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Development of CRISPR/Cas9-based Plasmid System for Targeted Gene Knockout to Enhance L-tryptophan Production in *Lactobacillus* Bacteria

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Mokslinio darbo projektas rengtas Biochemijos instituto Biologinių modelių skyriuje

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Abbreviations

ackA - acetate kinase

CNS - central nervous system

EPS - exopolysaccharides

GABA - gamma-aminobutyric acid

GI - gastrointestinal

HDR - homology-directed repair

HPLC - high-performance liquid chromatography

IBS - irritable bowel syndrome

IBD - inflammatory bowel disease

LAB - lactic acid bacteria

SCFAs - short-chain fatty acids

Trp - tryptophan

ZFNs - Zinc-finger nucleases

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INTRODUCTION

The possible therapeutic effects of probiotics on mental health and their capacity to produce components that are crucial for the gut-brain axis functionality have gained recognition over the years (Abouelela and Helmy, 2024). As described by health organizations, probiotics are termed to be “live microorganisms that, when given in sufficient quantities, have been shown to be beneficial” for gut health and general well-being of the host (WHO, 2001). Specifically *Lactobacillus* species are noted among them due to their strong capacity to alter the microbiome, improve metabolic processes, and strengthen immunological responses. Numerous fermented foods and nutritional supplements include these bacteria, which have potential uses in both preventative and therapeutic medicine (Ouwehand *et al.*, 2002).

The beneficial properties of probiotics stem not only from their ability to balance the gut microbiota by producing bioactive compounds but also by supplementing the lost essential microbes in the gut. Bioactive compounds include short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which play crucial roles in maintaining gut health by serving as energy sources for colonic epithelial cells, reducing inflammation, and strengthening the intestinal barrier (Canani *et al*., 2011). Probiotics also synthesize bacteriocins that inhibit the growth of pathogenic bacteria, thereby contributing to microbiome stability (Cotter *et al*., 2013). Furthermore, certain strains of probiotics produce exopolysaccharides (EPS), which have been shown to enhance immune modulation and promote gut healing (Patel & Prajapati, 2013). Other key bioactive metabolites include vitamins (e.g., folate and vitamin K), neurotransmitter precursors such as L-tryptophan, and anti-inflammatory cytokines, all of which contribute to systemic health benefits (Hill *et al*., 2014). Studies have shown that probiotics can also mitigate symptoms of irritable bowel syndrome, alleviate diarrhea, and enhance nutrient absorption by maintaining intestinal barrier integrity (Vighi *et al*., 2008). Moreover, the gut-brain axis—a delicate bidirectional communication system between the gut and the central nervous system—highlights probiotics' potential in mental health. Specific strains of probiotics, such as *Lactobacillus plantarum* and *Lacticaseibacillus paracasei*, have been associated with reducing symptoms of depression and anxiety through the modulation of serotonin pathways (Cryan *et al*., 2019).

These findings draw attention to the growing importance of probiotics in addressing complex health conditions.

In recent years, there has been a surge of interest in enhancing the natural properties of probiotics through genetic engineering (Ma *et al*., 2022). While traditional probiotic strains are limited by their innate metabolic capabilities, modern molecular tools such as CRISPR/Cas9 allow for the precise modification of bacterial genomes, expanding new approaches for therapeutic applications (Huang *et al*., 2019). For instance, engineered *Lactobacillus* strains have demonstrated increased production of neurotransmitter precursors such as L-tryptophan, which is a critical substrate for serotonin synthesis (Zhou *et al*., 2020). Enhanced production of L-tryptophan could be particularly beneficial in addressing eating disorders, gut dysbiosis, and depression—conditions often linked to imbalances in gut microbiota and neurotransmitter availability (Kumar *et al*., 2023).

Gut health plays a pivotal role in tackling eating disorders such as anorexia and bulimia, which are often accompanied by gastrointestinal distress and inflammation (Monteleone *et al*., 2021). Probiotics engineered to produce anti-inflammatory compounds or essential nutrients could support recovery by restoring gut homeostasis and reducing systemic inflammation. Similarly, depression, a leading cause of mental disability worldwide, has been increasingly linked to gut microbiota composition (Irum *et al*., 2023). By modulating serotonin levels via enhanced L-tryptophan biosynthesis, engineered probiotics hold the potential to serve as adjunctive treatments in mental health care (Carabotti *et al*., 2015). Furthermore, in cases of irritable bowel syndrome or chronic gut disorders, genetically modified strains could be tailored to produce specific metabolites that alleviate symptoms and promote gut healing (Jandhyala *et al*., 2015).

Despite these promising applications, several challenges remain in the development and deployment of engineered probiotics. These include ensuring the stability of genetic modifications, maintaining safety for human consumption, and optimizing delivery methods to preserve bacterial viability during administration (O'Toole *et al*., 2017). Nevertheless, advancements in genetic engineering tools, such as CRISPR/Cas systems, offer precise and efficient methods to overcome these barriers, paving the way for next-generation probiotics with enhanced therapeutic properties (Al-Fakhrany *et al*., 2024).

This research focuses on engineering a CRISPR/Cas9-based plasmid system designed to enhance the production of L-tryptophan in *Lactobacillus plantarum* and *Lacticaseibacillus paracasei*. By integrating genetic engineering for probiotic function enhancement, the study

aims to contribute to the development of targeted microbial therapeutics, addressing not only gut health but also broader systemic conditions such as mental health disorders.

The aim of the research:

This research aimed to investigate L-tryptophan production capabilities of multiple strains of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei,* with the main focus on constructing a CRISPR-Cas9-based plasmid system that would enhance L-tryptophan production in the highest performing strain.

The objectives of the research:

- Identify and characterize the genetic diversity of isolated bacteria strains;
- Assess the production levels of tryptophan by *L. plantarum* and *L. paracasei* strains;
- Design and assemble an engineering plasmid that would be utilized to increase L-tryptophan production in the highest performing strain;
- Perform transformation of *Lactobacillus* with newly constructed engineering and helper plasmid pairs.

1. LITERATURE REVIEW

1.1. Human gut microbiome

The human gut microbiome is a complex ecosystem and is collectively referred to as the second human genome (Pan *et al*., 2020; Ferranti *et al*., 2014). It is unprecedentedly diverse and complex, with bacteria from the four phyla Bacteroides, Firmicutes, Proteobacteria, and Actinobacteria accounting for more than 98% of the microorganisms (Pan *et al*., 2020; Collins *et al*., 2018). Its components, functions, homeostasis, and interactions with the host can all have a significant impact on human health (Pan et al., 2020).

The gut microbiome begins to develop at birth, influenced by factors such as delivery mode and feeding practices. Infants born vaginally tend to acquire microbiota resembling the maternal vaginal microbiome, dominated by *Lactobacillus* and *Prevotella* species, while those born via cesarean section often harbor microbiota resembling maternal skin, dominated by *Staphylococcus* and *Corynebacterium* species (Dominguez-Bello *et al*., 2010). Similarly, breastfed infants typically exhibit higher levels of *Bifidobacterium*, which thrives on human milk oligosaccharides, compared to formula-fed infants whose microbiota is more diverse but includes potentially pathogenic taxa (Walker *et al*., 2011). Over time, the microbiome diversifies and stabilizes, achieving a more adult-like composition by 3–5 years of age, shaped by environmental exposures, diet, and genetics (Yatsunenko *et al*., 2012).

In an ideal situation, the human host coexists with its diverse gut flora, promoting physiological resilience (Dethlefsen and Relman, 2011). However, the microbiome varies not only across life stages but also among individuals and populations globally. Geographic location and cultural practices significantly influence microbiome composition (Mallott *et al*., 2023). For example, populations with traditional diets rich in fiber, such as rural African communities, have microbiota enriched with fiber-degrading species like *Prevotella*, whereas Westernized populations with diets high in fat and sugar tend to have microbiota dominated by *Bacteroides* (De Filippo *et al*., 2010). These dietary influences contribute to notable differences in microbiome-mediated production of short-chain fatty acids (SCFAs), which play a crucial role in intestinal and systemic health (Canani *et al*., 2011).

Dysbiosis, a persistent imbalance of the gut's microbial community, can be caused by a variety of factors, including medications, infections, ageing, stressful or harmful lifestyle, surgeries, and poor nutrition, leading to a variety of disorders (Gagliardi *et al*., 2018;

Belizário and Faintuch, 2018). Stress, for instance, triggers the release of glucocorticoids and catecholamines, which alter gut motility and mucosal barrier function, allowing pathogenic bacteria to thrive and beneficial microbes to diminish (Moloney *et al*., 2014). Additionally, stress-induced changes in microbial metabolites such as SCFAs and tryptophan-derived compounds can disrupt the gut-brain axis, potentially contributing to mental health conditions like depression and anxiety (Cryan *et al*., 2019). Depression, in particular, has been linked to gut microbiota imbalances. Studies show that individuals with depression often exhibit reduced microbial diversity and altered levels of key bacterial species, such as decreased *Lactobacillus* and *Bifidobacterium* populations, which are associated with SCFA production and anti-inflammatory properties (Jiang *et al*., 2015). Furthermore, dysbiosis in depression has been correlated with increased levels of pro-inflammatory cytokines and altered production of serotonin, a neurotransmitter synthesized partly from the gut-derived precursor L-tryptophan (Foster & Neufeld, 2013). These findings highlight the bidirectional nature of the gut-brain axis and underscore the importance of targeting the microbiome as a potential therapeutic avenue.

Diet is a major modulator of the gut and so has a substantial impact on the functionality of the microbiota (Hansen *et al*., 2018). High-fat, high-sugar diets have been shown to reduce microbial diversity and decrease levels of beneficial microbes such as *Lactobacillus* and *Bifidobacterium*. These dietary patterns also promote the overgrowth of pro-inflammatory species like *Desulfovibrio*, which produces hydrogen sulfide, a compound that impairs epithelial cell integrity and exacerbates inflammation (Murphy *et al*., 2015). Conversely, fiber-rich diets support the growth of fiber-degrading microbes, enhancing the production of SCFAs like butyrate, which strengthens intestinal barrier function and reduces inflammation (Canani *et al*., 2011). As a result, several strategies, including faecal microbiota transplant and probiotic administration, have been tested and demonstrated to be practical and effective in restoring human gut microbiota (Kelly *et al*., 2021). Faecal microbiota transplants (FMT) have proven particularly effective in treating recurrent *Clostridioides difficile* infections, as they introduce a diverse and functional microbiota capable of outcompeting pathogenic strains (van Nood *et al*., 2013). Probiotic supplementation, particularly with strains like *Lactobacillus rhamnosus* and *Bifidobacterium longum*, has shown promise in alleviating depressive symptoms by restoring gut microbial balance and modulating the gut-brain axis (Romijn & Rucklidge, 2015). These findings suggest that microbiome-targeted therapies could serve as innovative treatments for both gut-related and systemic conditions, including mental health disorders.

1.2. Gut-brain axis

The gut-brain axis is referred to as a bidirectional communication pathway between the gastrointestinal (GI) tract and the central nervous system (CNS), involving complex interactions between the gut microbiota, immune system, and neural networks (Carabotti *et al*., 2015). The gut, which accommodates a plethora of microorganisms, collectively known as the gut microbiota, plays a vital role in this axis (Lozupone *et al*., 2012; Carabotti *et al*., 2015). Recent research has demonstrated that the gut microbiota, in addition to producing short-chain fatty acids (SCFAs) and bile acids, also generate neurotransmitters like glutamate, GABA, serotonin, and dopamine through their metabolic activities (Strandwitz *et al*., 2019; Luqman *et al*., 2018). Notably, certain bacteria possess genes encoding specific enzymes capable of catalyzing the conversion of substrates into neurotransmitters or their precursors (Yano *et al*., 2015; Williams *et al*., 2014). Furthermore, bacterial metabolites can function as signaling molecules, stimulating the synthesis and release of neurotransmitters by enteroendocrine cells (Yano *et al*., 2015). Since neurotransmitters are unable to penetrate the blood-brain barrier, their synthesis within the brain relies on local neurotransmitter precursor pools (Chen *et al*., 2021). Many of these precursors are amino acids, including tyrosine and tryptophan, which originate from the diet, enter the bloodstream, traverse the blood-brain barrier, and are subsequently taken up by corresponding neurotransmitter-producing cells (Chen *et al*., 2021; Richard *et al*, 2009).

The gut-brain axis has been implicated in numerous neuropsychiatric and metabolic disorders, including depression, anxiety, irritable bowel syndrome (IBS), and obesity (Foster & Neufeld, 2013). One of the most extensively studied pathways within the gut-brain axis involves serotonin, a neurotransmitter critical for mood regulation, sleep, and cognitive functions. Approximately 90% of serotonin is synthesized in the gut, with gut-resident bacteria influencing its production by metabolizing tryptophan into serotonin precursors (Yano *et al*., 2015). Dysbiosis, characterized by reduced microbial diversity and altered microbial composition, has been linked to disrupted serotonin pathways and heightened vulnerability to mood disorders (Jiang *et al*., 2015). For instance, studies have observed decreased populations of *Lactobacillus* and *Bifidobacterium* in individuals with depression, coupled with increased pro-inflammatory cytokines and oxidative stress markers, which may exacerbate neuroinflammation and serotonin dysfunction (Strandwitz *et al*., 2019). Emerging evidence highlights that chronic stress not only disrupts the gut microbiota but also exacerbates its impact on mental health.

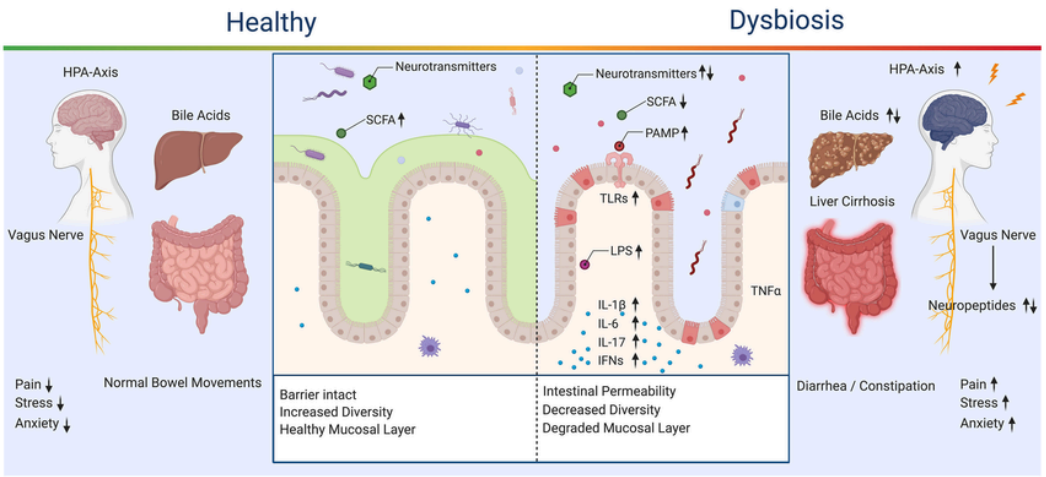

Figure 1.1. Gut-Brain Axis States: Healthy and Dysbiosis (Simpson *et al*, 2021).

Stress-induced alterations in the hypothalamic-pituitary-adrenal (HPA) axis result in the overproduction of cortisol, which can impair gut permeability and foster an inflammatory milieu (Moloney *et al*., 2014). These changes are compounded by a decline in beneficial SCFA-producing bacteria, further weakening the gut barrier and contributing to systemic inflammation (Bander *et al*., 2020). Such processes underline the importance of targeting the gut microbiota for therapeutic interventions in mental health disorders.

The therapeutic potential of modulating the gut-brain axis has gained considerable interest, particularly through the administration of probiotics, prebiotics, and dietary interventions (Fekete *et al*., 2024). Probiotic strains like *Lactobacillus rhamnosus* and *Bifidobacterium longum* have demonstrated the ability to alleviate symptoms of anxiety and depression by modulating the gut microbiota and restoring SCFA levels (Romijn & Rucklidge, 2015). Moreover, interventions targeting the tryptophan-serotonin pathway via engineered probiotics offer a promising avenue for enhancing serotonin bioavailability, potentially mitigating mood disorders (Jenkins *et al*., 2016). For example, the addition of tryptophan-enriched diets or probiotics capable of producing tryptophan precursors may address serotonin deficits more effectively than conventional treatments alone (Foster & Neufeld, 2013). Recent advancements in microbiome-targeted therapies emphasize the interplay between diet, microbial composition, and host neurochemistry. Dietary fibers, for instance, promote the growth of beneficial microbes that produce SCFAs, subsequently enhancing serotonin production through enterochromaffin cell stimulation (Yano *et al*., 2015). Such findings underscore the complex yet modifiable nature of the gut-brain axis and its far-reaching implications for both gut and brain health.

1.3. Probiotics

For the past several decades, there has been a rise in scientific interest in the potential health advantages of probiotics. These supplements are recognized as living nonpathogenic bacteria that, when prescribed in sufficient doses, enhance the host's microbial balance (WHO, 2001; Williams, 2010). Evidence from both animal and human studies has shown that probiotics may have several positive effects, such as increasing the quantity and diversity of the "good" bacteria in the gut (Irwin *et al.*, 2018)(Ferrario *et al.*, 2014), reducing the symptoms of numerous gastrointestinal (GI) disorders (Guarino et al., 2015)(Ford *et al.*, 2014), lowering blood cholesterol levels (Rowland *et al.*, 2018), reducing mycotoxins (Thursby and Juge, 2017), lowering blood pressure (hypertension) (Khalesi *et al.*, 2014), improving blood glucose tolerance (Sun and Buys, 2016)(Nikbakht *et al.*, 2018), enhancing mental health and cognitive function (Foster *et al.*, 2016). In a strain-specific and dose-dependent manner, probiotics can improve the nonspecific cellular immune response, which is characterised by the activation of macrophages, natural killer (NK) cells, antigen-specific cytotoxic T-lymphocytes, and the production of numerous cytokines (Ashraf and Shah, 2014). This is particularly crucial as probiotics may boost immune function by reducing the need for antibiotics during infections, therefore lowering the danger of antibiotic resistance - one of the biggest global risks of the last ten years (Ouwehand *et al.*, 2016).

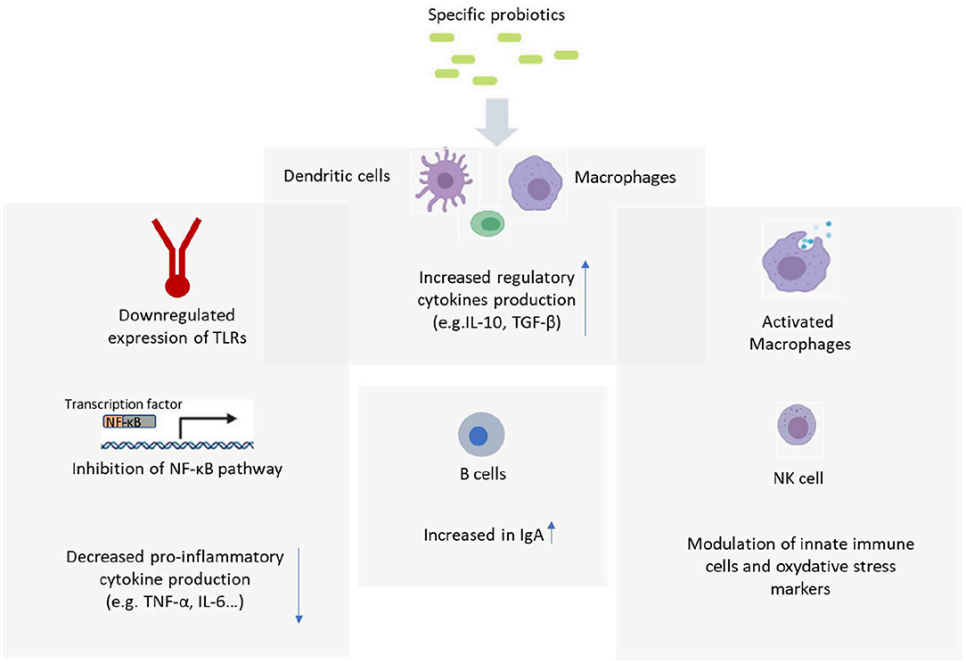

Figure 1.2. Probiotic mechanism of action (Kwok *et al.*, 2022).

Despite the fact that other bacterial species, like *Escherichia coli*, have been identified to possess advantageous traits, lactic acid bacteria (LAB), particularly the *Lactobacillus* species, are currently one of the most utilised probiotics (Williams 2010). Research has shown, that *lactobacilli* have the ability to inhibit the growth of pathogenic bacteria, produce lactic acid enantiomers used for bioplastics as well as 1,3-propanediol, which is a starting ingredient used for biomedicines, cosmetics, adhesives and plastics (Reddy *et al*., 2008). Hence, many of the *Lactobacillus* species have gained recognition as Generally Recognized as Safe (GRAS) by Food and Drug Administration (FDA) and a Qualified Presumption of Safety by the European Food Safety Authority (EFSA) (Sun *et al*., 2015). It is essential that probiotic advantages often apply only to a specific strain, thus even within a single species, a health benefit attributed to one strain may not necessarily apply to another (Williams 2010). In general, *Lactobacilli* are a varied collection of Gram-positive bacteria that inhabit nutrient-rich environments (Sun *et al*., 2015). They are extensively employed in the biotechnology and food preservation sector, and their potential as therapeutics is being investigated (Sun *et al*., 2015).

1.4. Probiotics as therapeutics

Probiotics have been extensively studied for their therapeutic potential in various health conditions, encompassing type 2 diabetes, obesity, irritable bowel syndrome, asthma, cancers, arthritis, and mental health disorders (Azad *et al*., 2018; Chen et al., 2010; Aponte *et al*., 2020). Furthermore, the exploration of the gut microbiome and microbial manipulation using probiotics has expanded to encompass severe and debilitating mental disorders, such as major depressive disorder, bipolar disorder, and schizophrenia (Capuco *et al*., 2020; Nguyen *et al*., 2018). The field of probiotics in mental health has witnessed a remarkable surge in research projects and clinical trials within the past decade (Johnson *et al*., 2020; Aponte *et al*., 2020). Notably, probiotics offer undeniable advantages over conventional psychiatric treatments, making them highly favorable for the management of psychiatric disorders (Johnson *et al*., 2020).

Probiotic supplementation has demonstrated the capacity to replenish and enhance the diminished levels of key neurotransmitters associated with depression, namely tryptophan (5-HT), dopamine (DA), norepinephrine (NE), and gamma-Aminobutyric acid (GABA) (Johnson *et al*., 2020). This specific mechanistic characteristic of probiotics bears

resemblance to the mode of action observed in certain antidepressant medications, exhibiting comparable effectiveness (Wei *et al*., 2018; Liang *et al*., 2015).

Among the most promising probiotic species, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* have gained attention for their broad-spectrum health benefits. These strains exhibit remarkable resilience in the gastrointestinal tract, withstanding acidic and bile conditions, which enables their effective colonization and interaction with the host (Kiousi *et al.*, 2022). *L. plantarum* has been shown to support gut barrier integrity by enhancing the expression of tight junction proteins, thereby reducing intestinal permeability and preventing systemic inflammation (Jeong *et al*., 2019). Similarly, *L. paracasei* has demonstrated immunomodulatory effects, such as reducing pro-inflammatory cytokines and promoting anti-inflammatory markers, which are crucial for maintaining gut homeostasis (Chong *et al*., 2019).

In recent years, *L. plantarum* has gathered considerable attention for its advantageous impact on conditions such as diabetes, obesity, liver dysfunction, and stress (Lee *et al*., 2018; Chong *et al*., 2019; Jeong *et al*., 2019). Emerging research has highlighted the presence of L-tryptophan-encoding genes in strains of *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* (Jeong *et al*., 2021; Kiousi *et al*., 2022). L-tryptophan serves as a precursor for serotonin, a neurotransmitter involved in mood regulation, sleep, and various physiological functions (Jenkins *et al*., 2016). The presence of L-tryptophan-encoding genes in these probiotic strains suggests the potential for these bacteria to enhance serotonin production within the human body (Jenkins *et al*., 2016). Furthermore, supplementation with these strains has been associated with alleviating symptoms of anxiety and depression, likely mediated by their role in regulating tryptophan metabolism and restoring serotonin balance (Wei *et al*., 2018; Kiousi *et al*., 2022).

The tryptophan synthesis pathway, also known as the direct pathway, is utilized by bacteria that possess tryptophan (Trp) encoding genes, such as *B. subtilis*, *L. plantarum*, or *L. paracasei* strains (Jeong *et al*., 2021; Kiousi *et al*., 2022). The operon consists of a cluster of genes transcribed together under the control of a single promoter (Lott, 2020). The pathway begins with the enzyme anthranilate synthase or anthranilate phosphoribosyltransferase (trpE), which converts chorismate, derived from the shikimate pathway, to anthranilate (Parker, 2017).

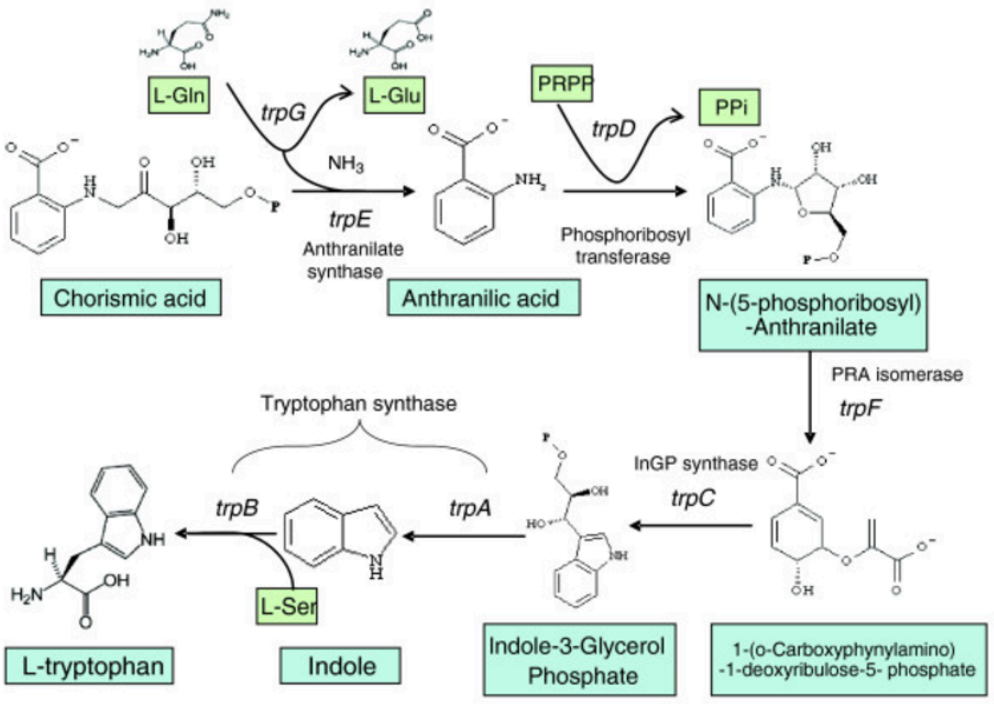

Figure 1.3. The biochemical pathway of tryptophan synthesis in probiotic bacteria (Kagan *et al*., 2008).

Subsequently, the enzyme indole-3-glycerol phosphate synthase (trpC) catalyzes the conversion of anthranilate and phosphoribosyl pyrophosphate (PRPP) into indole-3-glycerol phosphate (IGP) (Kagan *et al.*, 2008). The final step of L-tryptophan synthesis is mediated by the multifunctional enzyme, known as tryptophan synthase (trpA and trpB), which combines IGP and serine to produce L-tryptophan (Kagan *et al*., 2008).

The ability of *L. plantarum* and *L. paracasei* to synthesize L-tryptophan not only highlights their relevance in gut-brain axis modulation but also underscores their potential as therapeutic agents for managing mood disorders and improving intestinal barrier function (Montgomery *et al*., 2022). By enhancing the availability of L-tryptophan, these strains contribute to serotonin production and gut homeostasis, with broader implications for systemic health (Friedman, 2018). In the field of molecular biology, *Lactobacillus* species are undergoing genetic modifications to enhance their beneficial properties further. Advances in molecular tools, such as CRISPR/Cas9 systems, have enabled precise modifications of these strains, allowing researchers to optimize their tryptophan biosynthesis pathways and tailor their applications for targeted therapies (Huang *et al.*, 2019).

1.4. Genetically modified probiotics

Bacterial metabolic engineering has emerged as a powerful tool to enhance the production of crucial compounds, including amino acids like tryptophan. By targeting and knocking out specific pathways that compete with or inhibit the biosynthesis of tryptophan, researchers can redirect metabolic flux toward its production, significantly increasing yield (Li *et al*., 2024). This approach, although extensively explored in model organisms such as *Escherichia coli* (E. coli), demonstrates immense potential for application in other bacterial species, including probiotics like *Lactobacillus plantarum* and *Lacticaseibacillus paracasei* (Liu *et al.*, 2017).

One of the primary strategies to enhance tryptophan production involves eliminating or down-regulating genes associated with pathways that compete for precursors or cofactors required for tryptophan biosynthesis. Tryptophan is synthesized via the shikimate pathway, which also produces other aromatic amino acids, such as phenylalanine and tyrosine, as well as secondary metabolites like quinones and folates (Bongaerts *et al*., 2001). The elimination of competing pathways not only conserves precursors like chorismate but also increases the availability of enzymes and energy for tryptophan biosynthesis (Jiang *et al*., 2016). In a study by Wang *et al.* (2019), *E. coli* strains were engineered to optimize tryptophan production by targeting key competing pathways. The researchers first eliminated genes encoding tyrosine aminotransferase (TyrB) and phenylalanine-specific prephenate dehydratase (PheA), which divert precursors such as chorismate and prephenate toward tyrosine and phenylalanine biosynthesis, respectively (Wang *et al*., 2019). The knockout of these genes resulted in a dramatic redirection of flux, leading to a 50% increase in tryptophan titers compared to wild-type strains. Additionally, feedback inhibition of enzymes within the tryptophan pathway was relieved by mutating the allosteric binding sites of key enzymes like anthranilate synthase (TrpE) and tryptophan synthase (TrpAB) (Wang *et al.*, 2019). This multifaceted approach effectively demonstrated the synergistic benefits of removing competing pathways and relieving pathway bottlenecks.

Another example of this approach was described in a study by Bongaerts *et al*. (2001), where *E. coli* strains were engineered for enhanced tryptophan production through metabolic pathway optimization. By deleting genes involved in aromatic secondary metabolite synthesis, such as ubiC (involved in ubiquinone biosynthesis), researchers minimized the diversion of shikimate pathway intermediates into secondary metabolism (Bongaerts *et al*., 2001). In addition, the deletion of competing aromatic amino acid pathways, combined with

overexpression of the tryptophan biosynthetic operon, resulted in a 16-fold increase in tryptophan production compared to non-engineered strains. This study underscored the importance of both removing competing pathways and fine-tuning pathway regulation to maximize production.

Although these studies were conducted in *E. coli*, the principles are highly relevant to other bacteria, including *Lactobacillus* species. *Lactobacillus plantarum* and *Lacticaseibacillus paracasei* naturally possess genes for tryptophan biosynthesis, making them promising candidates for metabolic engineering to enhance tryptophan yields (Jeong *et al*., 2021). However, these probiotics face unique challenges, such as limited precursor availability and strong regulatory mechanisms that maintain metabolic balance (Mu *et al*., 2022). Knocking out pathways that compete for essential intermediates like phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) could redirect flux toward the shikimate pathway, thereby boosting tryptophan biosynthesis.

Recent advances in molecular engineering tools have made such modifications increasingly feasible in non-model organisms. CRISPR-Cas9, for instance, enables precise knockouts of competing pathways with minimal off-target effects, while homologous recombination facilitates the seamless integration of regulatory elements to fine-tune gene expression (Huang *et al*., 2019). In *Lactobacillus*, these tools can be used to knock out pathways such as acetate or lactate production, which compete for metabolic precursors like pyruvate, thereby increasing the pool of precursors available for tryptophan synthesis (Mu *et al*., 2022). Additionally, engineered strains could be optimized to overcome bottlenecks in the shikimate pathway by introducing mutations that relieve feedback inhibition or by overexpressing key enzymes. While metabolic engineering for tryptophan production in *Lactobacillus* is still in its infancy, the success of these strategies in *E. coli* highlights the potential for similar applications in probiotics. Traditional *lactobacilli* and *bifidobacteria* genome engineering tools were first based on non-replicative (O'Connell Motherway *et al.*, 2009)(Hirayama *et al*., 2012) and temperature-sensitive plasmid-mediated homologous recombination (Sakaguchi *et al*., 2012). Recently, however, scientific technologies have achieved progress and the CRISPR-Cas system has been used to modify the genomes of several *Lactobacillus* species (Zuo and Marcotte, 2021). By leveraging various molecular tools, researchers can unlock the metabolic potential of *Lactobacillus plantarum* and *Lacticaseibacillus paracasei*, paving the way for their use as biofactories for tryptophan production and therapeutic applications.

1.5. Genome engineering tools

The development of genome editing tools has revolutionized molecular biology, allowing precise modifications to be made in microbial genomes, including those of probiotics such as *Lactobacillus*. Over the years, these tools have evolved from protein-based systems, such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), to RNA-guided technologies like CRISPR-Cas9 (Jiang *et al*., 2013). Each tool has unique mechanisms, advantages, and limitations, but CRISPR-Cas9 has emerged as the most versatile and efficient system, particularly when paired with homologous recombination for gene knockout strategies (Xue and Greene, 2021).

ZFNs were one of the first targeted genome editing tools developed, combining DNA-binding zinc finger domains with the non-specific DNA cleavage domain of the restriction enzyme FokI (Kim *et al*., 1996). Zinc fingers are small protein motifs that recognize and bind specific 3-bp sequences of DNA. By engineering arrays of zinc fingers, researchers can create proteins capable of targeting unique genomic sites.

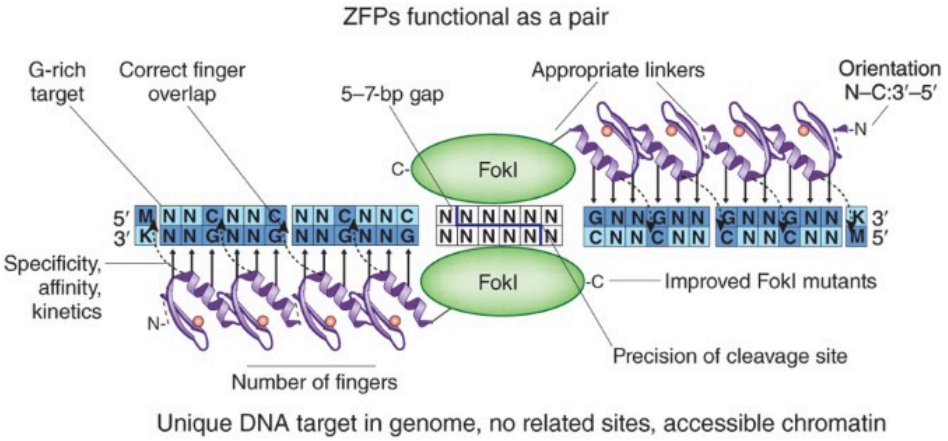

Figure 1.4. Engineering of functional zinc-finger nucleases (Isalan, 2011).

Once bound to the target DNA sequence, the FokI nuclease domains dimerize and induce a double-strand break (DSB) at the target site (Urnov *et al*., 2010). The cell's repair mechanisms then act on these breaks, either via homology-directed repair (HDR) or non-homologous end joining (NHEJ). HDR enables precise modifications using a donor template, while NHEJ often introduces insertions or deletions (indels), resulting in gene disruption (Urnov *et al*., 2010). The main advantages of employing ZFNs are these: they can be tailored to recognize almost any DNA sequence and have been successfully used in various organisms, including bacteria, plants, and humans (Kim *et al*., 1996). However,

engineering zinc finger arrays is time-consuming and technically challenging. To add, the high complexity of zinc finger domains increases the likelihood of off-target cleavage. Lastly, designing ZFNs for large-scale editing projects is impractical due to high costs and complexity. Although ZFNs paved the way for genome editing, their limitations spurred the development of more efficient tools, such as TALENs (Gupta and Musunuru, 2013).

TALENs (transcription activator-like effector nucleases), introduced in 2009, improved upon ZFNs by simplifying the process of designing DNA-binding domains (Boch *et al*., 2009). TALENs are composed of transcription activator-like effector (TALE) proteins, which naturally occur in *Xanthomonas* bacteria. These proteins recognize specific DNA sequences through repeat-variable di-residues (RVDs) in their structure, each of which targets a single nucleotide (Joung and Sander., 2013).

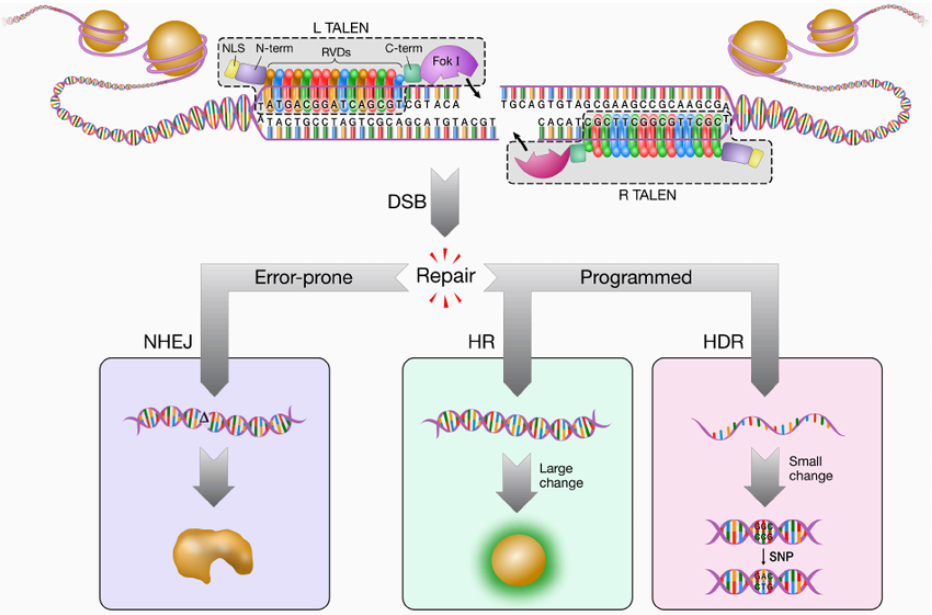

Figure 1.5. Overview of transcription activator-like effector nuclease (TALEN) genome engineering (Campbell *et al*., 2013).

Similar to ZFNs, TALENs pair their DNA-binding domains with FokI nuclease domains to induce DSBs. The repair of these breaks via HDR or NHEJ enables precise or disruptive genomic modifications (Becker and Boch., 2021). The positive side of TALEN implementation for genetic engineering consists of these points: ALE proteins are easier to engineer compared to ZFNs, the one-to-one relationship between RVDs and DNA bases ensures greater accuracy and TALENs have been used in various organisms, including microbes, plants, and mammals. Yet, as every tool, TALENs show a lack of efficiency in a few aspects: they are large, making delivery into cells challenging, particularly in bacteria

with restrictive cell walls like *Lactobacillus* and have lower activity compared to modern tools like CRISPR-Cas9 (Becker and Boch., 2021). While TALENs addressed some of the challenges posed by ZFNs, their inefficiency in bacterial systems limited their widespread adoption in *Lactobacillus* engineering (Vaid *et al.*, 2022).

1.5. CRISPR-Cas-based genetic modifications

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas protein system (Kozovska *et al*., 2021) has progressed through the years and is currently in its third generation. CRISPR-Cas9 technology is the most effective, practicable, and least expensive when compared to first-generation Zinc Finger Nucleases (ZFNs) and second-generation Transcription Activator-like Effector Nucleases (TALENs) (Randhawa and Sengar, 2021; Yin *et al*., 2017; Joung and Sanger, 2013). The CRISPR/Cas system is an adaptive immune system used by prokaryotes to combat the invasion of exogenous genetic elements such as bacteriophages or plasmids (Barrangou *et al.*, 2007). Since its discovery, CRISPR-Cas has been adapted for targeted genome editing across a wide range of organisms, including bacteria, plants, and mammals (Barrangou *et al.*, 2007; Makarova *et al*., 2011).

CRISPR systems are classified into two major classes, Class 1 and Class 2, based on the composition of the effector complexes used to target foreign DNA. Class 1 CRISPR systems employ multi-subunit effector complexes, such as those containing Cascade (CRISPR-associated complex for antiviral defense), which are more complex and less commonly utilized in genome editing (Makarova *et al.,* 2020). In contrast, Class 2 CRISPR systems rely on single, multidomain effector proteins, making them simpler and more adaptable for biotechnological applications. Within these classes, CRISPR systems are further subdivided into six types (I–VI) and multiple subtypes based on the structure and function of the effector proteins (Makarova *et al*., 2015). Among the various CRISPR-Cas types, Type II systems (Class 2) are the most extensively studied and widely used in genome editing, largely due to the simplicity and efficiency of the Cas9 effector protein (Jinek *et al*., 2012). Type II systems utilize a single guide RNA (sgRNA) to direct Cas9 to a specific DNA sequence, where it introduces double-strand breaks (DSBs) at the target site. The simplicity of this mechanism, coupled with its precision and versatility, has made Cas9 a cornerstone of genetic engineering.

CRISPR-Cas9 gene editing method is primarily divided into two phases, namely, DNA cleavage and DNA repair (Fig. 1.6).

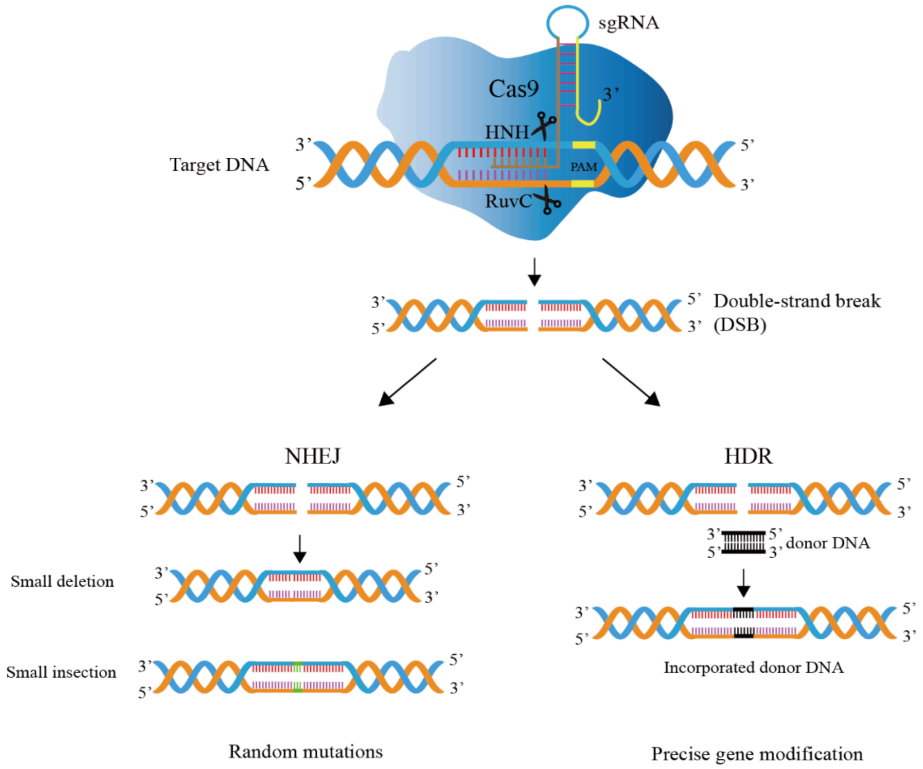

Figure 1.6. Mechanism of CRISPR/Cas9 genome editing system (Mu *et al.*, 2022).

In the DNA cleavage process, Cas9 is recruited by guide RNA and binds to it to form a complex, Cas9 nuclease activity is enabled, and then the created complex may begin to locate complementary target DNA locations for recognition and cleavage (Gasiunas *et al.*, 2012; Nishimasu *et al.*, 2014). The presence of a conserved PAM motif in the target attachment is required not only for complementary pairing of the original 20-nt spacer sequence in the target DNA and the spacer on the guide RNA, but also for target identification and cleavage (Anders *et al.*, 2014). The cell's DNA repair machinery repairs double-strand breaks (DSBs) and alters the DNA sequence during the repair process (Anders *et al.*, 2014). There are two types of DNA repair mechanisms, homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Hsu *et al.*, 2014). HDR needs repair templates to provide accurate and regulated editing, while NHEJ repair does not use repair templates and splices two DNA ends directly (Xue and Greene, 2021). However, base insertion or deletion (indels) may occur during the splicing process, preventing precise editing. *Lactobacillus* DSBs are often repaired using HDR rather than NHEJ (Mu *et al.*, 2022).

Compared to earlier genome editing tools, such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), CRISPR-Cas9 offers several advantages. First, the design and synthesis of sgRNAs are straightforward and cost-effective, whereas ZFNs and TALENs require labor-intensive protein engineering (Gupta and Musunuru, 2014). Second, Cas9 exhibits higher specificity and efficiency due to its reliance on RNA-DNA base pairing, which reduces the likelihood of off-target effects. Lastly, the ability to multiplex sgRNAs allows for the simultaneous editing of multiple genomic loci, a feature that is challenging to achieve with ZFNs or TALENs (Doudna & Charpentier, 2014). In the context of *Lactobacillus* species, CRISPR-Cas9 has been instrumental in overcoming challenges associated with traditional genetic engineering methods. The rigid cell walls and low transformation efficiencies of *Lactobacillus* strains often limit the uptake of exogenous DNA (Mu *et al*., 2022). However, CRISPR-Cas9 circumvents these limitations by directly inducing DSBs in the bacterial genome, enabling precise knockouts and targeted modifications. When coupled with homologous recombination, CRISPR-Cas9 enables the seamless integration of genetic constructs into the genome, further expanding its utility in engineering probiotics for therapeutic applications (Mu *et al*., 2022).

1.6. Bacterial recombination

Bacterial genome editing is typically accomplished by homologous recombination. It occurs between the target gene and an editing substrate, which can be either circular or linear DNA, with the latter consisting of single-stranded DNA (ssDNA) oligonucleotides (oligos) or double-stranded PCR products (dsDNA) (Fels *et al*., 2020). These editing substrates infiltrate bacteria via transformation, conjugation, or transduction (Fels *et al*., 2020). Homologous recombination between the target gene and the editing substrate can be accomplished by endogenously expressed recombination genes, the use of recombination-proficient strains, or alternatively, by phage recombination systems (recombineering) (Fels *et al*., 2020). A general model to describe homologous recombination was proposed (Fig. 1.7) (Holliday, 1964). Holliday model, or Holliday junctions, are critical intermediates for homologous, site-specific recombination, DNA repair, and replication (Holliday, 1964; Fels *et al*., 2020).

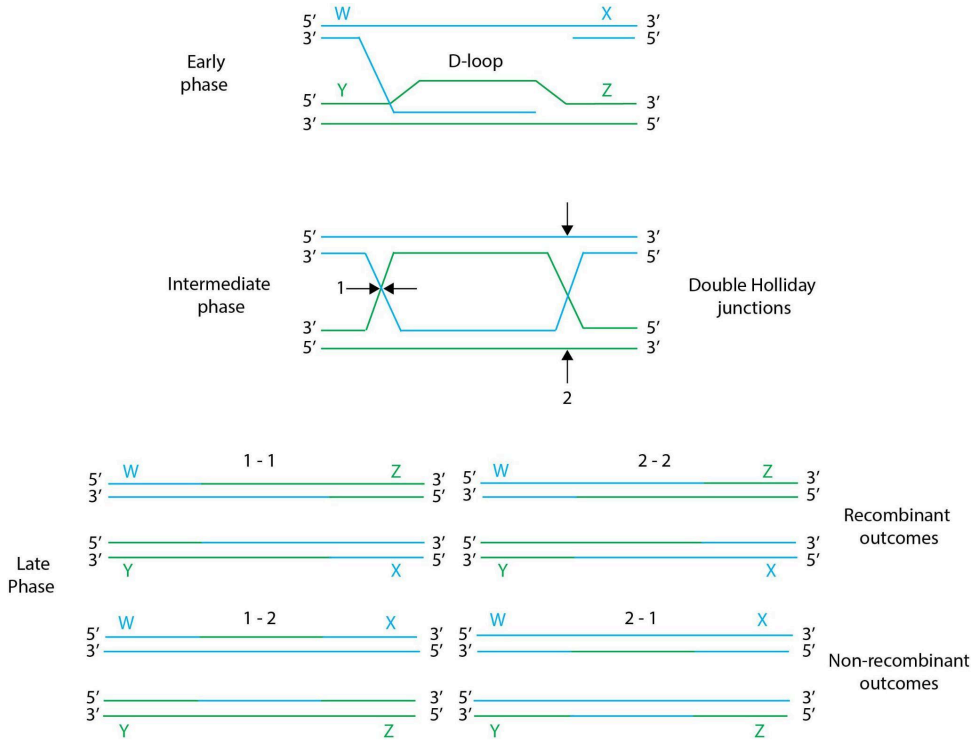

Figure 1.7. General scheme of homologous recombination (Fels *et al*., 2020).

The DNA exchange processes involved in homologous recombination are divided into early, intermediate, and late stages (Kuzminov, 2011). The development of branched DNA that eventually forms recombination intermediates known as Holliday junctions makes up the intermediate phase, which contrasts with the early phase, which involves the end-processing and invasion of ssDNA from one DNA duplex into another to produce a D-loop (Kuzminov, 2011). The Holliday junctions are severed by resolvases in the last step to create recombinant chromosomes, either with or without flanking sequence crossover (Kuzminov, 2011). Four alternative results emerge from different resolution cuts made to the left or right of the junctions, two recombinant molecules with flanking sequence exchange, or two patch recombinants without flanking sequence exchange (Kuzminov, 2011). Although this strategy possesses certain drawbacks, including unstable mutations, transformation efficiency dependence, a limited host range, and non-seamless editing, its application greatly facilitated chromosomal gene replacement and the development of knockouts and is essential for reverse genetic-assisted discoveries (Zuo and Marcotte, 2021).

2. MATERIALS AND METHODS

2.1. Bacterial cultures

Lactobacillus isolates were obtained from fermented foods, specifically yogurt, pickles, tomatoes and kombucha, using the following procedure. Samples of yogurt were aseptically collected and streaked onto Man, Rogosa, and Sharpe (MRS) agar plates using the quadrant streaking method. The plates were then incubated at 37°C for 48 hours to allow bacterial growth. After incubation, individual bacterial colonies were selected based on their morphological characteristics and purified by repeated streaking on fresh MRS agar plates. This process was performed to obtain pure cultures of the isolated bacteria.

2.2. Engineering plasmids

Escheria coli DH5α cells, containing an engineering plasmid pHSP02 (plasmid ID #117259) and a recombination helper plasmid pLH01 (plasmid ID #117261) were ordered via Addgene (Watertown, MA, USA). Bacterial cells carrying pHSP02 were grown in Luria broth (LB) media at 30°C for 24 hours with the recommended kanamycin concentration of 25 μg/mL. Similarly, cells with pLH01 plasmid were grown in 12.5 μg/mL chloramphenicol infused liquid LB media at 37°C for 24 hours. Cells were stored at 4°C until further use.

2.3. Whole-genome sequencing

Lactobacillus DNA from samples was isolated using the QIAGEN DNeasy PowerSoil Pro Kit, according to the manufacturer's protocol. DNA samples were quantified using the GloMax Plate Reader System (Promega) using the QuantiFluor® dsDNA System (Promega) chemistry. DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes with total DNA input of 1 ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Unique dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMpure magnetic Beads (Beckman Coulter) and eluted in QIAGEN EB buffer. DNA libraries were quantified using Qubit 4 fluorometer and

Qubit dsDNA HS Assay Kit. Libraries were then sequenced on Illumina NovaSeq platform 2x150bp. Quality control checks were conducted by the company to ensure the integrity and accuracy of the sequencing data. Following the sequencing, bioinformatics analysis was performed by CosmosID® using their in-house pipelines or software. This analysis involved *de novo* genome assembly, assembly statistics of assembled isolates, genome quality assessment using Check-M, SNP tree based on core genome phylogeny, AniM dendrogram based on MUMmer, antimicrobial resistance and virulence factors characterization, functional annotation of assembled isolates, MLST characterization.

2.4. Tryptophan production measurement by HPLC-MS analysis

A total of nine bacterial strains, encoding tryptophan genes, namely *Lactiplantibacillus plantarum* PN36, PN51Y, 33, 40, 48, 66, LAB25 and LAB66, as well as *Lacticaseibacillus paracasei* 11w, were selected for this study. The bacterial strains were cultured in de Man, Rogosa and Sharpe (MRS) medium and maintained on MRS agar plates.

For tryptophan measurement by high-performance liquid chromatography-mass spectrometry (HPLC-MS), bacterial supernatants were prepared. *L. plantarum* and *L. paracasei* strains were cultured at 37°C under aerobic conditions in MRS broth for 16 h. The cultured broth was then centrifuged for 10 min at 8,000 × g. The supernatants were filtered with a 0.22 μm filter and stored at -20°C until further use. MRS broth was prepared as a control under the same conditions. This broth served as the base medium for the control sample in the analysis.

In the samples, concentrations of tryptophan were determined by HPLC-MS according to the calibration curve prepared using the L-tryptophan standard. This process was performed by Dr. Jonita Stankevičiūtė of the Molecular Microbiology and Biotechnology department in Vilnius University Life Sciences Centre. First, the samples were mixed with an equal volume of acetonitrile and centrifuged for 10 min at 10,000 rpm. The samples were analyzed using HPLC-MS system (Shimadzu, Japan) equipped with a photodiode array (PDA) detector (Shimadzu, Japan) and mass spectrometer (LCMS-2020; Shimadzu, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro C18 column (3 × 150 mm; YMC, Japan) at 40°C and a mobile phase that consisted of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) delivered in the 5–95% gradient elution mode. Mass scans were measured from m/z 50 up to

m/z 2,000 at a 350°C interface temperature, 250°C desolvation line (DL) temperature, ± 4,500 V interface voltage, and neutral DL/Qarray, using N_2 as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions liquid chromatography-mass spectrometry (LCMS) software.

2.5. Design of gRNA sequences and homologous arms

Lacticaseibacillus paracasei 11w strain was selected for engineering work due to the highest performance in L-tryptophan production. An energetic pathway gene acetate kinase (*ackA*) was chosen as a knock-out target. gRNAs were designed with the help of a few bioinformatic tools. Proksee.ca (https://proksee.ca/) was used to identify target gene locus, while CHOPCHOP CRISPR web toolbox v3 (https://chopchop.cbu.uib.no/) and Benchling (www.benchling.com) (San Francisco, CA, 94107, USA) tools were applied for sequence designs following these steps. A sequence of *Lacticaseibacillus paracasei* ackA gene was uploaded into the CHOPCHOP system, which generated gRNA variants - DNA target sequences in the exon area of the gene. gRNA sequences were chosen according to such factors as location in the genome, GC content (%), self-complementarity and efficiency. A total of two variants were chosen per gene for best efficiency testing.

In order to provide the recombination material following CRISPR-Cas9 induced knock-out, homologous arms were designed via Benchling, namely *lpLHA_A*, *lpRHA_B1* and *lpRHA_B2*. *lpLHA_A* was a ~1.0 kb sequence found upstream the *ackA_1* gene and functioned as a left homologous arm. *lpRHA_B1* and *lpRHA_B2* fragments were ~1.2 kb and found downstream. Notably, the latter fragments are identical in structure with the only difference being gRNA sequences for most efficient gRNA testing. All three sequences were designed to carry appropriate digestion enzyme sites - ApaI and XhoI for *lpLHA_A,* XhoI and XbaI for *lpRHA_B1* and *lpRHA_B*. Sequences were ordered and synthesized by Twist Bioscience (USA). DNA was resuspended in a nuclease free Tris-EDTA (TE) buffer, pH 8.0 solution and stored at -20 °C.

2.6. Molecular manipulation

Engineering pHSP02 and helper pLH01 plasmids were isolated from *E. coli* DH5α cells using Zymo Research BAC DNA Miniprep Kit (Irvine, CA, USA). Extraction and purification steps were accomplished by utilizing manufacturer's protocols. DNA concentration and purity were measured by NanoDrop One^{C} (Thermo Fisher Scientific, Watertown, MA, USA). Restriction reaction components, including FastDigest enzymes ApaI, XbaI, XhoI and 10X Restriction Buffer, as well as ligation reagents T4 DNA ligase and 10X T4 DNA Ligase Buffer (with ATP) were all obtained from Thermo Fisher Scientific (Watertown, MA, USA).

A desired engineering pHSP02 plasmid was constructed by the following steps. Plasmid was first digested with ApaI and XbaI enzymes to remove homologous arms (~2kb fragment), which had been previously inserted by the original authors Huang *et al*, 2019. Identically, *lpLHA_A* was digested with ApaI and XhoI enzymes, while *lpRHA_B1* and *lpRHA_B2* fragments were digested with XhoI and XbaI. The digested components were run on 1% agarose gel electrophoresis under 100 V for 1 hour and then purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Watertown, MA, USA). Linearized DNA fragment concentration and purity were measured by NanoDrop One^{C}.

Sticky-end ligation was performed to assemble two engineered plasmid constructs, referred to as *pLcasei_AB1* and *pLcasei_AB2*. Then, *E.coli* Top10 cells were transformed with engineered constructs of *pLcasei_AB1* and *pLcasei_AB2.* To verify the ligation of the ~2 kb inserts into the 12 kb linearized plasmid backbone, plasmid DNA was extracted from transformants, digested with ApaI and XhoI restriction enzymes and analyzed using gel electrophoresis. These enzymes were specifically chosen because in case of a positive result, their combined action would produce a distinct ~1 kb band, which could only be visible if the fragment was successfully inserted due to the presence of the XhoI site in the fragments. Ligation was completed by employing the 3:1 (insert to vector) ratio to design a total of two final constructs, referred to as *pLcasei_AB1* (consisting of pHSP02 backbone, lpLHA_A and lpRHA_B1 inserts) and *pLcasei_AB2* (consisting of pHSP02 backbone, lpLHA_A and lpRHA_B2 inserts). Reaction was performed on ice by gently mixing the reagents together. The reaction mix was incubated overnight at 16°C for maximum efficiency, as recommended by the supplier. Lastly, inactivation was performed at 65°C for 10 minutes.

2.7. Bacterial transformation and electroporation

As a plasmid-amplifying host - One Shot™ TOP10 Electrocompetent *E. coli* cells were obtained from Thermo Fisher Scientific (Waltham, MA, USA), prepared according to manufacturer's protocol and kept at -70 °C until further usage. The following steps describe the arrangement of the transformation reaction. Recovery medium (SOC medium) was thawed to room temperature, and 0.1 cm gap electroporation cuvettes (Bio-rad, CA, USA) were pre-chilled on ice. Electrocompetent *E. coli* Top10 cells were retrieved from storage at -70 °C and thawed on ice for 10 minutes. For each transformation, 2-4 µL of constructed plasmid DNA (10 pg to 100 ng) was gently mixed with thawed cells. The mixture was transferred to a pre-chilled cuvette and electroporated using the P1 setting of 1.8 kV on an Eppendorf Eporator®. Immediately after electroporation, 975 µL of pre-warmed SOC medium was added to the cells. The mixture was then transferred to a sterile tube and incubated in a shaker at 37°C for 1 hour. Transformation mix was plated on LB agar plates in 2 ways, one being 45 µL and another - a 3x concentrate. A positive control was established by transforming pLH01 plasmid and plated onto the LB agar plate with 12.5 ng/mL of chloramphenicol. In terms of a negative control, engineered pHSP02 plasmids were also plated on LB agar plates containing 12.5 ng/mL of chloramphenicol.

As for final *Lactiplantibacillus paracasei* 11w transformation with *pLcasei_AB1* + pLH01 and *pLcasei_AB2* + pLH01 plasmids, an optimized protocol suggested by Fristot et al., 2023 was followed. Cells were grown overnight in 5 mL of MRS medium at 37°C. The following day, 975 µL of fresh MRS medium was inoculated with 25 µL of the overnight culture and incubated at 37°C without shaking until the optical density (OD) reached 0.5–0.6 (approximately 4–5 hours). The cells were harvested by centrifugation at 5,000 g for 5 minutes, the supernatant was discarded. The cell pellets were resuspended in 150 µL of ice-cold 3X SMEB electroporation buffer (298 mM sucrose, 1 mM $MgCl_2$ in cold sterile water) and centrifuged at 5,000 g for 6 minutes. This washing step was repeated twice to ensure thorough cleaning. The final cell pellets were concentrated in 200 µL of ice-cold 3X SMEB buffer. These aliquots of 200 µL were either used immediately for electroporation (within one hour) or stored at -80°C for long-term use. For electroporation, 200 µL of competent cells were thawed on ice and transferred to a 0.2 cm electroporation cuvette (Bio-rad Laboratories, Richmond, CA). Up to one µg of plasmid DNA was added and mixed gently. The mixture was incubated on ice for 10 minutes. Electroporation was performed following parameters: 12.5 kV/cm, 400 Ohm, and 25 µF capacitance. Immediately after the

electric pulse, 800 µL of pre-warmed (37°C) antibiotic-free MRS medium was added to the cuvette, and the cells were gently resuspended. The resuspended cells were then transferred to culture tubes and incubated at 37°C for 3 hours without agitation. Following incubation, 100 µL of the culture was spread onto MRS agar plates (1% agar) supplemented with 25 µg/mL kanamycin and 12.5 µg/mL chloramphenicol. If necessary, cells were concentrated by centrifugation at 4,000 g for 5 minutes and resuspended in 100 µL of MRS medium prior to plating.

2.8. Statistical methods

Numeric values are presented as the arithmetic mean with standard deviation for normal data distribution. Categorical variables are presented as absolute and relative numbers (percentages).

3. RESULTS

3.1. Phylogenetic analysis

A comprehensive analysis was conducted on a collection of bacterial strains isolated from yogurt and other fermented foods. Whole-genome sequencing was performed to determine the identities of the strains and explore their genetic composition. Utilizing SNP (single-nucleotide polymorphisms) Tree based on Core Genome Phylogeny, eight strains were identified as *Lactiplantibacillus plantarum*, with a remarkable core genome coverage of 74.3% (Fig. 3.1).

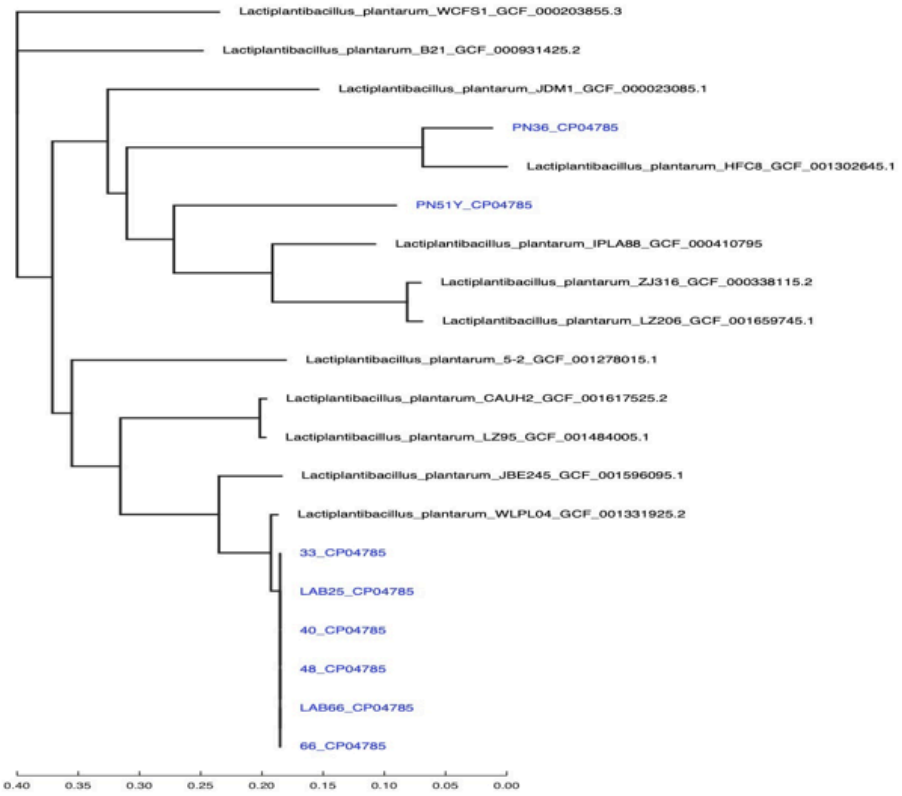

Figure 3.1. SNP Tree based on Core Genome Phylogeny.

Additionally, one strain was identified as *Lacticaseibacillus paracasei*, exhibiting a core genome coverage of 68.3% (Fig.3.1). Notably, analysis revealed the absence of antimicrobial genes in all the isolates through antibiotic Microbial Genes Characterization, suggesting that these strains may not possess intrinsic resistance to antibiotics. Furthermore, the Virulence

Factors Characterization analysis demonstrated the absence of virulence factor genes in the isolates, indicating their safety for use.

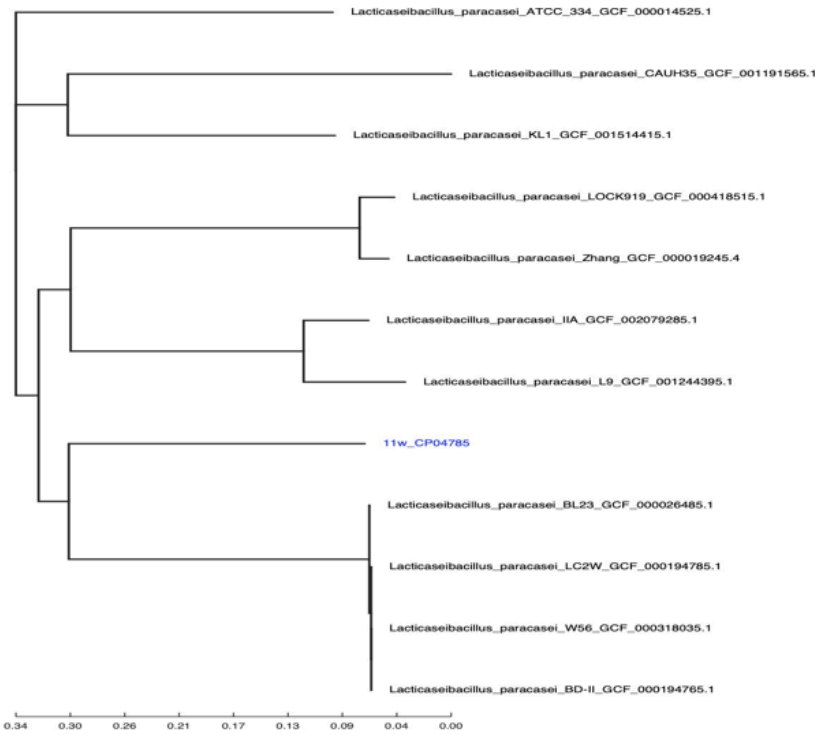

Figure 3.2. SNP Tree based on Core Genome Phylogeny.

3.2. Annotation of functional L-tryptophan synthesis genes

Among the analyzed bacterial strains, it was found that nine out of the total isolated strains carried tryptophan encoding genes, specifically *trpA*, *trpB*, and *trpC* (Table 1). These genes are integral components of the tryptophan biosynthetic pathway, enabling bacteria to produce tryptophan.

Table 1. Functional L-tryptophan genes in *L. plantarum* and *L. paracasei* strains.

Bacterial strains	Genes associated with L-tryptophan synthesis	Gene name	Gene size (bp)	Gene function
Lactiplantibacillus plantarum				
PN36				
PN51Y				
33				
40	*trpA*	Tryptophan synthase alpha chain	795	
48	*trpB*	Tryptophan synthase alpha chain	1200	Tryptophan synthesis
66	*trpC*	Indole-3-glycerol phosphate synthase	783	
LAB25				
LAB66				
Lacticaseibacillus paracasei				
11w				

3.3. Design of gRNAs and homologous arms using bioinformatics tools

Genomic analysis was conducted using Proksee (proksee.ca) to examine the scaffolds obtained from sequencing data. The primary objective was to identify potential target genes and confirm their presence within the genome. The gene of interest - ackA_1 of 1,206 bp was located, and its corresponding locus was determined (refer to Fig. 3.3).

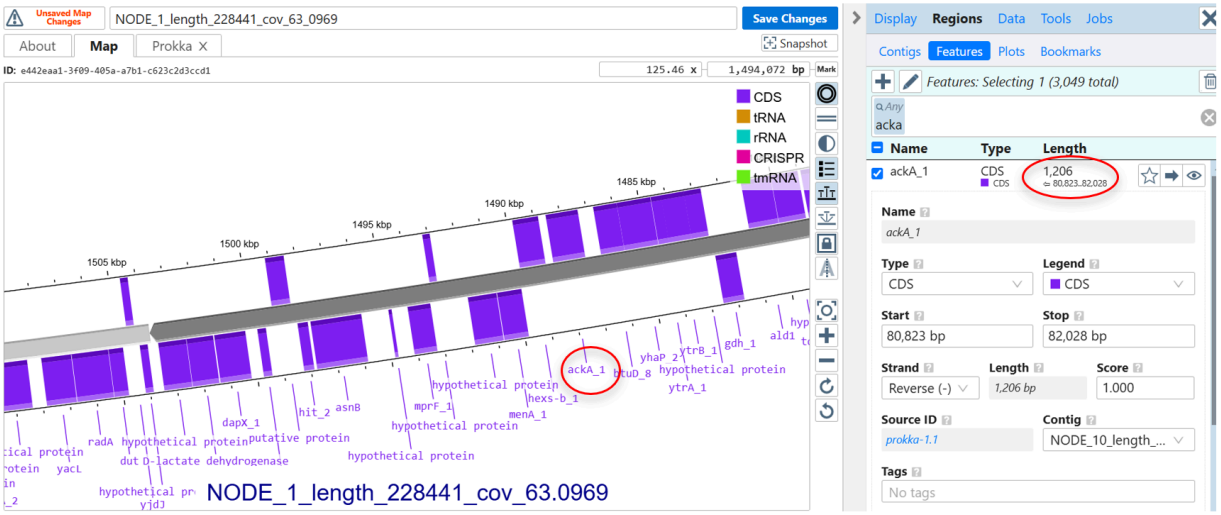

Figure 3.3. Image portraying annotated features of the genome, specifically the node of the ackA_1 gene (proksee.ca).

The scaffold sequence containing the gene was subsequently uploaded to Benchling (benchling.com) for annotation.

Figure 3.4. Image of annotated acka_1 gene and homologous arm sequences in the scaffold (Benchling).

In addition to annotating the target gene, homologous arms were defined by marking a 1 kb region upstream (left homologous arm) and a 1 kb region downstream (right homologous arm) of the gene to facilitate future experimental procedures (as shown in Fig. 3.4).

Guide RNAs (gRNAs) were designed for CRISPR-based targeting using the online tool CHOPCHOP (https://chopchop.cbu.uib.no/). The sequence of the identified target gene (ackA_1) was submitted to the platform, and multiple gRNA variations were generated. Selection criteria included the genomic location of the gRNAs, their GC content (%), self-complementarity, and predicted targeting efficiency scores. From the initial set of candidates, two gRNA variants were selected for further testing. The first candidate was excluded due to its location outside the coding region of the gene. Among the remaining options, gRNAs 2 and 6 were selected based on their favorable characteristics, especially self-complementarity and their GC content, which fell within the optimal range (framed in red, Fig. 3.5).

Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	GGCTTAAGGACATGACGTCGCGG	seq:24	+	55	0	0	0	0	0	70.07
2	ATCATCAATGACAATTGGGGCGG	seq:895	+	40	0	0	0	0	0	67.73
3	ATCGACACCGTCCATCAAGGCGG	seq:244	+	55	3	0	0	0	0	70.71
4	TTGCAGGTTAGAGAGCAGGTTGG	seq:994	+	50	0	0	0	0	0	66.91
5	TGGGGCGGGTTCAAGCATCACGG	seq:567	-	60	0	0	0	0	0	66.24
6	CATCGTGACACCGGACAAAGGGG	seq:502	+	55	0	0	0	0	0	65.36
7	GAACAGCTTCCATTTGAGTGAGG	seq:1150	+	45	0	0	0	0	0	65.28
8	GTCACGATAATTGCAAATTGGGG	seq:1024	+	35	0	0	0	0	0	64.95
9	AATGATTGTGATGCATCTTGGGG	seq:585	-	35	0	0	0	0	0	64.88
10	ACTCAAATCCACGATAACGCAGG	seq:116	-	45	0	0	0	0	0	64.70

Figure 3.5. Image exhibiting potential target sequences for CRISPR-Cas9 knock out (CHOPCHOP).

A GC content of 40–80% was prioritized, as higher GC content is known to enhance the stability of RNA-DNA duplex formation. This stabilization improves on-target binding while simultaneously reducing off-target hybridization. By selecting gRNAs with these characteristics, the design aimed to optimize the efficiency and specificity of the CRISPR-based targeting approach.

3.4. Tryptophan assessment in bacterial supernatant samples by HPLC analysis

To investigate the capacity of the bacterial strains to produce tryptophan, high-performance liquid chromatography (HPLC) analysis was performed. As a control, MRS broth was utilized as a baseline for comparison. Notably, while all the bacterial strains examined in this study possessed tryptophan encoding genes, only one strain, specifically *Lacticaseibacillus paracasei* 11w, exhibited a significant increase in tryptophan levels compared to the control sample (Fig. 3.6). The total concentration of tryptophan by *L. paracasei* 11w strain was 101.05 ± 4.26 µM (mean ± standard deviation), while control was 68.69 ± 3.88 µM. Thus, meaning that the concentration of L-tryptophan in the sample of *L. paracasei* 11w strain was 32.36 ± 5.12 µM. The average concentration of L-tryptophan was ± 53.37 µM, with the lowest being 41.63 ± 3.67 µM (in *L. plantarum* 40 strain).

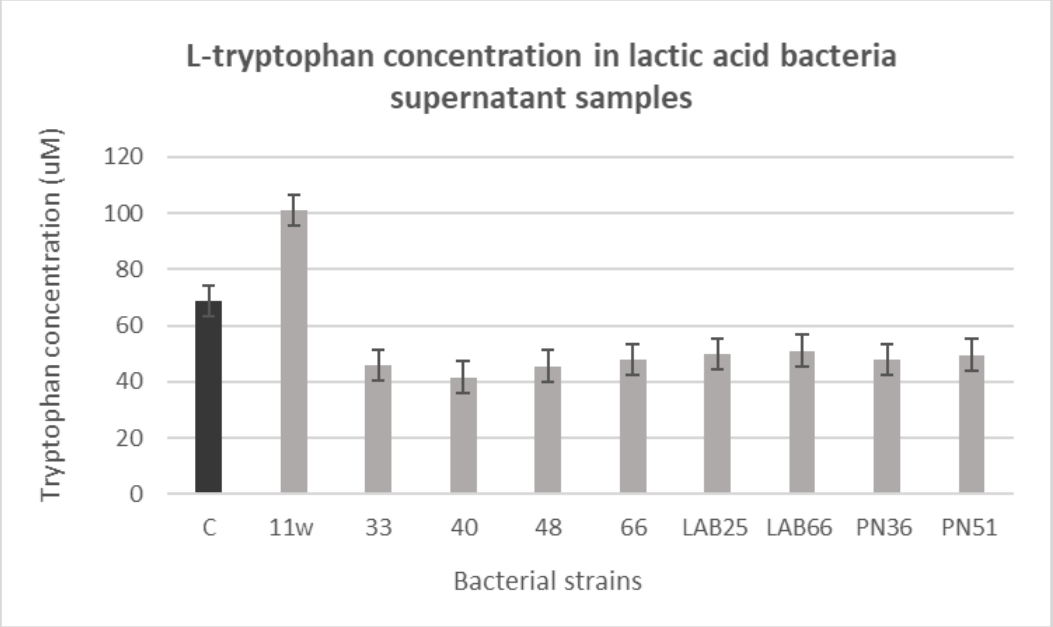

Figure 3.6. L-tryptophan production in bacterial supernatant samples measured by HPLC analysis after fermentation. Control – MRS broth; 11w – *L. paracasei* 11w strain. 33 - *L. plantarum* 33, 40 - *L. plantarum* 40, 48 - *L. plantarum* 48, 66 - *L. plantarum* 66, LAB25 - *L. plantarum LAB*25, LAB66 - *L. plantarum* LAB66, PN36 - *L. plantarum* LAB66, PN51 – *L. plantarum* PN51 strain.

The HPLC analysis revealed that *L. paracasei* 11w demonstrated a higher concentration of tryptophan than the MRS control sample, suggesting its capability for efficient tryptophan biosynthesis.

3.4. Digestion of the engineering pHSP02 plasmid and insert sequences

The original pHSP02 plasmid was extracted from *E.coli* DH5α cells. Concentration and absorbance measurements were evaluated using NanoDrop One^{C}. The concentration of the plasmid DNA was 34.2 ng/µL, while A260/280 was 1.96 and A260/230 - 2.23. The values indicated that DNA is of high purity with minimal protein, phenolic or organic compound contamination. Digestion with ApaI and XbaI restriction endonucleases created sticky ends for new inserts of *lpLHA_A*, *lpRHA_B1* and *lpRHA_B2,* and removed homologous arms from the previous study (Fig. 3.7).

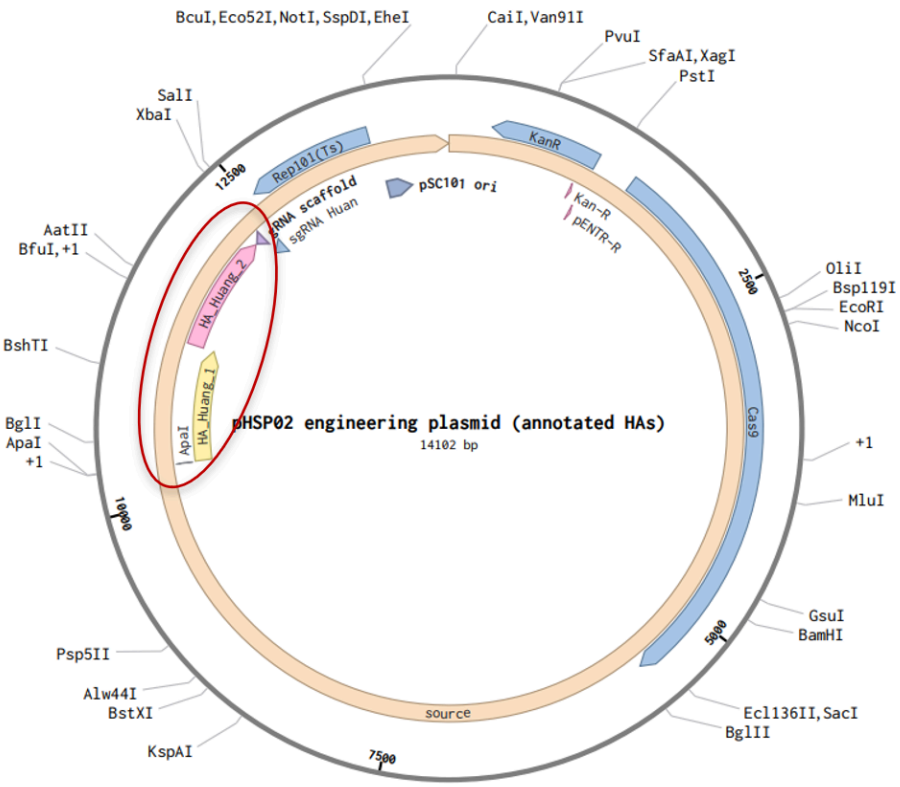

Figure 3.7. Original pHSP02 plasmid with annotated primary homologous arms (Addgene and Benchling).

The digestion resulted in creation of a linearized plasmid backbone suitable for the insertion of desired fragments. The digestion products were determined by agarose gel electrophoresis and are presented in Fig. 3.8.

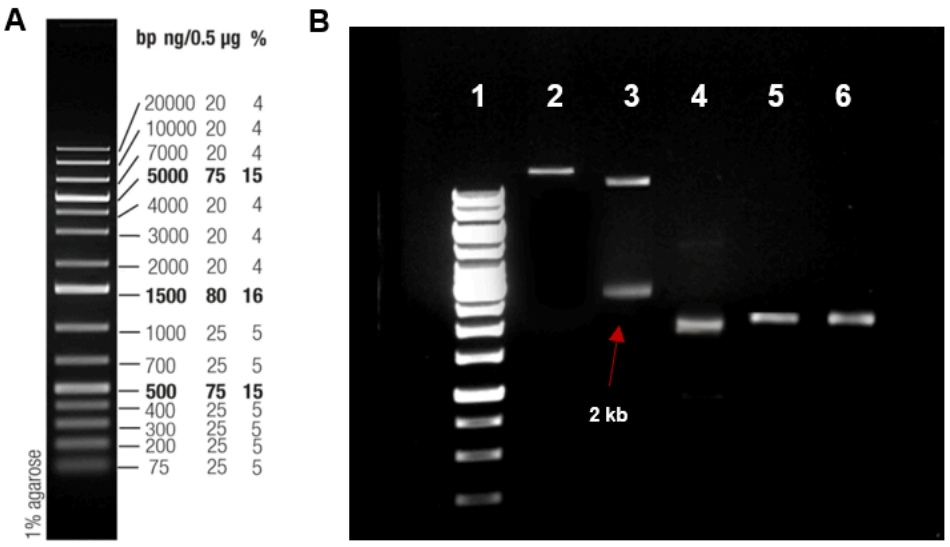

Figure 3.8. Agarose gel electrophoresis image of digested pHSP02 plasmid and DNA fragments. On the left side - A, a reference of the 10 kb DNA ladder marker is shown. On the right in B, starting from 1 - 10 kb DNA Ladder, 2 - uncleaved pHSP02, 3 - linearized pHSP02 of 12 kb and a fragment of 2 kb containing original homologous arms, 4 - *lpLHA_A* insert, 5 - *lpRHA_B1* insert, 6 - *lpRHA_B2*.

The digestion of the pHSP02 plasmid yielded the anticipated ~2 kb fragment, confirming precise cleavage at the ApaI and XbaI sites. Similarly, the clear and distinct bands observed for fragments *lpLHA_A, lpRHA_B1* and *lpRHA_B2* confirm successful enzymatic digestion, with no evidence of degradation. These results validate the preparation of all components for subsequent ligation.

3.5. Transformation of *E.coli* Top10 with *pLcasei_AB1*, *pLcasei_AB2* and pLH01

To assess the transformation efficiency of the plasmid constructs, two ligated variants of pHSP02 - *pLcasei_AB1* and *pLcasei_AB1* were first transformed into *E. coli* TOP10 cells and plated onto LB agar containing 25 ng/µL kanamycin. From 45 µL of the post-transformation mixture, three colonies grew on each plate (Fig. 14 pictures D and E), indicating successful uptake and expression of the kanamycin resistance marker. In a separate transformation, a concentrated cell suspension containing pHSP02, prepared via centrifugation, was plated (Fig. 3.10 pictures A and B). This resulted in a dense lawn of growth across the plate, with distinct colonies visible near the periphery. This pattern is

consistent with plating from concentrated transformation mixtures (as seen in the Fig. 3.9, pictures A and B).

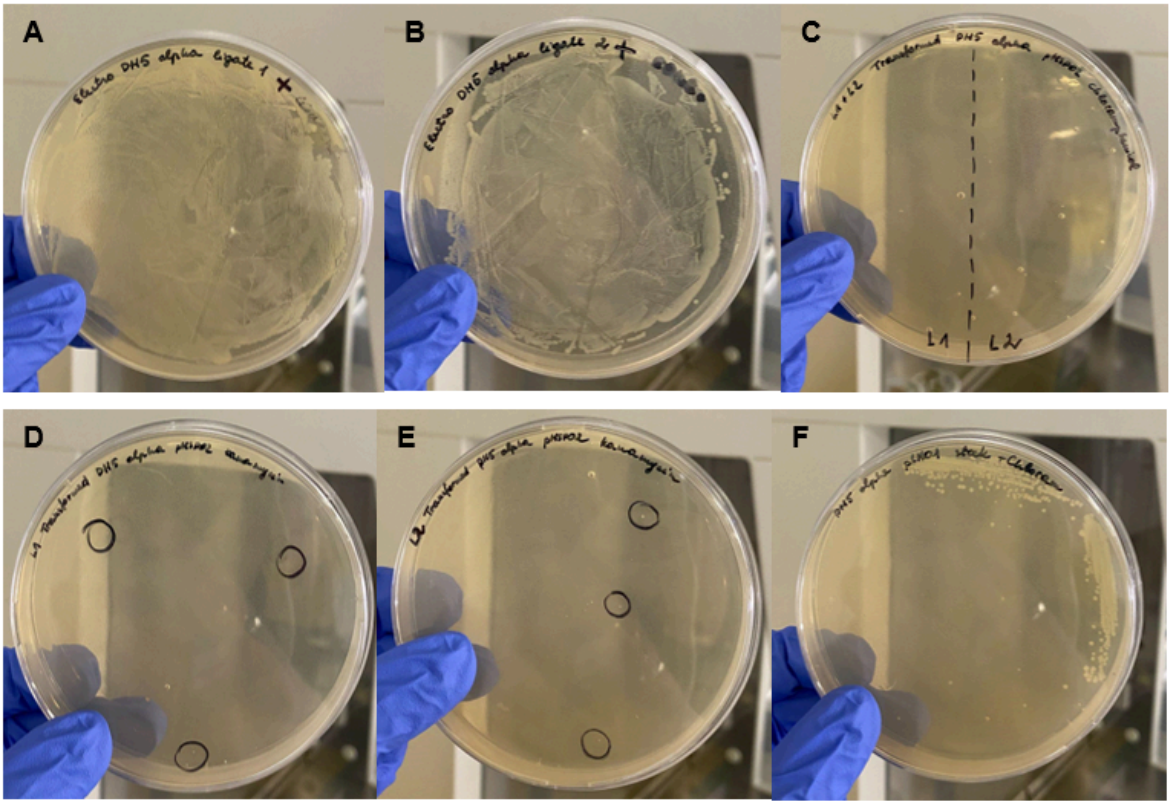

Figure 3.9. Confirmation of *E.coli* Top10 transformation growth using antibiotic selection. *A* - 3x concentrate of *pLcasei_AB1* transformants on kanamycin, *B - 3x* concentrate of *pLcasei_AB2* transformants on kanamycin, *C - pLcasei_AB1* and *pLcasei_AB2* transformants on chloramphenicol induced *LB agar, D - 45* µL transformation mix of *pLcasei_AB1, E - 45* µL transformation mix of *pLcasei_AB2, F - E.coli Top10* transformed with pLH01 and plated on 12.5 ng/µL chloramphenicol.

A positive control transformation using the plasmid pLH01 was performed in *E. coli* TOP10, followed by plating onto LB agar supplemented with 12.5 ng/µL chloramphenicol. Growth along the peripheral was observed, confirming the competence of the cells and the functionality of the transformation protocol (Fig. 3.9. picture F). As a negative control, *E. coli* TOP10 cells transformed with the two pHSP02 constructs were plated onto LB agar containing 12.5 ng/µL chloramphenicol (Fig. 3.9 picture C). No colonies could be seen on these plates, as expected, since the pHSP02 plasmid does not carry a chloramphenicol resistance cassette.

3.6. Confirmation of ligation efficiency via restriction enzyme digestion

The uncut plasmid (well 2) displays characteristic plasmid DNA form of circular conformation. In contrast, the digested plasmids (Lanes 3 and 4) show two distinct bands: the 12 kb linearized plasmid backbone and the 1 kb insert, corresponding to the expected sizes and confirming the successful ligation.

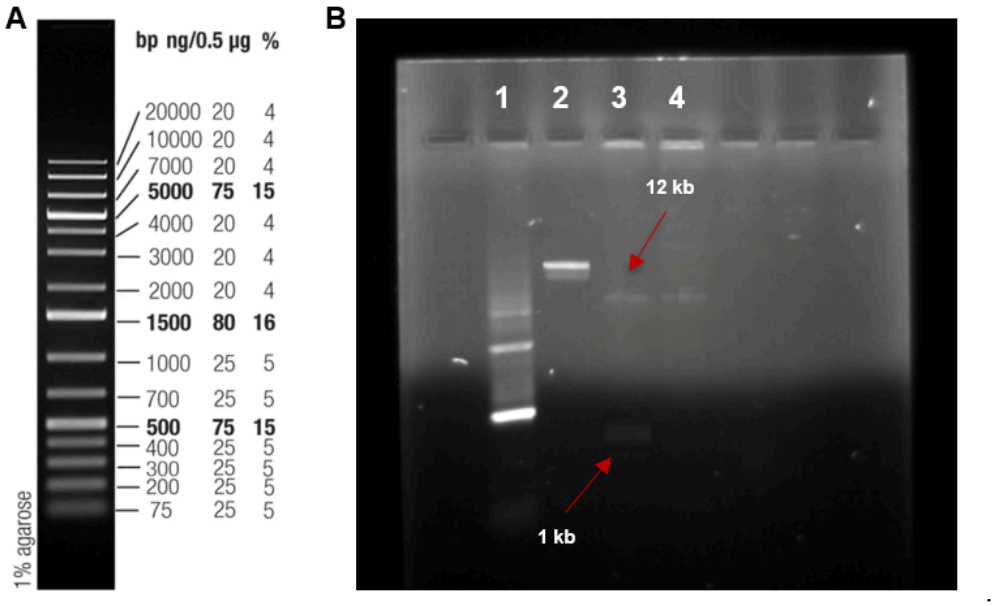

Figure 3.10. Agarose gel electrophoresis image of ligation products after digestion with ApaI and XhoI restriction enzymes. A - reference photo of 10 kb DNA ladder. Picture B represents ligation products. 1 - 10 kb DNA ladder marker, 2 - uncut pHSP02 plasmid, 3 - ApaI and XhoI digested *pLcasei_AB1* showing a 1 kb product, 4 - ApaI and XhoI digested *pLcasei_AB2* showing only the linearized 12 kb vector.

However, the 1 kb desired product could not be identified in the 4th well, which could be a result of limited DNA concentration due to low copy DNA digestion. Therefore, only the *pLcasei_AB1 construct* exhibited positive results. Repetition of digestion process would be necessary to fully confirm the findings.

3.7. Transformation of *L.paracasei 11w* with *pLcasei_AB1* and *pLcasei_AB2* and pLH01

The transformation of *L. paracasei* 11w was conducted using the engineering plasmids pLcasei_AB1 and pLcasei_AB2 in combination with the control plasmid pLH01. Plasmid concentrations and purity were tested prior. pLcasei_AB1 concentration was 21.3

ng/µL, A260/280 - 1.76 and A260/230 - 1.92, while pLcasei_AB1 concentration was 18.5 ng/µL, A260/280 - 1.81 and A260/230 - 1.95. Measurements indicate protein contamination and organic compound residue. pLH01 concentration was 43.2 ng/µL, A260/280 - 1.94 and A260/230 - 2.02, with plasmid of higher purity. Initial attempts to transform *L. paracasei* 11w with either pLcasei_AB1 or pLcasei_AB2 alone did not yield any detectable growth on selective media (Fig. 3.11. A and B), indicating insufficient transformation efficiency for these plasmids under the conditions tested.

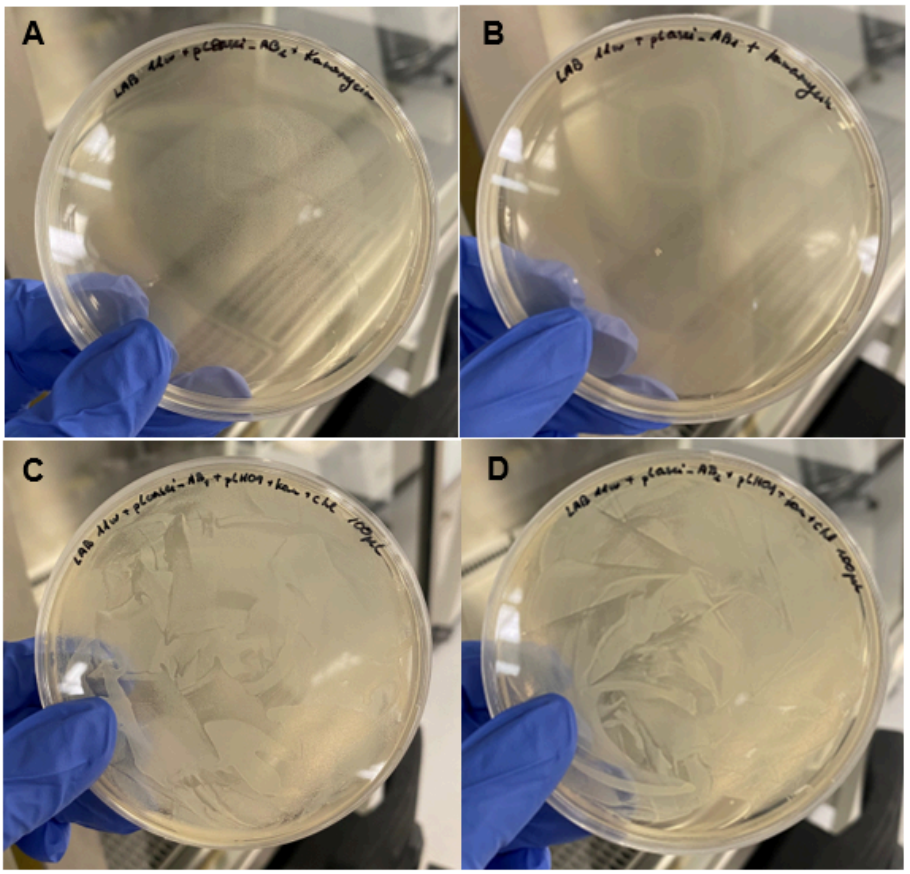

Figure 3.11. Confirmation of *L.paracasei* 11w transformation growth using antibiotic selection. A - pLcasei_AB1 transformants on kanamycin. B - pLcasei_AB2 transformants on kanamycin. C - pLcasei_AB1 + pLH01 transformants on kanamycin and chloramphenicol. D - pLcasei_AB2 + pLH01 transformants on kanamycin and chloramphenicol.

Co-transformation experiments were performed with pLcasei_AB1 and pLH01, as well as pLcasei_AB2 and pLH01 pairs. In both cases, lawns of growth were observed on selective kanamycin and chloramphenicol plates (Fig. 3.11. C and D**)**. This indicated that cells were successfully transformed; however, it is likely that the observed growth was due primarily to the uptake of the pLH01 plasmid. The results suggest that pLH01, which is smaller in size

and was present at a higher concentration during the transformation process, was preferentially taken up by the cells. In contrast, the low concentrations and larger sizes of pLcasei_AB1 and pLcasei_AB2 likely contributed to their reduced transformation efficiency.

4. DISCUSSION

The design and assembly of a CRISPR/Cas9-based plasmid system for gene knockout represents a step in developing tools for microbial engineering. This study focused on developing and constructing a plasmid system to enable targeted genetic modifications, particularly for enhancing *L-tryptophan* production in *Lactobacillus* species. While the system was not implemented experimentally, the methodological insights contribute to advancing molecular cloning strategies.

Previous studies have demonstrated the utility of CRISPR/Cas9 systems in engineering *Escherichia coli* for enhanced amino acid production, such as tryptophan (Wang *et al*., 2019; Bongaerts *et al*., 2001). However, applications in *Lactobacillus* species remain underexplored, primarily due to the challenges associated with low transformation efficiencies and the rigid cell walls of these bacteria (Mu et al., 2022). This study addressed these challenges by applying optimized electroporation protocols (Fristot *et al.,* 2023) and employing plasmid designs tailored for *L. paracasei*, thereby providing a replicable framework for similar research in other probiotic strains. The transformation efficiency observed in this study revealed a limitation when co-transforming larger plasmids (e.g., pLcasei_AB1 and pLcasei_AB2) with a smaller helper plasmid (pLH01). The preferential uptake of pLH01 likely stemmed from its smaller size and higher concentration, corroborating earlier observations that plasmid size significantly impacts transformation success (O'Toole *et al.*, 2017). These results underscore the need for careful optimization of plasmid ratios in co-transformation experiments, particularly when employing systems reliant on multiple plasmids.

While earlier research on *Lactobacillus plantarum* demonstrated enhanced tryptophan biosynthesis through native metabolic pathways (Jeong et al., 2021), this study's focus on targeted gene knockout adds a novel dimension. The deletion of the *ackA* gene, a critical component of the acetate kinase pathway, is hypothesized to redirect metabolic flux toward tryptophan biosynthesis. This approach leverages metabolic engineering principles previously validated in *E. coli* (Wang et al., 2019) but adapts them for the unique physiological context of *L. paracasei*.

Despite these advancements, the low transformation efficiency of larger plasmids remains a significant limitation. Future studies should explore strategies to improve transformation rates, such as optimizing electroporation parameters further or employing

alternative delivery methods like conjugation. Additionally, while this study focused on the *ackA* gene, the metabolic landscape of *L. paracasei* is complex, and additional targets may yield synergistic effects on tryptophan production. Finally, the potential applications of this engineered strain in therapeutic contexts warrant further exploration. Enhanced tryptophan production could support gut-brain axis modulation, offering new avenues for addressing disorders such as depression or irritable bowel syndrome (Carabotti et al., 2015; Foster & Neufeld, 2013). Future studies should validate these strains in in vivo models to establish their efficacy and safety.

CONCLUSION

This study investigated key steps towards developing a CRISPR/Cas9-based plasmid system to enhance L-tryptophan production with the focus on *Lacticaseibacillus paracasei*. The work focused on constructing an engineering plasmid while evaluating the genetic diversity and tryptophan production capabilities of various bacterial strains. The genetic characterization of isolated *L. plantarum* and *L. paracasei* strains revealed a high degree of diversity, offering valuable insights into their metabolic potential. Among the strains tested, significant differences in L-tryptophan production levels were observed, highlighting candidates for genetic modification. These findings emphasize the importance of strain-specific approaches in probiotic optimization.

The primary goal of designing and assembling a CRISPR/Cas9-based engineering plasmid was achieved in *pLcasei_AB1* construct, with evidence pointing to successful incorporation of homologous arms and guide RNA sequences. Despite challenges, such as low plasmid yield during purification and limited colony recovery post-transformation, restriction digestion and gel electrophoresis results confirmed the successful assembly of the engineered plasmid *pLcasei_AB1*. Recommendations for optimizing DNA purification methods and validation steps, such as employment of PCR purification kits and implementing standard PCR for ligation verification, as well as adjusting protocols for *Lactobacillus* transformation, were proposed to improve future workflows.

By successfully integrating advanced genome editing techniques with metabolic pathway optimization, it paves the way for the development of next-generation probiotics with enhanced therapeutic potential. The findings emphasize the importance of continued innovation in probiotic engineering to address both fundamental and applied challenges in microbial biotechnology.

VILNIUS UNIVERSITY

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Master's thesis

Development of CRISPR/Cas9-based Plasmid System for Targeted Gene Knockout to Enhance L-tryptophan Production in *Lactobacillus* Bacteria

ABSTRACT

Probiotics have emerged as vital components in promoting health and preventing disease, particularly through their capacity to modulate the gut microbiota and produce bioactive compounds. Among these, tryptophan, a precursor to serotonin, plays a critical role in the gut-brain axis, influencing mood regulation, sleep, and overall mental health. Recent evidence highlights the potential of probiotic strains, such as *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei*, to enhance tryptophan biosynthesis, offering therapeutic promise for conditions like depression, anxiety, and gastrointestinal disorders. However, natural limitations in bacterial metabolic pathways often restrict the production of such beneficial compounds. With the development of molecular engineering tools, such as the CRISPR-Cas9 system, the capacity to accurately alter bacterial genomes has been transformed. This research leverages CRISPR-Cas9 technology to construct an innovative plasmid system aimed at knocking out competing metabolic pathways in *Lactobacillus* strains, thereby enhancing tryptophan production. The study focuses on designing and assembling an engineered system to optimize the biosynthetic capabilities of these probiotic strains, addressing a significant gap in the field of microbial therapeutics.

VILNIAUS UNIVERSITETAS

GYVYBĖS MOKSLŲ CENTRAS

Ugnė Ledovskojūtė

Magistro baigiamasis darbas

CRISPR/Cas9 pagrįstos plazmidžių sistemos kūrimas tiksliniam genų išmušimui, siekiant padidinti L-triptofano gamybą *Lactobacillus* bakterijose

SANTRAUKA

Probiotikai tapo itin svarbiais sveikatos stiprinimo ir ligų prevencijos komponentais, ypač dėl jų gebėjimo reguliuoti žarnyno mikrobiotą ir gaminti biologiškai aktyvius junginius. Iš jų triptofanas, serotonino pirmtakas, atlieka svarbų vaidmenį žarnyno ir smegenų ašyje, daro įtaką nuotaikos reguliavimui, miegui ir bendrai psichikos sveikatai. Naujausi duomenys rodo, kad probiotinės padermės, pavyzdžiui, *Lactiplantibacillus plantarum* ir *Lacticaseibacillus paracasei*, gali sustiprinti triptofano biosintezę, o tai teikia terapinių vilčių tokių ligų kaip depresijos, nerimo ir virškinimo trakto sutrikimų gydymui. Tačiau natyvūs bakterijų medžiagų apykaitos keliai dažnai riboja tokių naudingų junginių gamybą dėl skirtingo išteklių pasiskirstymo. Visgi, molekulinės inžinerijos priemonių atradimas, pavyzdžiui, CRISPR-Cas9 sistema, atvėrė kelius tikslingam bakterijų genomų redagavimui. Šiame tyrime CRISPR-Cas9 įrankis buvo pritaikytas siekiant sukurti plazmidžių sistemą, kurią pritaikius būtų pažeistos su triptofano sinteze konkuruojančių genų struktūros. *Lactobacillus* padermių medžiagų apykaitos kelius ir taip padidinti triptofano gamybą. Tyrime dėmesys skiriamas inžinerinės sistemos, skirtos šių probiotinių padermių biosintezės gebėjimams optimizuoti, kūrimui ir surinkimui.

REFERENCES

1. Abouelela, M. E., & Helmy, Y. A. (2024). Next-Generation Probiotics as Novel Therapeutics for Improving Human Health: Current Trends and Future Perspectives. *Microorganisms*, *12*(3), 430. https://doi.org/10.3390/microorganisms12030430
2. Al Bander, Z., Nitert, M. D., Mousa, A., & Naderpoor, N. (2020). The Gut Microbiota and Inflammation: An Overview. *International journal of environmental research and public health*, *17*(20), 7618. https://doi.org/10.3390/ijerph17207618
3. Al-Fakhrany, O. M., & Elekhnawy, E. (2024). Next-generation probiotics: the upcoming biotherapeutics. *Molecular biology reports*, *51*(1), 505. https://doi.org/10.1007/s11033-024-09398-5
4. Anders, C., Niewoehner, O., Duerst, A., & Jinek, M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature, 513(7519), 569–573. https://doi.org/10.1038/nature13579
5. Aponte, M., Murru, N., & Shoukat, M. (2020). Therapeutic, Prophylactic, and Functional Use of Probiotics: A Current Perspective. *Frontiers in microbiology*, *11*, 562048. https://doi.org/10.3389/fmicb.2020.562048
6. Azad, M. A. K., Sarker, M., Li, T., & Yin, J. (2018). Probiotic Species in the Modulation of Gut Microbiota: An Overview. *BioMed research international*, *2018*, 9478630. https://doi.org/10.1155/2018/9478630
7. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science, 315(5819), 1709–1712. https://doi.org/10.1126/science.1138140
8. Berger, M., Gray, J. A., & Roth, B. L. (2009). The expanded biology of serotonin. *Annual review of medicine*, *60*, 355–366. https://doi.org/10.1146/annurev.med.60.042307.110802
9. Best, J., Nijhout, H. F., & Reed, M. (2010). Serotonin synthesis, release and reuptake in terminals: a mathematical model. *Theoretical biology & medical modelling*, *7*, 34. https://doi.org/10.1186/1742-4682-7-34

10. Blier, P., & El Mansari, M. (2013). Serotonin and beyond: therapeutics for major depression. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *368*(1615), 20120536. https://doi.org/10.1098/rstb.2012.0536
11. Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., ... & Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. Science, 326(5959), 1509–1512. https://doi.org/10.1126/science.1178811
12. Bombardi, C., Grandis, A., Pivac, N., Sagud, M., Lucas, G., Chagraoui, A., Lemaire-Mayo, V., De Deurwaerdère, P., & Di Giovanni, G. (2021). Serotonin modulation of hippocampal functions: From anatomy to neurotherapeutics. *Progress in brain research*, *261*, 83–158. https://doi.org/10.1016/bs.pbr.2021.01.031
13. Bongaerts, J., Kramer, M., Müller, U., Raeven, L., & Wubbolts, M. (2001). Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metabolic Engineering, 3(4), 289–300. https://doi.org/10.1006/mben.2001.0201
14. Campbell, Jarryd & Campbell, Katherine & Nelson, Timothy & Xu, Xiaolei & Ekker, Stephen. (2013). New and TALENted genome engineering toolbox. Circulation research. 113. 571-87. 10.1161/CIRCRESAHA.113.301765.
15. Canani, R. B., Costanzo, M. D., Leone, L., Pedata, M., Meli, R., & Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World Journal of Gastroenterology, 17(12), 1519–1528. https://doi.org/10.3748/wjg.v17.i12.1519
16. Capuco, A., Urits, I., Hasoon, J., Chun, R., Gerald, B., Wang, J. K., Ngo, A. L., Simopoulos, T., Kaye, A. D., Colontonio, M. M., Parker-Actlis, T. Q., Fuller, M. C., & Viswanath, O. (2020). Gut Microbiome Dysbiosis and Depression: a Comprehensive Review. *Current pain and headache reports*, *24*(7), 36. https://doi.org/10.1007/s11916-020-00871-x (Retraction published Curr Pain Headache Rep. 2023 May;27(5):129)
17. Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of gastroenterology*, *28*(2), 203–209.
18. Chen, Y. S., Jan, R. L., Lin, Y. L., Chen, H. H., & Wang, J. Y. (2010). Randomized placebo-controlled trial of lactobacillus on asthmatic children with allergic rhinitis. *Pediatric pulmonology*, *45*(11), 1111–1120. https://doi.org/10.1002/ppul.21296

19. Chen, Y., Xu, J., & Chen, Y. (2021). Regulation of Neurotransmitters by the Gut Microbiota and Effects on Cognition in Neurological Disorders. *Nutrients*, *13*(6), 2099. https://doi.org/10.3390/nu13062099
20. Cryan, J. F., O'Riordan, K. J., Cowan, C. S., Sandhu, K. V., Bastiaanssen, T. F., Boehme, M., et al. (2019). The microbiota-gut-brain axis. Physiological Reviews, 99(4), 1877–2013.
21. Cui, Y., Wang, M., Zheng, Y., Miao, K., & Qu, X. (2021). The Carbohydrate Metabolism of Lactiplantibacillus plantarum. International journal of molecular sciences, 22(24), 13452. https://doi.org/10.3390/ijms222413452
22. Dahiya, D., & Nigam, P. S. (2023). Biotherapy Using Probiotics as Therapeutic Agents to Restore the Gut Microbiota to Relieve Gastrointestinal Tract Inflammation, IBD, IBS and Prevent Induction of Cancer. *International Journal of Molecular Sciences*, *24*(6), 5748. https://doi.org/10.3390/ijms24065748
23. De Filippo, C., Cavalieri, D., Di Paola, M., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences, 107(33), 14691–14696.
24. Dominguez-Bello, M. G., Costello, E. K., Contreras, M., et al. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences, 107(26), 11971–11975.
25. Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science, 346*(6213), 1258096. https://doi.org/10.1126/science.1258096
26. Fekete, M., Lehoczki, A., Major, D., Fazekas-Pongor, V., Csípő, T., Tarantini, S., Csizmadia, Z., & Varga, J. T. (2024). Exploring the Influence of Gut–Brain Axis Modulation on Cognitive Health: A Comprehensive Review of Prebiotics, Probiotics, and Symbiotics. *Nutrients*, *16*(6), 789. https://doi.org/10.3390/nu16060789
27. Foster, J. A., & Neufeld, K. A. (2013). Gut–brain axis: How the microbiome influences anxiety and depression. Trends in Neurosciences, 36(5), 305–312.
28. Fristot E, Bessede T, Camacho Rufino M, Mayonove P, Chang H-J, Zuniga A, et al. (2023) An optimized electrotransformation protocol for *Lactobacillus jensenii*. PLoS ONE 18(2): e0280935. https://doi.org/10.1371/journal.pone.0280935
29. Gupta, R. M., & Musunuru, K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *The Journal of clinical investigation*, *124*(10), 4154–4161. https://doi.org/10.1172/JCI72992

30. Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology, 11(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66
31. Huang, H., Song, X., & Yang, S. (2019). Development of a RecE/T-Assisted CRISPR-Cas9 Toolbox for Lactobacillus. *Biotechnology journal*, *14*(7), e1800690. https://doi.org/10.1002/biot.201800690
32. Huang, H., Zheng, G., Jiang, W., Hu, H., & Lu, Y. (2019). One-step high-efficiency CRISPR/Cas9-mediated genome editing in *Lactobacillus plantarum*. Frontiers in Microbiology, 10, 294.
33. Irum N, Afzal T, Faraz MH, Aslam Z and Rasheed F (2023) The role of gut microbiota in depression: an analysis of the gut-brain axis. *Front. Behav. Neurosci.* 17:1185522. doi: 10.3389/fnbeh.2023.1185522
34. Isalan, M. Zinc-finger nucleases: how to play two good hands. *Nat Methods* 9, 32–34 (2012). https://doi.org/10.1038/nmeth.1805
35. FAO/WHO. (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Food and Agriculture Organization of the United Nations/World Health Organization.
36. Fels, U., Gevaert, K., & Van Damme, P. (2020). Bacterial genetic engineering by homologous recombination. Molecular Biology Reports, 47(7), 5497–5510. https://doi.org/10.1007/s11033-020-05512-7
37. Foster, J. A., & Neufeld, K.-A. M. (2013). Gut-brain axis: How the microbiome influences anxiety and depression. *Trends in Neurosciences, 36*(5), 305–312. https://doi.org/10.1016/j.tins.2013.01.005
38. Friedman M. (2018). Analysis, Nutrition, and Health Benefits of Tryptophan. *International journal of tryptophan research : IJTR*, *11*, 1178646918802282. https://doi.org/10.1177/1178646918802282
39. Jandhyala, S. M., Talukdar, R., Subramanyam, C., & Reddy, D. N. (2015). Role of the normal gut microbiota. World Journal of Gastroenterology, 21(29), 8787–8803.
40. Jeong, M., Park, Y., Kim, H., & Yoon, H. (2021). Probiotic Lactobacillus plantarum strains with potential role in alleviating metabolic syndrome via producing tryptophan metabolites. Journal of Functional Foods, 79, 104387. https://doi.org/10.1016/j.jff.2021.104387

41. Jiang, M., Chen, M., Guo, Z., Wang, Z., & Zhang, H. (2016). Metabolic engineering of Escherichia coli for L-tryptophan production by manipulating global regulators. World Journal of Microbiology and Biotechnology, 32(5), 82. https://doi.org/10.1007/s11274-016-2025-4
42. Jiang, H., Ling, Z., Zhang, Y., et al. (2015). Altered fecal microbiota composition in patients with major depressive disorder. Brain, Behavior, and Immunity, 48, 186–194.
43. Jiang, W., Bikard, D., Cox, D., Zhang, F., & Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature Biotechnology, 31(3), 233–239. https://doi.org/10.1038/nbt.2508
44. Jeong, H., Park, J., Kim, Y. H., & Choi, S.-K. (2021). Enhancing tryptophan biosynthesis in *Lactobacillus plantarum* using metabolic engineering. *Microbial Cell Factories, 20*(1), 123. https://doi.org/10.1186/s12934-021-01604-3
45. Jeong, J., Lee, Y., Yoon, S., Kim, J. H., & Kim, W. (2021). *Lactiplantibacillus plantarum* LRCC5314 includes a gene for serotonin biosynthesis via the tryptophan metabolic pathway. *Journal of microbiology (Seoul, Korea)*, *59*(12), 1092–1103. https://doi.org/10.1007/s12275-021-1472-2
46. Jenkins, T. A., Nguyen, J. C., Polglaze, K. E., & Bertrand, P. P. (2016). Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis. *Nutrients*, *8*(1), 56. https://doi.org/10.3390/nu8010056
47. Johnson, D., Thurairajasingam, S., Letchumanan, V., Chan, K. G., & Lee, L. H. (2021). Exploring the Role and Potential of Probiotics in the Field of Mental Health: Major Depressive Disorder. *Nutrients*, *13*(5), 1728. https://doi.org/10.3390/nu13051728
48. Kagan, Juliana & Sharon, Itai & Beja, Oded & Kuhn, Jonathan. (2008). The tryptophan pathway genes of the Sargasso Sea metagenome: New operon structures and the prevalence of non-operon organization. Genome biology. 9. R20. 10.1186/gb-2008-9-1-r20.
49. Kelly, C. R., Yen, E. F., Grinspan, A. M., Kahn, S. A., Atreja, A., Lewis, J. D., Moore, T. A., Rubin, D. T., Kim, A. M., Serra, S., Nersesova, Y., Fredell, L., Hunsicker, D., McDonald, D., Knight, R., Allegretti, J. R., Pekow, J., Absah, I., Hsu, R., Vincent, J., … Laine, L. (2021). Fecal Microbiota Transplantation Is Highly Effective in Real-World Practice: Initial Results From the FMT National Registry. *Gastroenterology*, *160*(1), 183–192.e3. https://doi.org/10.1053/j.gastro.2020.09.038
50. Kiousi, D. E., Efstathiou, C., Tegopoulos, K., Mantzourani, I., Alexopoulos, A., Plessas, S., Kolovos, P., Koffa, M., & Galanis, A. (2022). Genomic Insight Into

Lacticaseibacillus paracasei SP5, Reveals Genes and Gene Clusters of Probiotic Interest and Biotechnological Potential. *Frontiers in microbiology*, *13*, 922689. https://doi.org/10.3389/fmicb.2022.922689

51. Kim, Y. G., Cha, J., & Chandrasegaran, S. (1996). Hybrid restriction enzymes: Zinc finger fusions to FokI cleavage domain. Proceedings of the National Academy of Sciences, 93(3), 1156–1160. https://doi.org/10.1073/pnas.93.3.1156
52. Kiro, R., Shitrit, D., & Qimron, U. (2014). Efficient engineering of a bacterial genome using the CRISPR/Cas9 system. Bioengineering, 5(4), 230–235. https://doi.org/10.1089/ind.2014.0035
53. Kumar, A., Pramanik, J., Goyal, N., Chauhan, D., Sivamaruthi, B. S., Prajapati, B. G., & Chaiyasut, C. (2023). Gut Microbiota in Anxiety and Depression: Unveiling the Relationships and Management Options. *Pharmaceuticals (Basel, Switzerland)*, *16*(4), 565. https://doi.org/10.3390/ph16040565
54. Kwok, K. O., Fries, L. R., Silva-Zolezzi, I., Thakkar, S. K., Iroz, A., & Blanchard, C. (2022). Effects of Probiotic Intervention on Markers of Inflammation and Health Outcomes in Women of Reproductive Age and Their Children. *Frontiers in nutrition*, *9*, 889040. https://doi.org/10.3389/fnut.2022.889040
55. Liang, S., Wang, T., Hu, X., Luo, J., Li, W., Wu, X., Duan, Y., & Jin, F. (2015). Administration of Lactobacillus helveticus NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. *Neuroscience*, *310*, 561–577. https://doi.org/10.1016/j.neuroscience.2015.09.033
56. Lin, S. H., Lee, L. T., & Yang, Y. K. (2014). Serotonin and mental disorders: a concise review on molecular neuroimaging evidence. *Clinical psychopharmacology and neuroscience : the official scientific journal of the Korean College of Neuropsychopharmacology*, *12*(3), 196–202. https://doi.org/10.9758/cpn.2014.12.3.196
57. Lott J. S. (2020). The tryptophan biosynthetic pathway is essential for Mycobacterium tuberculosis to cause disease. *Biochemical Society transactions*, *48*(5), 2029–2037. https://doi.org/10.1042/BST20200194
58. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230. https://doi.org/10.1038/nature11550
59. Luqman, A., Nega, M., Nguyen, M. T., Ebner, P., & Götz, F. (2018). SadA-Expressing Staphylococci in the Human Gut Show Increased Cell Adherence and Internalization. *Cell reports*, *22*(2), 535–545. https://doi.org/10.1016/j.celrep.2017.12.058

60. Ma, J., Lyu, Y., Liu, X. *et al.* Engineered probiotics. *Microb Cell Fact* 21, 72 (2022). https://doi.org/10.1186/s12934-022-01799-0

61. Mallott, E. K., Sitarik, A. R., Leve, L. D., Cioffi, C., Camargo, C. A., Jr, Hasegawa, K., & Bordenstein, S. R. (2023). Human microbiome variation associated with race and ethnicity emerges as early as 3 months of age. *PLoS biology*, *21*(8), e3002230. https://doi.org/10.1371/journal.pbio.3002230

62. Marraffini, L. A., & Sontheimer, E. J. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nature Reviews Genetics, 11(3), 181–190. https://doi.org/10.1038/nrg2749

63. Monteleone, A. M., Troiani, M. R., & Cascino, G. (2021). The interaction between inflammation and eating disorders: A narrative review. Nutrients, 13(12), 4114.

64. Montgomery, T. L., Eckstrom, K., Lile, K. H., Caldwell, S., Heney, E. R., Lahue, K. G., D'Alessandro, A., Wargo, M. J., & Krementsov, D. N. (2022). Lactobacillus reuteri tryptophan metabolism promotes host susceptibility to CNS autoimmunity. *Microbiome*, *10*(1), 198. https://doi.org/10.1186/s40168-022-01408-7

65. Mu, D., Zhou, Y., Ji, J., Xie, J., & Zhu, Y. (2022). Recent advances in genetic tools for *Lactobacillus* species: Molecular toolbox and its applications. *Frontiers in Microbiology, 13*, 854497. https://doi.org/10.3389/fmicb.2022.854497

66. Mu, L., Feng, Y., Yu, S., Zhang, H., & Chen, W. (2022). Genome editing tools in *Lactobacillus*: State-of-the-art and future perspectives. Applied Microbiology and Biotechnology, 106, 7415–7433. https://doi.org/10.1007/s00253-022-12225-3

67. Murphy, E. A., Velazquez, K. T., & Herbert, K. M. (2015). Influence of high-fat diet on gut microbiota: A driving force for chronic disease risk. Current Opinion in Clinical Nutrition & Metabolic Care, 18(5), 515–520.

68. Nguyen, T. T., Kosciolek, T., Eyler, L. T., Knight, R., & Jeste, D. V. (2018). Overview and systematic review of studies of microbiome in schizophrenia and bipolar disorder. *Journal of psychiatric research*, *99*, 50–61. https://doi.org/10.1016/j.jpsychires.2018.01.013

69. O'Toole, G. A., Kaplan, H. B., & Kolter, R. (2017). Biofilm formation as microbial development. *Annual Review of Microbiology, 54*(1), 49–79. https://doi.org/10.1146/annurev.micro.54.1.49

70. O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: The spectrum from probiotics to live biotherapeutics. Nature Microbiology, 2, 17057.

71. Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotic and other functional microbes: From markets to mechanisms. Current Opinion in Biotechnology, 13(5), 483–487.
72. Parker E.J. (2017) The biosynthesis of tryptophan In *The Handbook of Microbial Metabolism of Amino Acids* and (D'Mello J.P.F., ed.), pp. 256–266, CAB International; ISBN-13: 978 1 78064 723 4
73. Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2018). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell, 152(5), 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022
74. Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *International journal of tryptophan research : IJTR*, *2*, 45–60. https://doi.org/10.4137/ijtr.s2129
75. Romijn, A. R., & Rucklidge, J. J. (2015). Systematic review of evidence to support the theory of psychobiotics. Nutrition Reviews, 73(10), 675–693.
76. Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., & Burnet, P. W. J. (2016). Psychobiotics and the Manipulation of Bacteria-Gut-Brain Signals. *Trends in neurosciences*, *39*(11), 763–781. https://doi.org/10.1016/j.tins.2016.09.002
77. Shvartsman, E., Richmond, M. E. I., Schellenberg, J. J., Lamont, A., Perciani, C., Russell, J. N. H., Poliquin, V., Burgener, A., Jaoko, W., Sandstrom, P., & MacDonald, K. S. (2022). Comparative analysis of DNA extraction and PCR product purification methods for cervicovaginal microbiome analysis using cpn60 microbial profiling. *PloS one*, *17*(1), e0262355. https://doi.org/10.1371/journal.pone.0262355
78. Simpson, Sierra & Mclellan, Rio & Wellmeyer, Emma & Matalon, Frederic & George, Olivier. (2021). Drugs and Bugs: The Gut-Brain Axis and Substance Use Disorders. Journal of Neuroimmune Pharmacology. 17. 1-29. 10.1007/s11481-021-10022-7.
79. Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J., & Lewis, K. (2019). GABA-modulating bacteria of the human gut microbiota. *Nature microbiology*, *4*(3), 396–403. https://doi.org/10.1038/s41564-018-0307-3

80. Urnov, F., Miller, J., Lee, YL. *et al.* Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435, 646–651 (2005). https://doi.org/10.1038/nature03556
81. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. *Nature reviews. Genetics*, *11*(9), 636–646. https://doi.org/10.1038/nrg2842
82. zinc-finger nucleases. Nature, 435(7042), 646–651. https://doi.org/10.1038/nature03556
83. van Nood, E., Vrieze, A., Nieuwdorp, M., et al. (2013). Duodenal infusion of donor feces for recurrent *Clostridium difficile*. New England Journal of Medicine, 368(5), 407–415.
84. Vighi, G., Marcucci, F., Sensi, L., Di Cara, G., & Frati, F. (2008). Allergy and the gastrointestinal system. Clinical & Experimental Immunology, 153(S1), 3–6.
85. Wang, J., Zheng, P., Tang, W., Wang, Y., & Zou, J. (2019). Rational metabolic engineering of *E. coli* for enhanced tryptophan production. *Biotechnology and Bioengineering, 116*(3), 542–552. https://doi.org/10.1002/bit.26881
86. Wang, W., Liu, J., Shen, X., Wang, Z., Zhang, H., & Chen, T. (2019). Metabolic engineering of Escherichia coli for the production of L-tryptophan. Synthetic and Systems Biotechnology, 4(2), 71–79. https://doi.org/10.1016/j.synbio.2019.03.001
87. Wei, C. L., Wang, S., Yen, J. T., Cheng, Y. F., Liao, C. L., Hsu, C. C., Wu, C. C., & Tsai, Y. C. (2019). Antidepressant-like activities of live and heat-killed Lactobacillus paracasei PS23 in chronic corticosterone-treated mice and possible mechanisms. *Brain research*, *1711*, 202–213. https://doi.org/10.1016/j.brainres.2019.01.025
88. Williams, B. B., Van Benschoten, A. H., Cimermancic, P., Donia, M. S., Zimmermann, M., Taketani, M., Ishihara, A., Kashyap, P. C., Fraser, J. S., & Fischbach, M. A. (2014). Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell host & microbe*, *16*(4), 495–503. https://doi.org/10.1016/j.chom.2014.09.001
89. Xu, L., Han, F., Dong, Z., & Wei, Z. (2020). Engineering Improves Enzymatic Synthesis of L-Tryptophan by Tryptophan Synthase from Escherichia coli. Microorganisms, 8(4), 519. https://doi.org/10.3390/microorganisms8040519
90. Xue, C., & Greene, E. C. (2021). DNA Repair Pathway Choices in CRISPR-Cas9-Mediated Genome Editing. *Trends in genetics : TIG*, *37*(7), 639–656. https://doi.org/10.1016/j.tig.2021.02.008
91. Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K., & Hsiao, E. Y. (2015). Indigenous bacteria from the

gut microbiota regulate host serotonin biosynthesis. *Cell*, *161*(2), 264–276. https://doi.org/10.1016/j.cell.2015.02.047

92. Yatsunenko, T., Rey, F. E., Manary, M. J., et al. (2012). Human gut microbiome viewed across age and geography. Nature, 486(7402), 222–227.

93. Zhou, J., Zhu, X., Cai, Z., et al. (2020). Metabolic engineering of *Escherichia coli* for high-efficiency production of *L-tryptophan*. Applied Microbiology and Biotechnology, 104, 4055–4065. https://doi.org/10.1007/s00253-020-10543-2

94. Zuo, Z., & Marcotte, E. M. (2021). Engineering probiotics with CRISPR-Cas9 for enhanced metabolic output. Nature Communications, 12, 3924. https://doi.org/10.1038/s41467-021-24295-y