGGGTGAACCGTGGCAAACGATTTCGTTAATTTCTGA ATTTTACCCGGAAGACAGCGGTCTGTTCTCCCCGCTATTG CTGAATGTGGTGAAATTGAACCCTGGCGAAGCGATGTTT CTGTTGCTGAAACACCGCACGCTTACCTGCAAGGCGTG GCGCTGGAAGTGATGGCAAACCTCCGATAACGTGCTGCGT GCGGGTCTGACGCCTAAATACATTGATATTCCGGAACGTG GTTGCCAATGTGAAATTCGAAGCCAAACCGGCTAACCAG TTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGAC TTCCCGATTCCAGTGGATGATTTTGCCTTCTCGCTGCATG ACCTTAGTGATAAAGAAACCACCATTAGCCAGCAGAGTG CCGCCATTTTGTCTGCGTCGAAGGCGATGCAACGTTGT GGAAAGGTTCTCAGCAGTTACAGCTTAAACCGGGTGAAT CAGCGTTTATTGCCGCCAACGAATCACCGGTGACTGTCA AAGGCCACGGCCGTTTAGCGCGTGTTTACAACAAGCTGT AA
<i>manB</i>	ATGCTAACTTGCTTTAAAGCTTATGATATTCGCGGGGAAA CTAGGCGAAGAAGTGAATGAAGATATCGCCTGGCGCATT GGGCGTGCCTATGGCGAATTTCTCAAACCGAAAACCAT GTGTTAGGCGGTGATGTCCGCCTCACCAGCGAAACCTTA AAACTGGCGCTGGCGAAAGGTTTACAGGATGCGGGGCGTC GATGTGCTGGATATCGGTATGTCCGGCACCGAAGAGATC TATTTGCGCCACGTTCCATCTCGGCGTGGATGGCGGCATTG AAGTTACCGCCAGCCATAATCCGATGGATTATAACGGCA TGAAACTCGTGCGCGAGGGGGCTCGCCCGATCAGCGGG GATACCGGACTGCGCGATGTCCAGCGTCTGGCAGAAGCC AACGACTTCCCTCCCGTTGATGAAACCAAACGCGGTGCT TATCAGCAAATCAATCTGCGTGACGCTTACGTTGATCAC CTGTTGCGTTATATCAACGTCAAAAACCTCACGCCGCTC AAGCTGGTGATTAACTCCGGGAATGGCGCGGCGGGGCC GGTGGTGGACGCCATTGAAGCCCGCTTTAAAGCCCTCGG CGCACCGGTGGAATTAATCAAAGTACACAACACGCCGG ACGGCAATTTCCCCAACGGTATTCCTAACCCGTTGCTGCC GGAATGCCGCGACGACACCCGTAATGCGGTCATCAAACA CGGCGCGGATATGGGCATTGCCTTTGATGGCGATTTTGA CCGCTGTTTCCTGTTTGACGAAAAAGGGCAGTTTATCGA GGGCTACTACATTGTGCGCCTGCTGGCAGAAGCATTCCT CGAAAAAAATCCCGGCGCGAAGATCATCCACGATCCGC GTCTCTCCTGGAACACCGTTGATGTGGTGACCGCCGCGG GCGGCACTCCGGTGATGTCGAAAACCGGACACGCCTTA

	TTAAAGAACGTATGCGCAAGGAAGACGCCATCTACGGTG GCGAAATGAGCGCCCACCACTATTTCCGTGATTTCGCTT ACTGCGACAGCGGCATGATCCCGTGGCTGCTGGTCGCCG AACTGGTGTGTCTGAAAGGAAAAACGCTGGGCGAACTG GTGCGCGACCGGATGGCGGCGTTTCCGGCAAGCGGTGAG ATCAACAGCAAACCTGGCGCACCCCGTTGAGGCGATTAAT CGCGTCGAACAGCATTTTAGCCGCGAGGCGCTGGCGGTG GATCGCACCGATGGCATCAGCATGACCTTTGCCGACTGG CGCTTTAACCTGCGCTCCTCCAACACCGAACC GGTTGGTG CGGTTGAATGTGGAATCGCGCGGTGATGTACCGCTGATG GAAGAAAAGACAAAACCTTATCCTTGAGTTACTGAACAAG TAA
<i>manC</i>	ATGGCGCAGTCGAAACTCTATCCAGTTGTGATGGCAGGT GGCTCCGGTAGCCGCTTATGGCCGCTTTCCCGCGTACTTT ACCCCAAGCAGTTTTTTATGCCTGAAAGGCGATCTCACCA TGCTGCAAACCACCATCTGCCGCCTGAACGGTGTGGAGT GCGAAAGCCCGGTGGTGATTGTGCAATGAGCAGCACCGCT TTATTGTCGCGGAACAGCTGCGTCAACTGAACAAAACCTCA CCAAGAACATTATTCTCGAACC GGCGAGGGCGTAACACTG CACCTGCCATTGCGCTGGCGGCGCTGGCGGCAAAACGTC ATAGCCCGGAGAGCGACCCGTTAATGCTGGTCTTGCGGG CGGATCATGTGATTGCCGATGAAGACGCGTTCCGTGCCG CCGTGCGTAATGCCATGCCGTATGCCAAAGCGGGCAAGC TGGTGACCTTCGGCATTGTGCCGGATCTACCTGAAACCG GTTATGGCTATATTCGTCGCGGTGAAGTGTCGGCGGGTG AGCAGGATACGGTGGCCTTTGAAGTGGCGCAGTTTGTCTG AAAAACCGAATCTGGAAACCGCTCAGGCCTATGTGGCAA GCGGCGAATATTACTGGAACAGCGGTATGTTCTCTGTTCC GCGCCGGACGCTATCTCGAAGAACTGAAAAAATATCGCC CGGATATTCTCGATGCCTGTGAAAAAGCGATGAGCGCCG TCGATCCGGATCTCGATTTTATTCGTGTGGATGAAGAAG CGTTTCTCGCCTGCCCCGGAAGAGTCGGTGGATTACGCGG TCATGGAACGTACGGCAGATGCCGTTGTGGTGCCGATGG ATGCGGGCTGGAGTGATGTCGGTTCTTGGTCTTCATTATG GGAGATCAGCGCCACACCGCCGAGGGCAACGTTTGCCA CGGCGATGTGATTAATCACAAAACCTGAAAACAGCTATGT GTACGCCGAATCTGGCCTGGTCACCACCGTCGGGGTGAA AGATTTGGTGGTAGTGCAGACCAAAGATGCAGTGCTGAT TGCCGACCGTAACGCGGTGCAGGATGTGAAAAAAGTGG TCGAGCAGATCAAAGCCGATGGTCGCCATGAGCATCGGG TACATCGCGAAGTGTATCGTCCGTGGGGCAAATATGACT CTATCGACGCGGGCGACCGCTACCAGGTGAAACGCATCA CCGTGAAACCGGGCGAGGGCTTGTCCGTACAGATGCACC ATCACCGCGCGGAACACTGGGTAGTGGTCGCGGGAACG GCAAAAGTCACTATTGACGGTGATATCAAACCTGCTTGGT GAAAACGAGTCCATTTATATTCCGCTGGGGGCGACGCAC TGCCTGGAAAACCCGGGGAAAATTCCGCTCGATTTAATT GAAGTGCGGTCCGGCTCTTATCTCGAAGAGGATGATGTG GTGCGCTTCGCGGATCGCTACGGACGGGTGTAA

<i>fucT</i>	ATGGCTTTTAAGGTGGTGCAAATTTGCGGAGGGCTTGGG AATCAAATGTTTCAATACGCTTTCGCTAAAAGTTTGCAA AAACACTCTAATACGCCTGTGCTGTTAGATATCACTTCTT TTGATTGGAGCGATAGGAAAATGCAATTAGAACTTTTCC CTATTGATTTGCCCTATGCGAGCGCGAAAGAAATCGCTA TAGCTAAAATGCAACACCTCCCCAAGCTAGTAAGAGACG CGCTCAAATGCATGGGATTTGATAGGGTGAGTCAAGAAA TCGTTTTTTGAATACGAGCCTAAATTGCTAAAGCCAAGCC GCTTGACTTATTTTTTTTGGCTATTTCCAAGATCCACGATA CTTTGATGCTATATCCCCTTTAATCAAGCAAACCTTCACT CTACCACCACCACCAGAAAATAATAAGAATAATAATAA AAAAGAGGAAGAATATCAGTGCAAGCTTCTTTGATTTT AGCCGCTAAAAACAGCGTGTTTGTGCATATAAGAAGAGG GGATTATGTGGGGATTGGCTGTCAGCTTGGTATTGACTA TCAAAAAAAGGCGCTTGAGTATATGGCAAAGCGCGTGCC AAACATGGAGCTTTTTTGTGTTTTGCGAAGACTTAGAATTC ACGCAAAATCTTGATCTTGGCTACCCTTTTATGGACATGA CCACTAGGGATAAAGAAGAAGAGGCGTATTGGGACATG CTGCTCATGCAATCTTGTGTCAGCATGGCATTATCGCTAATA GCACTTATAGCTGGTGGGCGGCCTATTTGATAGAAAATC CAGAAAAAATCATTATTGGCCCCAAACACTGGCTTTTTG GGCATGAGAATATCCTTTGTAAGGAGTGGGTGAAAATAG AATCCCATTTTGAGGTAAAATCCCAAAGTATAACGCTT AA
<i>gmd</i>	ATGTCAAAGTCGCTCTCATCACCGGTGTAACCGGACAA GACGGTTCTTACCTGGCAGAGTTTCTGCTGGAAAAAGGT TACGAGGTGCATGGTATTAAGCGTCGTGCATCGTCATTC AACACCGAGCGCGTGGATCACATTTATCAGGATCCGCAC ACCTGCAACCCGAAATTCCATCTGCATTATGGCGACCTG AGTGATACCTCCAACCTGACACGCATTTTGCGTGAAGTG CAGCCGGATGAAGTGTATAACCTGGGCGCAATGAGCCAC GTTGCGGTCTCTTTTGAAGTCACCGGAATATACCGCAGAC GTTGATGCGATGGGTACGCTGCGCCTGCTCGAGGCGATC CGCTTCCTCGGTCTGGAAAAGAAAACCCGTTTTTATCAG GCTTCCACCTCTGAAGTGTACGGTCTGGTGCAGGAAATT CCGCAGAAAGAAACTACGCCGTTCTACCCGCGATCTCCG TATGCGGTGCGCAAACCTGTACGCCTACTGGATCACCGTT AACTACCGCGAATCCTACGGCATGTACGCCTGTAACGGT ATTCTCTTCAACCATGAATCCCCGCGCCGCGGTGAAACC TTCGTTACCCGCAAAATCACCCGCGCAATCGCCAATATC GCCCAGGGGCTGGAGTCGTGCCTGTACCTCGGCAATATG GATTCCCTGCGTGACTGGGGCCATGCCAAAGACTACGTA AAAATGCAGTGGATGATGCTGCAACAGGAACAGCCGGA AGATTCGTTATTGCTACCGGCGTTTCACTCCGTACGT CAGTTCGTGGAAATGGCGGCAGCACAGTTGGGCATCAAA CTGCGCTTTGAAGGCACGGGTGTTGAAGAGAAGGGCATT GTGGTTTCCGTCACCGGGCATGACGCGCCGGGCGTTAAA CCGGGTGATGTGATTATCGCCGTTGACCCGCGTTACTTCC GTCCGGCAGAAAGTTGAAACGCTGCTCGGCGACCCGACCA AAGCGCACGAAAAACTGGGCTGGAAACCGGAAATCACC

	CTCAGAGAGATGGTGTCTGAAATGGTGGCTAATGACCTC GAAGCGGCGAAAAAACACTCTCTGCTGAAATCTCACGGC TACGACGTGGCGATCGCGCTGGAGTCATAA
<i>wcaG</i>	ATGAGTAAACAACGAGTTTTTATTGCTGGTCATCGCGGG ATGGTCGGTTCTGCCATCAGGCGGCAGCTCGAACAGCGC GGTGATGTGGAACCTGGTATTACGCACCCGCGACGAGCTG AACCTGTTGGACAGCCGCGCGGTGCATGATTTCTTTGCC AGCGAACGCATTGACCAGGTCTATCTGGCGGGCGGCGAAA GTGGGCGGCATTGTTGCTAACAACACCTATCCGGCGGAT TTCATCTACCAGAACATGATGATTGAGAGCAACATCATT CACGCCGCGCATCAGAACGACGTGAACAAACTGCTGTTT CTCGGATCGTCCTGTATCTACCCGAAACTGGCAAAACAG CCGATGGCAGAAAGCGAGTTGTTGCAGGGGCACGCTGGA GCCGACTAACGAGCCTTATGCTATTGCCAAAATCGCCGG GATCAAACCTGTGCGAATCTTACAATCGCCAGTACGGACG AGATTACCGTTCAGTCATGCCGACCAACCTGTACGGGCC GCACGACAACTTCCACCCGAGTAATTCGCATGTGATCCC AGCATTGCTGCGCCGCTTCCACGAGGGCGACGGCACAGAA TGCACCGGACGTGGTGGTATGGGGCAGCGGTACACCGAT GCGTGAATTCCTGCACGTCGATGATATGGCGGCGGCGAG CATTCATGTCATGGAGCTGGCGCATGAAGTCTGGCTGGA GAACACCCAGCCGATGCTGTGCGCACATTAACGTCGGCAC GGGCGTTGACTGCACCATCCGTGAACTGGCGCAAACCAT CGCCAAAGTGGTGGGTACAAAAGGTCGGGTGGTTTTGA TGCCAGCAAACCGGATGGTACGCCGCGCAAACCTGCTGGA TGTGACGCGCCTGCATCAGCTTGGCTGGTATCACGAAAT CTCACTGGAAGCGGGGCTTGCCAGCACTTACCAGTGGTT CCTTGAGAATCAAGACCGCTTTCGGGGGTAA
<i>WJL_0209</i>	ATGGATGCAGAGACAAAGCAGAGTAAAGGTCGTAAAAT AACATCGCGACTTGCTTATTCGTTTGGGGCGTTTGGGCAC GATGCCTTCTATGCCACTTTATCAACTTATTTTATTATGTT TGTGACCTCCCACTTGTTTCGATAAGAGTAGTGGGGCTCA AGGAACACAAATGATTGCTTGGATCACCATGATTATTGC AGGTTTGCGGTTCGTGGAAGTAGCAATTGATCCTTTGATC GGGAATGCCATTGATAATACTAATTCTCGGTGGGGCCAC TTTAAACCATGGATTGTCATTGGTGGGATTATAGGTTCCG TCGTTTTAGCACTTTTATTCACGAGTTTAGGCGGATTAAA TACCTCAAACCCCACTTCTATATTTAATTATTTTGCATT TTATACATTACAATGGATATTTTCTATTTCGTTTAAAGACG TTGGTTTTTGGTCAATGATTCCAGCAATCTCATTGACTC AGCAGAACGTGAAAAGACGGCGACATACGCCCGGGTTG GCTCTAACATTGGTGCCAACATCGTTGGGGTTATCGTTAT GCCAATCGTCTTAATGTTTTCTGTAAAGTCTAACAGTGGT CAAGGTGACAACCGTGGTTGGATGGCATTGTTGGGATCATT ATTGCCGCAATCGCTTTGATTTCTGCCTTAGTTGTTGCAC TTGGAACCAAAGAAAATGATTCTGAATTGCGTAAGAACA CTGAAAAGACTAGCTTCAAGGAAGTCTTCAAAGTGTTAG CCCGTAACGATCAATTGATGTGGTTATCATTGATGTATG GGATCTACACGGCAGGGATTGCCATTACGAACTCACTAG AACTTTACTACTTCACCTATATTTTGGGTGACGCCTCGAA

	GTTTACACTATTAGGTTCACTAAATGCGATTATTGGGATT TTTGCCGTATTGGCCTTCCCACCATTAGCAAAGAAGTTTA GTCGTCGTAAAGTGTTCTTCTTAGCAGTTGTTATTATGAT GATTTCACTGATCATGTTTGCCTTTGCTGGTCAGTCATTA CCAATGATCTTAACAGCGGCCGTATTATTCTACATTCCAC AACCATTAATTTTCTTAGTTGTTTTGATGGTTTTGAGTGA TTCTGTTGAATATGGGCAGTTGAAGTTTGGTCATCGTGAC GAATCTTTAACCTTATCCGTTTCGGCCTTTACTTGATAAAT TAGGCGGTGCGATTCTAACGGTGTCGTTGGTTTAACTGC TGTTTGGGCTGGGATGACAGCTGGTGCCACTGCTGGTGA TGTGACTAGCCATGGCGCTGCCATCTTCAAGTTAATGAT GTTCGGTGTACCTGCTGCTATGATTTTGATTGGGACATTC ATCTTCTTCAAGAAGGTAAAGTTGGATGAAAAGATGCAT GCACAAATCGTTGATGAACTTGAAAAACATGGTCAACT CACTTGGAACCGATGAACTCCTGCTGAAGTTGAAGCA GAAGCCGATGCCAAGACGACGGTTTATCAAGATCCTGTT GCCGGGGAAGTATCAGTTTGAAAGATGTTGCTGACGAA TCCTTCGCAAACGGTAGTATGGGTAAAGTTTCGCCATT AAACCAACGGATGGTCGCGTTGTCGCACCATTTGATGGT GAAGTTGTTGCAACCTTCCCGACTCGCCATGCAATCGGA TTACGTTCTGATACCGGTATTTTAACTAATTCATATTG GGATTGGTACCGTTAAGATGCGTGGGACTGGATTGTTC AGTACGTTGAGAAGGGCGACCATGTCACACAAGGGCAA GAATTAATTGAATTCTGGCAACCAGCAATTGAAAAGGCT GGCTTGGATGATACCGTTATGGTCGTTATTACAACTAT AAAGATATGCCCGAATTTACGTATCTTAAAGATTCAGGA ACCACCGTACAAGATGATGAAGATGTCTTACAATTATCG AATGTCGGCTTACCGGATCAAGCATAG
P11 promoter	AGCGCTATAGTTGTTGACAGAATGGACATACTATGATATATT
Ptuf33 promoter	GGTACCAATTCAGCAATTTAGCCTTGATTTACAATCATA AGCGTGTGTAATAAGAATTTACTAACAAAAATTCAATTT TTTGAATAATATCTGTTTACAAATCAGATTAGGCTATATA TAATATTTAAGGATTCTCAGTGATGGGTGCGCGATTGG CCTTTTCACTAGGATGTAGTATAAT
Ptuf33 RBS	ACTAACTAAAGAATTGTTGAGACCATTTTGGCCTCGACG TTATTCTTGCGAAAATCTGTACAAGGAGGTTTTACAT
rrnB T1 terminator	GGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGG GCCTTTCGTTTTATCTGTTGTTTGTCTGGTGAACGCTCTCCT GAGTAGGACAAATCCGCCGCCCTAGA
RiboJ (with its own spacer)	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGT GAGGACGAAACAGCCTCTACAAATAATTTTGTTTAATTA ACTTTA
spacer sequence in the operon	AAATTTAC
P11 RBS	AAGGAGG
<i>alr</i> -gRNA1	GCCCGAAGAATTGGCAACGGCGG
<i>alr</i> -gRNA2	ACAGGATGAATGGATTGGCACGG

<i>argH</i> - gRNA1	TGGATGGCTTGAAAAACAGCGG
<i>argH</i> - gRNA2	TGAACCAGCAATATCTTCTGCGG
<i>upp</i> - gRNA1	ATCATTCGGGAAAAGAACTGTGG
<i>upp</i> - gRNA2	CATGATGGCTGAACCACCAGTGG



APPENDIX B

LIST OF PRIMERS

Table B. 1: Primer sequences used for cloning of constructs

Primer Name	Sequence (5'-3')
prLABP1	GGGAAACGCCTGGTATCTTT
prLABP2	CTTACTATGTTGGCACTGATGAG
prLABP3	AGAGTTTGATCCTGGCTCAG
prLABP4	CTACGGCTACCTTGTTACGA
prHMO5	TCTAGAATATATCATAGTATGTCCATTCTG
prHMO6	GGGCCCTAGTTTAGAAAAAG
prHMO7	CGGATCACATCTTTTTCTAAACTAGGGCCCCCTCAAAAA AAGCACCGAC
prHMO8	TGGACATACTATGATATATTCTAGAGCCCGAAGAATTG GCAACGGGTTTTAGAGCTAGAAATAGCAAG
prHMO9	TGGACATACTATGATATATTCTAGATGGATGGCTTGAA AAACAGGTTTTAGAGCTAGAAATAGCAAG
prHMO10	TGGACATACTATGATATATTCTAGATGAACCAGCAATA TCTTCTGGTTTTAGAGCTAGAAATAGCAAG
prHMO11	TGGACATACTATGATATATTCTAGAATCATTCGGGAAA AGAACTGGTTTTAGAGCTAGAAATAGCAAG

prHMO12	TGGACATACTATGATATATTCTAGACATGATGGCTGAA CCACCAGGTTTTAGAGCTAGAAATAGCAAG
prHMO13	TTGGAACGTTTTAACAATAATTCATGCTG
prHMO14	AGTCGGCGCAAACCTATCGTCTGATAAAACCACAAATT GCCTCTTTCCAATATAAC
prHMO15	GTTTTATCAGACGATAGGTTTGCGCCGAC
prHMO16	AGTACTGGACGCATGCCTC
prHMO17	TCATCAAGTTGGATCAGATTGCTG
prHMO18	CAATGGTCAAATTGATAAGCATTAAATAGGCGTGTGCCT TATCAG
prHMO19	TTAATGCTTATCAATTTGACC
prHMO20	AAGACGTAACCGAGTA
prHMO21	ACAGCCTGAGTTTAAGGTTTATG
prHMO22	CCTGGCGGTGCTACCTTTTGTC AATGCAACGTTGCTAC GCACACTCCTTAG
prHMO23	GTTGCATTGACAAAAGGTAGCAC
prHMO24	GCCGCTCAAATGGCTGATG
prHMO25	AATGGCGGGCCCAGTACTGGACGCATGCCTC
prHMO26	GATTTTTACGCATTTTGCAACGGCTGACCAG
prHMO27	GTCCCACAATAGTCAGCAATATCTTGCAAC
prHMO28	ATACTTCAGTCACCTCCTAGCTGAACATCATTGTCATT CATATTTTTCATTATATTTGGC
prHMO29	CAGCTAGGAGGTGACTGAAGTATATTTTAG

prHMO30	GAATGGACATACTATGATATATTTCTAGAAGCTGTCAC CGGATGTG
prHMO31	GTTTTTGCATGTAAATTCCTCCTTCTTAAAGTTAATTA AACAAAATTATTTGTAGAGGC
prHMO32	CCGGAAAGCACATCCGGTGACAGCTTTATTACAGCTTG TTGTAAACACG
prHMO33	GAAGGAGGAAATTTACATGCAAAAACTCATTAACTCA GTG
prHMO34	AGCTGTCACCGGATGTG
prHMO35	CTTAAAGTTAATTAAACAAAATTATTTGTAGAGGC
prHMO36	TTTGTTTAATTAACTTTAAGAAGGAGGAAATTTACATG CTAACTTGCTTTAAAGCTTATG
prHMO37	GGAAAGCACATCCGGTGACAGCTTTATTACTTGTTTCAG TAACTCAAGGATAAGTTTTG
prHMO38	GAGCCTTTCGTTTTATTTGATGCCACGCGTTTATTACAC CCGTCCGTAGC
prHMO39	AAATAATTTTGTTTAATTAACTTTAAGAAGGAGGAAAT TTACATGGCGCAGTCGAAACTC
prHMO40	GTAAATTTCTCCTTCTTAAAGTTAATTAAACAAAATT ATTTGTAGAGGC
prHMO41	ACGCGTGGCATCAAATAAAACGAAAG
prHMO42	AATTATTAGGGCCCTCTAGGGCGGCGGATTG
prHMO43	GATTTACAATCATAAGCGTGTGTAATAAGAATTTACTA ACAAAATTCAATTTTTTGAAT

prHMO44	ATTCAAAAAATTGAATTTTTGTAGTAAATTCTTATTAC ACACGCTTATGATTGTAAATC
prHMO54	CTTGCGAAAATCTGTACAAGGAGGTTTACATATGGTT GTAATTGGGGAG
prHMO55	CAGCCTTGGGCCCTTATTAATCTATATAAACTCTCGGC AC



APPENDIX C

PLASMID MAPS OF CONSTRUCTS

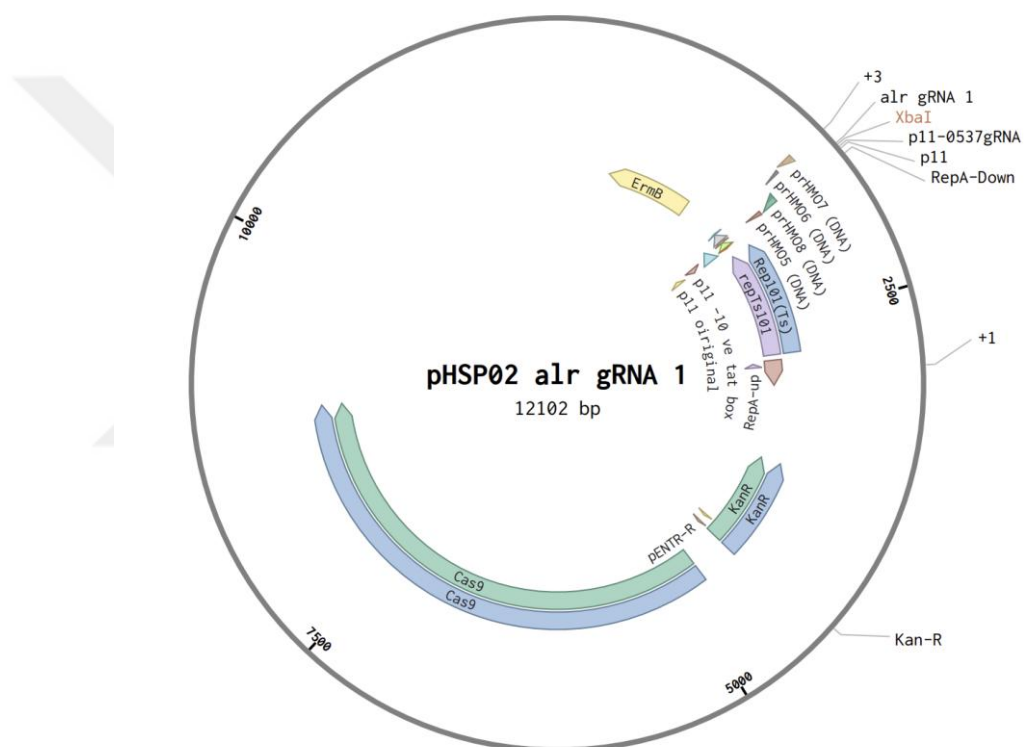


Figure C. 1: The schematic illustration of pHSP02-*alr*-gRNA1 plasmid (without the homology arms).

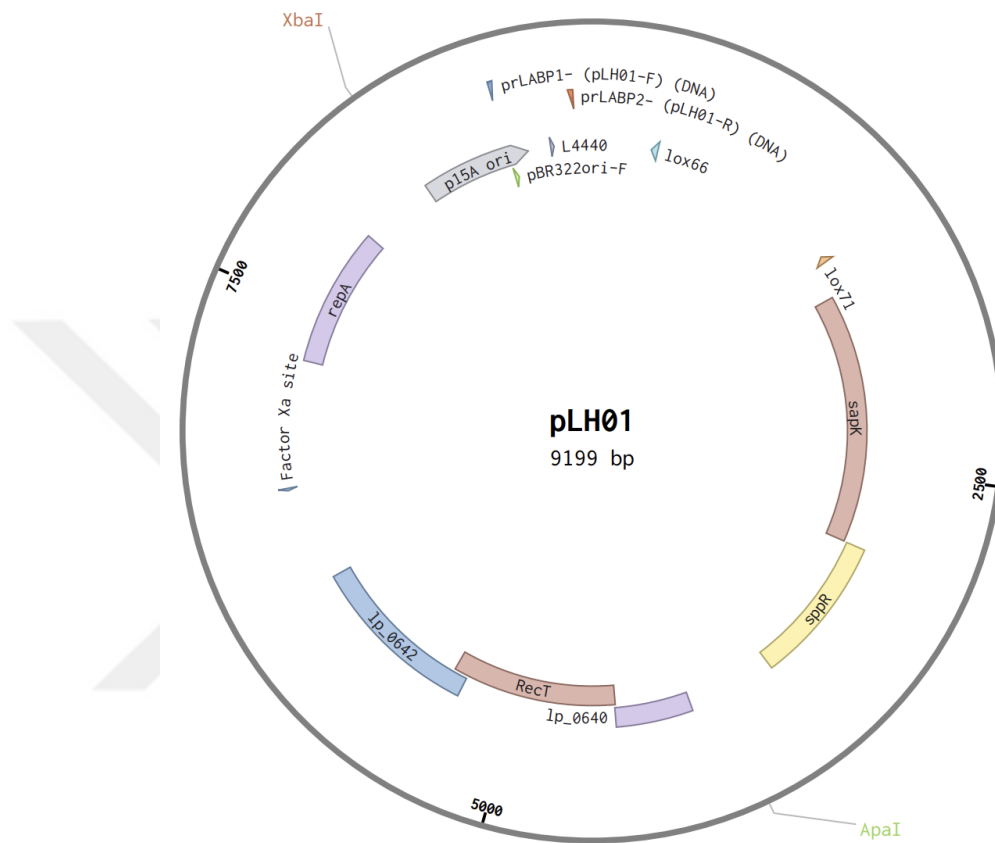


Figure C. 3: The schematic illustration of pLH01 plasmid (Addgene, Plasmid #117261).

manA-manB-manC in pHSP02 (under p11) (12308 bp)

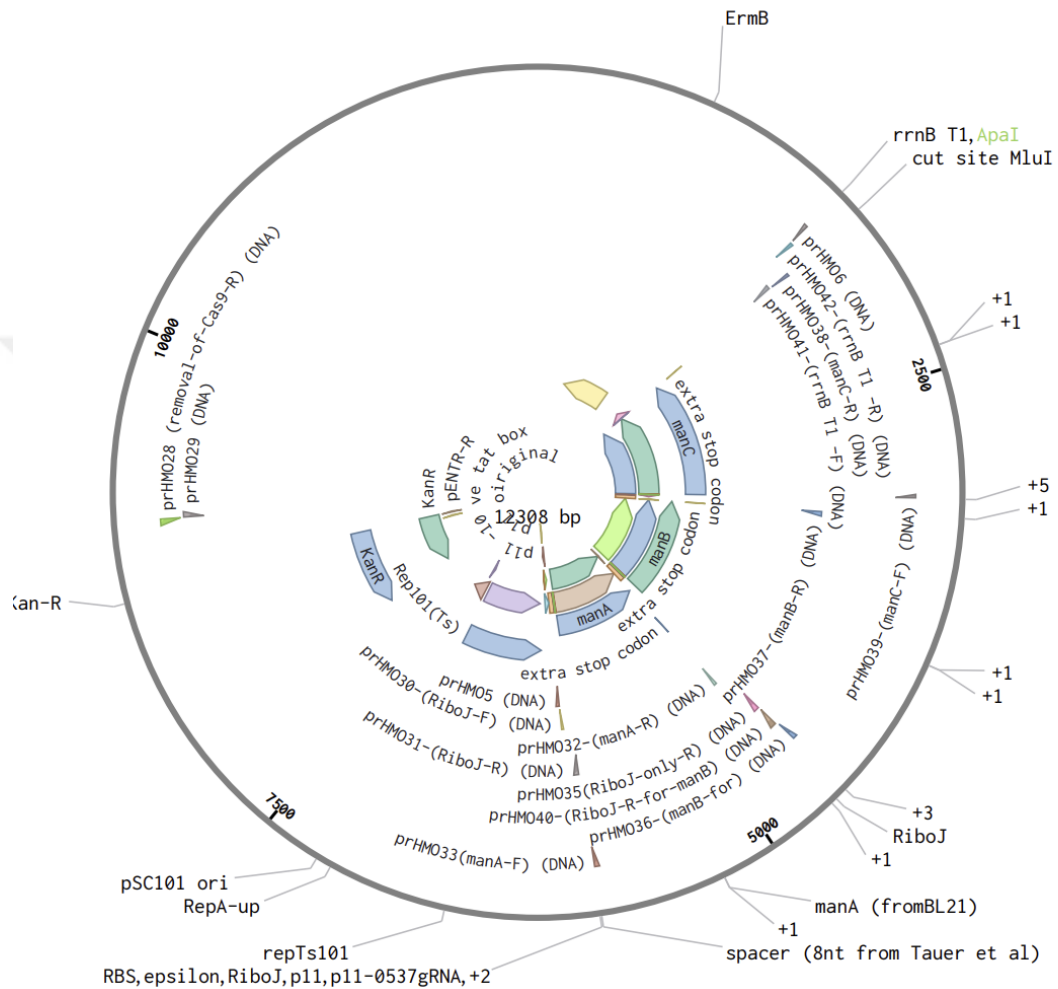


Figure C. 4: The schematic illustration of *manA-manB-manC* expression cassette plasmid in Cas9-free pHSP02 backbone

APPENDIX D

SEQUENCING RESULTS

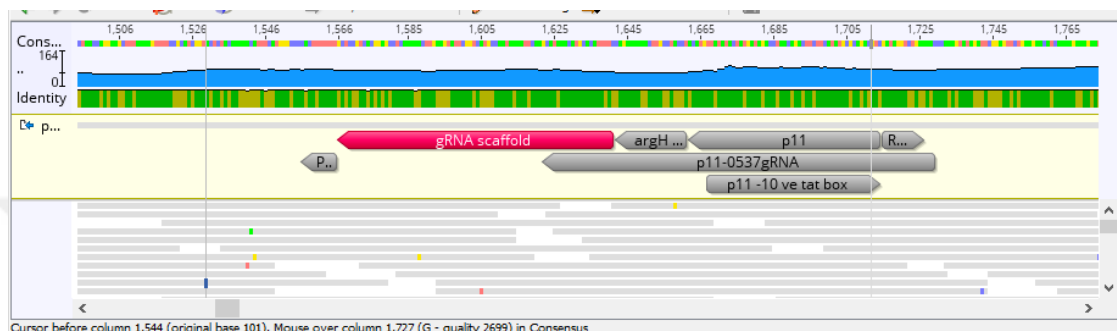


Figure D. 1: Next Generation Sequencing alignment result of *argH*-gRNA1 in pHSP02 backbone. Alignment was performed in Geneious application.

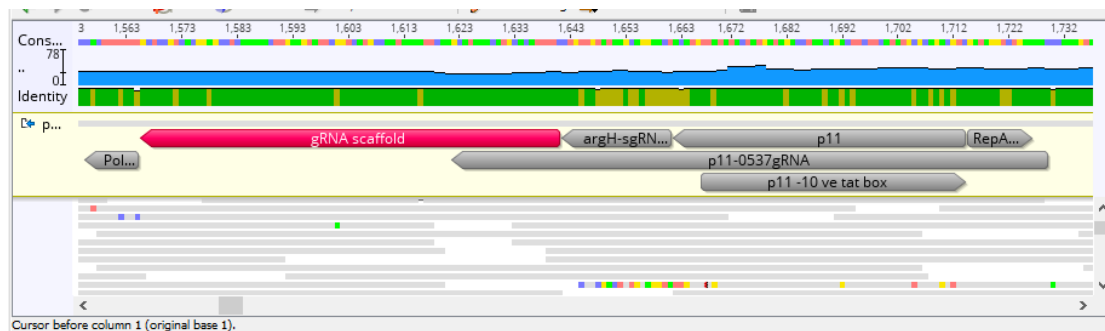


Figure D. 2: Next Generation Sequencing alignment result of *argH*-gRNA2 in pHSP02 backbone. Alignment was performed in Geneious application.

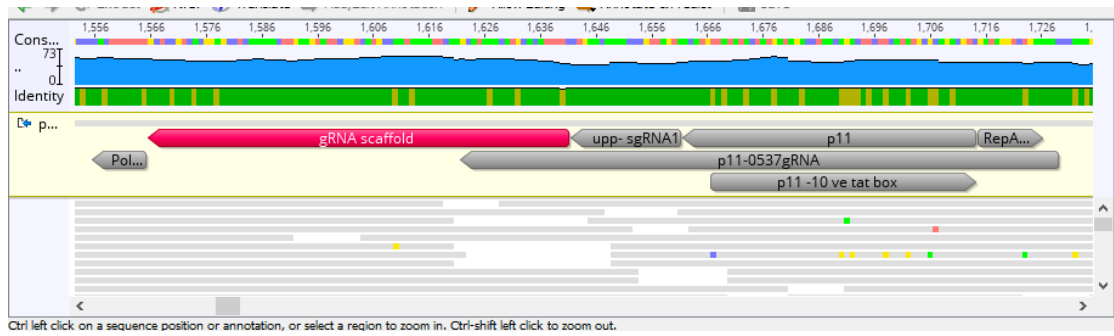


Figure D. 3: Next Generation Sequencing alignment result of *upp*-gRNA1 in pHSP02 backbone. Alignment was performed in Geneious application.

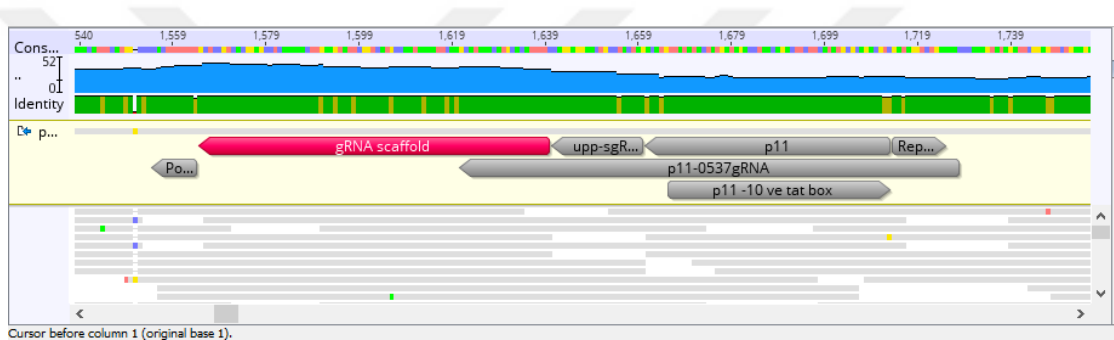


Figure D. 4: Next Generation Sequencing alignment result of *upp*-gRNA2 in pHSP02 backbone. Alignment was performed in Geneious application.

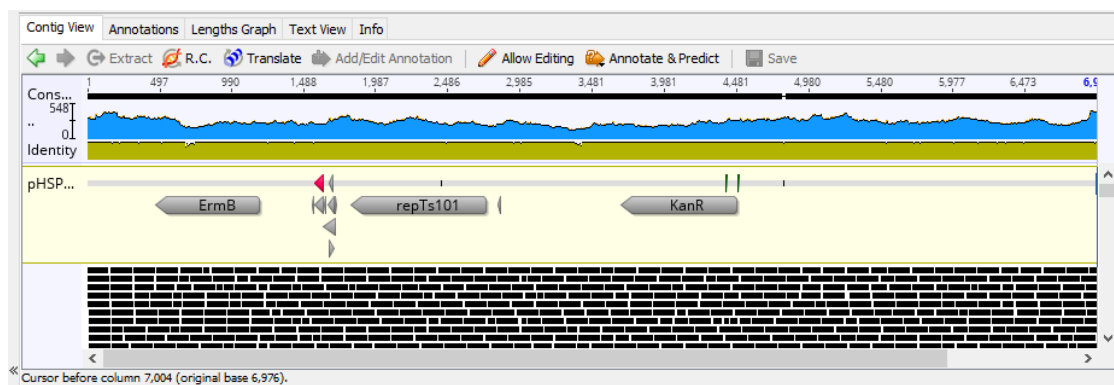


Figure D. 5: Next Generation Sequencing alignment result of Cas9-removed pHSP02 backbone for expression cassette clonings. Alignment was performed in Geneious application and black boxes represent high coverage.

APPENDIX E

ADDITIONAL REACTION RECIPES

Table E. 1 Lysogeny Broth (LB) Growth Medium and LB Agar Recipe

Component	Amount
Yeast Extract	5 g
Sodium Chloride (NaCl)	10 g
Tryptone	10 g
Agar (for LB agar)	15 g
Water	1000 ml

Table E. 2 PCR Reaction Mix Recipe with Q5

Component	Amount (for 25 μ l)
5X Q5 Reaction Buffer	5 μ l
10 mM dNTP	0.5 μ l
10 μ M Forward Primer	1.25 μ l
10 μ M Reverse Primer	1.25 μ l
Template DNA	0.5 ng – 0.5 μ g
Q5 DNA Polymerase	0.5 μ l
Nuclease Free Water	Up to 25 μ l

Table E. 3 Digestion Reaction

Component	Amount (for 50 μ l)
10x Cut Smart Buffer	5 μ l
DNA	2.5 μ g
Restriction Enzyme 1	1 μ l
Restriction Enzyme 2*	1 μ l*
Nuclease Free Water	Up to 50 μ l

*for double digestion reactions

Table E. 4 Gibson Assembly Mixture (1.33X)

Component	Amount
Taq Ligase (40 U/μl)	50 μl
5X Isothermal Buffer	100 μl
T5 Exonuclease (1 U/μl)	2 μl
Phusion Polymerase (2 U/μl)	6.25 μl
Nuclease Free Water	216.75 μl

Table E. 5 TSS Buffer Recipe

Component	Amount
PEG 8000	5 g
1M MgCl ₂	1.5 ml
2.5 ml DMSO	2.5 ml
LB Medium	Up to 50 ml

Table E. 6 PCR Reaction Conditions of Q5 DNA Polymerase

Step	Temperature	Duration
Initial Denaturation	98 °C	30 seconds
Denaturation	98 °C	5 - 10 seconds
Annealing	50 - 72 °C	10 - 30 seconds
Extension	72 °C	30 seconds / kb
Final Extension	72 °C	2 minutes
Hold	4 °C	∞

Table E. 7 T4 Ligation Reaction

Component	Amount
10x T4 DNA Ligase Buffer	2 μl
Vector DNA	50 ng
Insert DNA	Equal molar to vector DNA (1:1)
T4 DNA Ligase	1 μl
Nuclease Free Water	Up to 20 μl

Table E. 8 Suc-Gly Solution Recipe

Component	Amount	Note
50% (v/v) Glycerol (sterile)	20 mL	Pre-sterilized stock solution
Sucrose	34.23 g	MW = 342.3 g/mol
Distilled water (dH ₂ O)	60 mL	
Final volume	100 mL	Bring to final volume with dH ₂ O
Sterilization	Filter sterilize	Use 0.22 µm filter

Table E. 9 MRS Recovery Medium Composition

Component	Final Concentration
MRS Broth	As per manufacturer
Magnesium chloride (MgCl ₂)	80 mM

Table E. 10 Colony PCR Reaction Mixture (Total Volume: 20 µL)

Component	Volume (µL)	Final Concentration / Notes
10X Reaction Buffer	2.0	1X
dNTP Mix (10 mM each)	0.4	200 µM each
Forward Primer (10 µM)	1.0	0.5 µM
Reverse Primer (10 µM)	1.0	0.5 µM
DNA Template	~1.0	~10-100 ng (boiled colony lysate or plasmid)
Pfu DNA Polymerase (2.5 U/µL)	0.5	~1.25 U per reaction
Nuclease-Free Water	to 20.0	-

Table E. 11 Colony PCR Thermal Cycling Conditions

Step	Temperature (°C)	Duration	Notes
Initial Denaturation	95	2 minutes	-
Denaturation	95	30 seconds	Per cycle
Annealing	X (T _m -dependent)	30 seconds	Adjust based on primer T _m
Extension	72	2 minutes per kb	Adjust according to product length
Final Extension	72	≥5 minutes	Ensures complete product extension
Hold	4	∞	Until sample retrieval

Table E. 12 DNA Oligo Hybridization Protocol

Step	Component / Setting	Details
Reaction Mixture Preparation	Nuclease-Free Water	45.5 µL
	NaCl	2.5 µL (1 M stock)
	Forward oligo (5')	1.0 µL (100 µM)
	Reverse oligo (3')	1.0 µL (100 µM)
	Total Volume	50.0 µL
Thermal Cycling Program	Initial Denaturation	95 °C for 5 minutes
	Denaturation	95 °C for 1 minute
	Gradual Annealing (Ramp Down)	Decrease 1 °C per cycle for 72 cycles
	Final Hold	10 °C indefinitely