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Weissella paramesenteroides **UFTM 2.6.1:** Probiotic Potential and Biotechnological Applications

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Beatriz Macedo de Oliveira Rocha

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I dedicate this work to the most beautiful soul that has ever graced this Universe. This achievement is ours, Mom.

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This thesis marks the end of a long journey filled with extensive study, dedication, courage, perseverance, and consistency. I have been fortunate to share this path with remarkable people who inspired me to get here, and I am grateful to every single one of them. In this dedication, I wish to mention a few key figures who have been essential not only to this achievement but to my entire story.

Over the years, the greatest wealth I have accumulated is the love I have cultivated, especially the wonderful friendships that accompany me wherever I go, and since "happiness is only real when shared," I extend my deepest gratitude to each and every one of you. Family bonds are always so significant, but some are particularly memorable for their wisdom and care, such as my dearly missed grandfather João F. de Oliveira Neto. I will never forget all the encouragement to pursue my dreams, no matter how great the challenges I faced along the way, and I will always remember the prayer he taught me in the sauna: “Grant me the strength to change what can be changed, the serenity to accept what cannot be changed, and the wisdom to distinguish one from the other”. I hope I am making you very proud. I also dedicate this work to the person who shares the emptiness left in our hearts, my sister Carolina Macedo de Oliveira Rocha, who, as the eldest, has always been my reference, especially for her focus, intelligence, and determination, as well as being my great companion in everything I set out to do. Talking about "Beatriz" means understanding her origins, and despite our differences, we are very much alike, Dad. Pedro Manoel A. da Rocha was a human being with whom I often struggled to understand the complexity of our relationship, but his honesty, meticulous attention to detail, and serenity were qualities I have always clearly recognized. I am grateful for having had the example of so many virtues that I carry within my essence today.

Expressing in words the overwhelming feeling I have for the most important person in my life, to whom I dedicate all my achievements, including this work, I make this paragraph entirely yours, Mom. It took me several days to complete this tribute because, among the typed words, I had to wipe away tears, relive precious memories, and decide where to place the final period, as I could write an entire thesis about you. I had the privilege of sharing 23 years of my life with the most incredible, generous, and brave human being that will ever have inhabited this vast cosmos. Saying the name Martha Valéria Macedo de Oliveira Rocha emits the most sublime sound and touches my soul with the greatest love I feel, which expands over the years. My mother dedicated her entire life to her "girls," and the least I can do is repay her by living a

life of resilience and gratitude. I conclude this paragraph by saying that there is no greater love in the world than what I feel for you.

I am infinitely grateful to the Universe for all the opportunities I have been given so far, as well as for my health which allows me to achieve my goals. I am proud to have come this far. I would like to express my deep gratitude to the Federal University of Juiz de Fora (UFJF) for providing the resources and environment necessary to carry out this research. My sincere thanks also go to my advisor, Prof.ª Dr.ª Alessandra B. F. Machado, for her guidance, and to the examining committee for their dedication and essential contributions, which were fundamental for the improvement of this work. The opportunity to complete this important stage of my academic training would not have been possible without the support and trust of all of you.

Science is not only compatible with spirituality; it is a profound source of spirituality. When we recognize our place in an immensity of light-years and in the passage of ages, when we grasp the intricacy, beauty, and subtlety of life, then that soaring feeling, that sense of elation and humility combined, is surely spiritual. So are our emotions in the presence of great art or music or literature, or acts of exemplary selfless courage such as those of Mohandas Gandhi or Martin Luther King Jr. The notion that science and spirituality are somehow mutually exclusive does a disservice to both (SAGAN, 1997).

ABSTRACT

Lactic Acid Bacteria (LAB) constitute a diverse group of Gram-positive bacteria known for their production of lactic acid during carbohydrate fermentation. Among them, *Weissella* species have emerged as notable candidates for applications in the food and pharmaceutical industries due to their probiotic, biotechnological, and bacteriocinogenic potential. This study presents a comprehensive probiogenomic analysis of *Weissella paramesenteroides* UFTM 2.6.1 isolated from unpasteurized cow's milk from the Triângulo Mineiro region, exploring its probiotic potential through *in vitro* and *in silico* analyses and investigating biotechnological prospects. Taxonomic characterization confirmed the identity of *W. paramesenteroides*, revealing a genome with 1,926 protein-coding genes primarily linked to cell metabolism, information storage and processing, and cellular processes and signaling. The genome also harbors over 100 genes associated with probiotic traits, such as stress response, gastrointestinal persistence, and biosynthesis of vitamins. *In silico* analysis identified bacteriocin-related genes, including pediocin, and confirmed *in vitro* antimicrobial activity of *W. paramesenteroides* UFTM 2.6.1 against *Listeria* spp. Genomic analysis identified pLCK4 replicon and four prophage regions (one intact), without CRISPR-Cas systems, Cas proteins, resistance, or virulence genes, suggesting the strain's safety profile. Pan-genome analysis revealed 204 unique genes in *W. paramesenteroides* UFTM 2.6.1, encompassing metabolism and stress-related functions. These findings highlight its probiotic potential. To investigate relevant literature data on the use of LAB in vaccine strategies, we developed and published an integrative review on recombinant LAB for vaccine development, highlighting their efficacy as potent vectors for mucosal and systemic immunization in animal models. Given this biotechnological potential of LAB, we conducted plasmid design and selection for bacterial integration, resulting in the efficient cloning of the Receptor Binding Domain (RBD) gene of SARS-CoV-2 in *Escherichia coli*, demonstrating significant synthesis in both supernatant and cell lysate, and recognition by human antibodies. Additionally, we designed a plasmid optimized for LAB, incorporating cassettes for expressing the SARS-CoV-2 RBD gene and IS1223 insertion sequence. This design also features *cel*A as a non-toxic screening marker, avoiding antibiotic resistance concerns. These were preliminary assays that provide insights for future research into LAB, particularly *W. paramesenteroides* UFTM 2.6.1, requiring further validation to assess its potential as a viable vaccine platform and confirm its probiotic characteristics.

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LIST OF ABBREVIATIONS AND ACRONYMS

Amp	Ampicillin
ACE2	Angiotensin-Converting Enzyme 2
ANI	Average Nucleotide Identity
ARG	Antibiotic Resistance Genes
B2	Riboflavin
B12	Cobalamin
BALF	Bronchoalveolar Lavage Fluid
BSH	Bile Salt Hydrolase
CDS	Coding Sequence
CFU	Colony Forming Units
COVID-19	Coronavirus Disease
CoVs	Coronaviruses
CRISPRs	Clustered Regularly Interspaced Short Palindromic Repeats
Da	Dalton
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ENA	European Nucleotide Archive
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization
FDA	American Food and Drug Administration
GC	Guanine-Cytosine
GIT	Gastrointestinal Tract
GRAS	Generally Recognized as Safe
HCoVs	Human Coronaviruses
HMP	Human Microbiome Project
HPV	Human Papilloma Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-2	Interleucin-2
IL-4	Interleucin-4
IL-10	Interleucin-10

IFN-γ	Interferon Gamma
IVDC	National Institute of Viral Disease Control and Prevention
LAB	Lactic Acid Bacteria
LB	Luria Bertani
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MGE	Mobile Genetic Elements
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information
NGS	Next-generation Sequencing
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance Spectroscopy
PAMPs	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
QPS	Qualified Presumption of Safety
RBD	Receptor Binding Domain
rDNA	Ribosomal DNA
RGI	Resistance Gene Identifier
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
lncRNA	Long non-coding RNA
miRNA	Micro RNA
mRNA	Messenger RNA
rRNA	Ribosomal RNA
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SEM	Standard Error of the Mean
SOC	Super Optimal Broth With Catabolite Repression
TE	Tris-HCl and EDTA
Tetra	Tetra-nucleotide
tRNA	Transfer Ribonucleic Acid
VG	Virulence genes
VOC	Variants of Concern
VOI	Variants of Interest

WGS	Whole-genome sequencing
WHO	World Health Organization

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1 INTRODUCTION

Lactic Acid Bacteria (LAB) are a large group of Gram-positive bacteria that produce lactic acid as the main end product of carbohydrate fermentation (CHO; YIM; SEO, 2020; DA SILVA *et al.*, 2021; ONWUAKOR *et al.*, 2014). Genera of LAB include *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Vagococcus*, *Weissella*, *Tetragenococcus*, and *Bifidobacterium* (REUBEN *et al.*, 2019). They play a crucial role in food fermentation and health, contributing to intestinal integrity, immunomodulation, and resistance to pathogens (DE FILIPPIS; PASOLLI; ERCOLINI, 2020; SORNPLANG; PIYADEATSOONTORN, 2016). Additionally, certain species within this group are acknowledged for their probiotic properties (ZHENG *et al.*, 2020).

Probiotic bacteria, known for their health-promoting effects, have long been incorporated into various food products as live components (SANDERS; HUIS IN'T VELD, 1999; VANGAY *et al.*, 2018). Yet, there is still an incomplete understanding of the exact impact of probiotics on the gastrointestinal tract (GIT) in humans. Research into probiotics underscores the importance of precise identification and comprehensive assessment of their functional properties, highlighting their potential in disease prevention and treatment (FAO *et al.*, 2002). In addition to their traditional use in food production and preservation, and as probiotics, LAB have also been explored as potential carriers of compounds with therapeutic or prophylactic effects, particularly in the development of mucosal vaccines (SZATRAJ; SZCZEPANKOWSKA; CHMIELEWSKA-JEZNACH, 2017). This approach aims to develop vaccines that are more efficient in terms of production time and costs, require reduced labor, and are easier to administer (WELLS; MERCENIER, 2008).

Understanding the strain-specific mechanisms of action in probiotic research involves exploring their unique genetic and metabolic characteristics. This approach is crucial because traditional tests alone may not reliably determine probiotic safety and efficacy, thereby complicating predictions about their functionality (GUARNER; MALAGELADA, 2003). Additionally, probiotics do not universally share essential attributes, instead, they can exhibit diverse mechanisms that contribute to specific clinical benefits (ACETI *et al.*, 2018).

Probiogenomics is an emerging research field that enhances our understanding of probiotics and could facilitate their application in food production and therapeutic contexts by employing high-throughput techniques to explore the genetic and molecular mechanisms

underlying their health benefits and utilizing phenotypic characteristics to identify their probiotic potential for further investigation *in silico* (ABRIOUEL *et al.*, 2017).

In this context, the present study conducted in-depth genomic investigations of *Weissella paramesenteroides* UFTM 2.6.1, a potentially probiotic bacterium isolated from unpasteurized cow's milk from the Triângulo Mineiro region. This research provided valuable insights into essential and specific genetic elements for probiosis, and the probiotic potential of this bacterium was assessed to propose its use in biotechnological applications. For this final purpose, a preliminary study was conducted on the development of vaccine strategies using recombinant LAB against COVID-19.

To provide a comprehensive overview, this work is systematically organized into chapters, each addressing a specific aspect of the research. The first chapter corresponds to the article titled "Unlocking Probiotic Potential: Genomic Insights into *Weissella paramesenteroides* UFTM 2.6.1," submitted to “Probiotics and Antimicrobial Proteins”. This chapter results from extensive research on the probiogenomics of *W. paramesenteroides* UFTM 2.6.1, which was the primary focus of the doctoral research. The second chapter is based on a book chapter published in 2021 titled "Recombinant Lactic Acid Bacteria: An Integrative Review," which discusses the use of LAB as recombinant bacterial vectors and provides key insights for the subsequent research detailed in Chapter 3, titled "Expression and Quantification of the SARS-CoV-2 Receptor Binding Domain (RBD) Protein by Recombinant Bacteria: Exploring Biotechnological Potential for Vaccine Vector Development". This chapter presents preliminary results on the development of vaccine strategies using LAB against COVID-19, concluding the study with a focus on biotechnological applications.

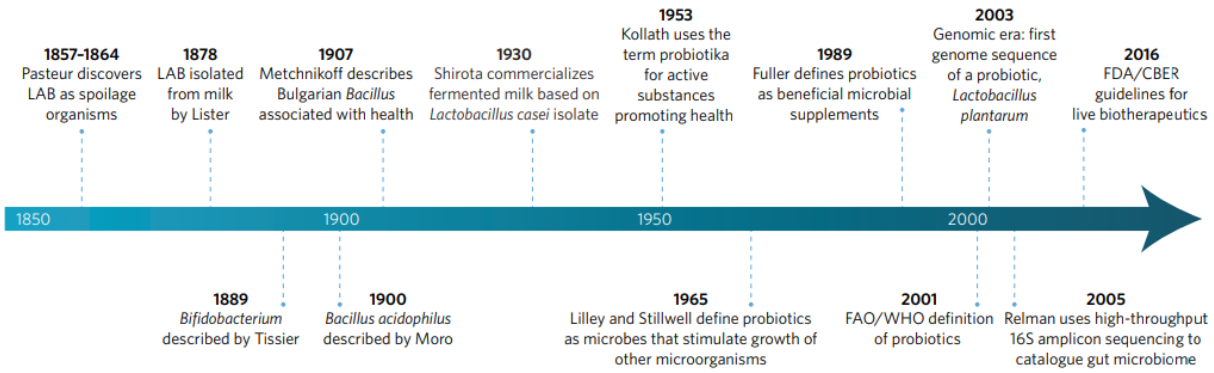
2 LITERATURE REVIEW

2.1 BENEFICIAL MICROORGANISMS: HISTORY AND HEALTH IMPACT

Over millennia, humans have used the fermentation process to enhance the sensory qualities of foods and prevent spoilage. This practice has driven scientists and the food and pharmaceutical industries to explore the beneficial effects of microorganisms on human health, with a focus on prophylactic and therapeutic applications (JOHNSON; KLAENHAMMER, 2014).

At the end of the 19th century, Louis Pasteur demonstrated that fermentation is carried out by microorganisms, establishing that their growth is not the result of spontaneous generation but biogenesis. Based on Pasteur's research, Elie Metchnikoff, a Nobel-winning Russian scientist and professor at the Pasteur Institute in Paris, postulated that consuming fermented foods offered health benefits leading to longevity. Supporting Metchnikoff, Michel Cohendy, a colleague at the Pasteur Institute, provided experimental data backing his hypothesis. In the early 20th century, the Pasteur Institute began selling *Lactobacillus* under the label "*Le Ferment*." However, in 1915, Leo Rettger showed that *L. bulgaricus* (a component of *Le Ferment*) does not survive gastric transit and proposed *L. acidophilus* as a suitable probiotic. Yet, due to technical and technological limitations of the time, *L. acidophilus* could not be distinguished from other aciduric lactobacilli. The growing evidence for probiotic bacteria prompted Japanese physician Minoru Shirota to isolate the *Lacticaseibacillus casei* Shirota species from human feces, which could survive gastrointestinal transit. Shirota developed and marketed Yakult®, one of the first fermented dairy products providing a pure and defined strain culture (JOHNSON; KLAENHAMMER, 2014). Since then, the interest in developing and commercializing fermented dairy products containing probiotic bacteria has grown exponentially (SANDERS; HUIS IN'T VELD, 1999). This market growth is correlated with technological advancements in sequencing, genomic analysis of probiotic bacteria, and the characterization of the human intestinal microbiome. Sequencing and bioinformatics tools have enabled a broader and deeper understanding of the diversity and functionality of the human microbiota (CULLEN *et al.*, 2020; HIDALGO-CANTABRANA *et al.*, 2017; O'CONNELL, 2020) (Figure 1).

Figure 1 – Timeline of selected milestones in the history of probiotics and next-generation probiotics.



Source: O'TOOLE; MARCHESI; HILL (2017).

Understanding the importance of the microbiome and the factors influencing the distribution and evolution of microorganisms in the human body led to the conception of the "Human Microbiome Project" (HMP), launched by the National Institutes of Health (NIH). This project aimed to characterize the human microbiome in detail and analyze its role in human health and disease (TURNBAUGH *et al.*, 2007). Currently, alterations in the human microbiome are associated with various diseases, potentially when a microbial community is deficient in beneficial functions or due to harmful microbial activity. For instance, alterations in the Firmicutes/Bacteroidetes ratio in the gut microbiome are associated with inflammatory bowel diseases like Crohn's disease, characterized by a significant decrease in Firmicutes compared to healthy individuals (MANICHANH *et al.*, 2006), and in ulcerative colitis, where lower Firmicutes levels correlate with active disease versus periods of remission (VESTER-ANDERSEN *et al.*, 2019). This is also evident when antibiotic-induced alterations in the microbiome composition result in increased susceptibility to other pathogens, immune system dysregulation, and the emergence of resistance genes (FRANCINO, 2016; RAYMOND *et al.*, 2016). This scenario raises the possibility that manipulating these communities could be used to treat these diseases (REQUENA; VELASCO, 2021; YOUNG, 2017). Therefore, beyond identifying new ways to determine health and disease predisposition, it is also possible to define the parameters necessary to design, implement, and monitor strategies to intentionally manipulate the human microbiota community to optimize its performance in the context of an individual's physiology and/or disease treatment (BRITTON *et al.*, 2020; BRITTON; FAITH, 2021; TOMKOVICH *et al.*, 2020).

2.2 LACTIC ACID BACTERIA (LAB)

Lactic Acid Bacteria (LAB) form a heterogeneous group of Gram-positive bacteria, including cocci, coccobacilli, or bacilli, most of which have a low GC content. They are non-pathogenic, non-spore-forming, catalase-negative, microaerophilic, or facultatively anaerobic, and produce lactic acid as the main end product of carbohydrate fermentation (CHO; YIM; SEO, 2020; DA SILVA *et al.*, 2021; ONWUAKOR *et al.*, 2014). Six families constitute the LAB group based on genetic similarity and phylogenetic classification: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae* (phylum Firmicutes) (MATTARELLI *et al.*, 2014). In terms of phenotypic characteristics, *Bifidobacterium* (phylum Actinobacteria) can also be considered part of the LAB group (CHO; YIM; SEO, 2020). The LAB group also includes the following genera: *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Vagococcus*, *Weissella*, *Tetragenococcus*, and *Bifidobacterium* (REUBEN *et al.*, 2019).

Microorganisms' successful adaptation and persistence depend on specific nutritional, environmental, and adhesion properties. These adaptive characteristics are observed in LAB, enabling their identification in various food matrices, including dairy products, meats, vegetables, and sourdough bread (DUAR *et al.*, 2017). Moreover, LAB frequently colonizes human mucosal surfaces, including the mouth, vagina, and gastrointestinal tract (DE FILIPPIS; PASOLLI; ERCOLINI, 2020). Among the organisms constituting the human microbiota, LAB are naturally found in the gastrointestinal tract (*Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactiplantibacillus plantarum, Streptococcus agalactiae* and *Enterococcus faecalis*), in the oral cavity (*Streptococcus mutans* and *Bifidobacterium longum*), and vaginal cavity (*B. longum*, *S. agalactiae*, and *Lactobacillus crispatus*) (KLAENHAMMER *et al.*, 2005). Many LABs occupy important niches in the human gastrointestinal tract (GIT), contributing significantly to intestinal integrity, immunomodulation, and pathogen resistance (DE FILIPPIS; PASOLLI; ERCOLINI, 2020; SORNPLANG; PIYADEATSOONTORN, 2016).

LAB are widely used in the production of fermented foods due to their ability to confer desirable organoleptic characteristics and preserve these products through various compounds (organic acids, hydrogen peroxide, and bacteriocins) produced by their metabolism (KLAENHAMMER *et al.*, 2005; PACHECO-MARTÍNEZ *et al.*, 2023). The safety assessment of LABs varies between regulatory agencies. In the United States, these species are generally

recognized as safe (GRAS) by the Food and Drug Administration (FDA) status, indicating their safety for human consumption under specific approved uses (MARKOWIAK; ŚLIZEWSKA, 2017). In contrast, the European Food Safety Authority (EFSA) introduced the Qualified Presumption of Safety (QPS) concept to assess the safety of microorganisms in food production more broadly. QPS evaluates factors such as taxonomy, scientific knowledge, safety characteristics (including virulence factors and antimicrobial resistance), and intended use (OF *et al.*, 2019; SANDERS *et al.*, 2010). This approach allows certain LAB species to gain QPS status, simplifying safety assessments for these organisms across different applications. Several LAB strains have GRAS status, with some considered "probiotic". Thus, through investigations into the historical use of LABs in food fermentation and their traditional applications, research has significantly advanced our understanding of selecting specific strains and their practical applications in both the food industry and therapeutic contexts (DOUILLARD; VOS, 2019).

In addition to probiotic role, many LAB have been studied as potential carriers of compounds with therapeutic or prophylactic effects. LAB are considered promising candidates for biotechnological applications, such as bacterial recombinant vectors in vaccination strategies (BERMÚDEZ-HUMARÁN *et al.*, 2011; WYSZYŃSKA *et al.*, 2015).

2.3 EXPLORING PROBIOTIC BACTERIA: CONCEPTS AND RESEARCH

The human body provides various environmental niches that offer the conditions necessary for microbial growth, such as nutrients, moisture, pH, and suitable temperature for their establishment and population growth. Therefore, humans are colonized both externally and internally by a diverse, complex, and dynamic collection of microorganisms that outnumber human somatic and germ cells, collectively representing a significantly greater genetic variety compared to their host genome (BÄCKHED *et al.*, 2005; REQUENA; VELASCO, 2021; VENTURA *et al.*, 2009). It is estimated that there are between 500 to 1,000 bacterial species in the human body and a much larger number of subspecies, resulting in considerable genetic diversity (GILBERT *et al.*, 2018).

Each individual has a unique microbiome shaped by multiple factors over time, influenced by lifestyle, genetics, birth mode, diet, environmental factors, geographic location, and age, which are crucial for its establishment and evolution (TAMBURINI *et al.*, 2016). These microorganisms interact with the host's physiology, contributing to homeostasis and playing essential roles in digestion, energy metabolism, immune development, neurological

function, disease susceptibility, and protection against potentially pathogenic microorganisms (DOMINGUEZ-BELLO *et al.*, 2016; UUSITUPA *et al.*, 2020).

The gut microbiome is composed of a highly diverse and complex microbial community consisting of bacteria, *archaea*, fungi, and viruses. These microorganisms establish essential relationships with the human host, and the combined total of their genomes corresponds to at least 100 times more genes than the human genome (BÄCKHED *et al.*, 2005). The human microbiota, particularly the intestinal microbiota, has been extensively studied in recent years due to its well-established role in promoting host health.

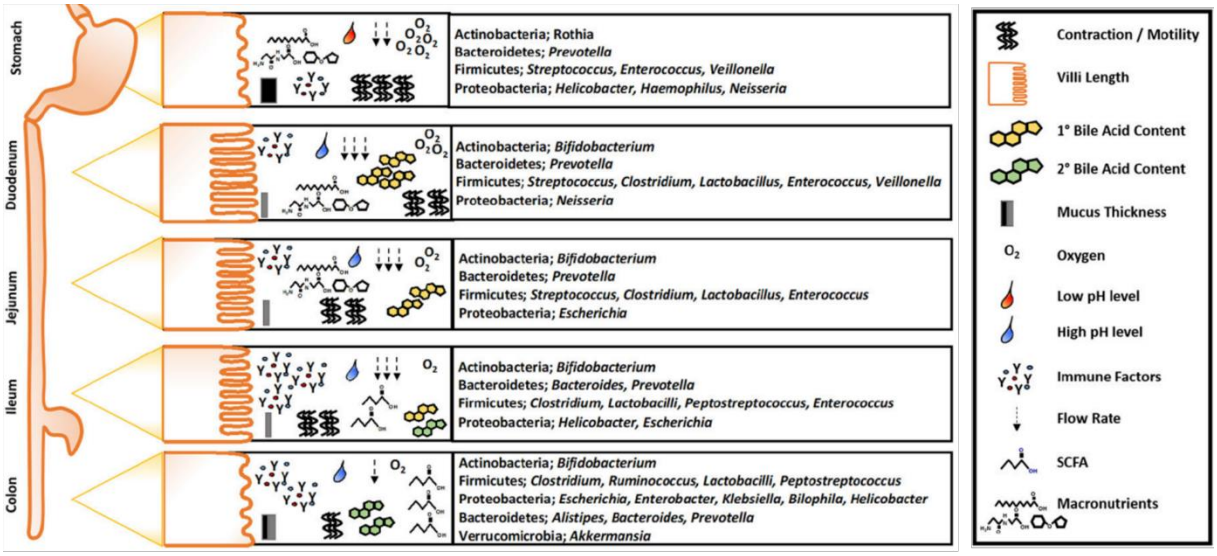
Maintaining or restoring the gastrointestinal tract microbiota using probiotic strains or their combinations has been shown to reduce the risk of various diseases, enhance the metabolic activity of resident microbiota, and support intestinal immune function, along with other essential physiological functions for human health (BRÜSSOW, 2019; DA SILVA *et al.*, 2020). Probiotic bacteria derive their name from the Greek term "pro", meaning "in favor" and "biotic", meaning "life" (PACHECO-MARTÍNEZ *et al.*, 2023). By definition, probiotics are "live strains of microorganisms rigorously selected to provide health benefits when administered in adequate doses to the host" (BIELECKA, 2006; FAO *et al.*, 2002).

Many probiotic strains can colonize the intestines and inhibit the proliferation of potential pathogens through competition for available nutrients, demonstrating various beneficial effects in preventing or treating numerous diseases (VANGAY *et al.*, 2018). Studies have consistently highlighted the role of probiotics in preventing conditions such as colon cancer, cardiovascular diseases, inflammatory bowel disease, type 2 diabetes, and metabolic syndrome (YADAV *et al.*, 2017). Therefore, studying the human microbiome is crucial for understanding the dynamics between microbial communities, their survival, and the promotion of host health. This knowledge can lead to the identification of specific strains with potential therapeutic applications.

To properly investigate probiotic candidates, studying the human intestinal microbiota is essential, given the taxonomic and functional differences across different parts of the gastrointestinal tract, which can also vary within individuals due to factors such as stages of infant development, age, and environmental factors like antimicrobial usage (RINNINELLA *et al.*, 2019). Each anatomical segment of the intestine harbors a unique microbiota due to variations in physiology, pH, digestion transit times, substrate availability, and host secretions, all of which influence its composition (FLINT *et al.*, 2012). The stomach and small intestine have short transit times (3-5 hours) and high bile concentrations, making microbial colonization challenging. In contrast, the large intestine, with slower transit rates and a pH ranging from

neutral to slightly acidic, supports the largest microbial community (FLINT *et al.*, 2012) (Figure 2).

Figure 2 – Representative host and microbial factors and community membership of the GI tract.

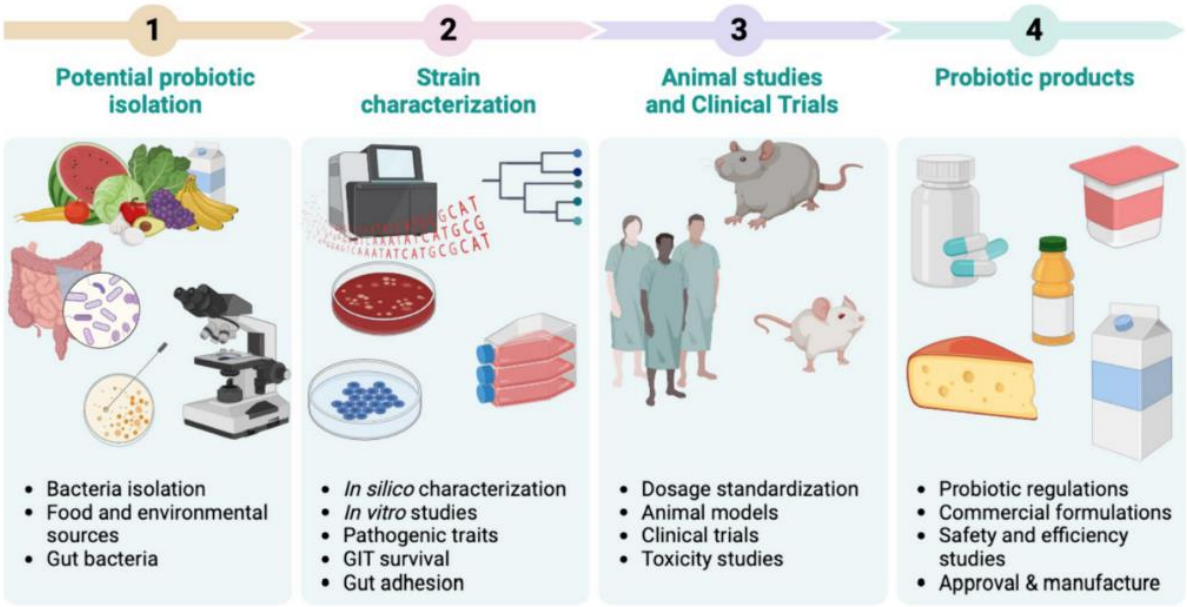


Source: Adapted from MARTINEZ-GURYN; LEONE; CHANG (2019)
Note: Schematic representation of the human stomach and intestine indicating their different regions, host/microbe factors, and phylum; genera.

Given that the effects of probiosis are strain-specific, the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) have issued guidelines for the evaluation of probiotics in food (FAO *et al.*, 2002). These guidelines underscore the necessity of precise identification and comprehensive assessment of potential probiotics through a series of *in vitro* tests to evaluate their functional properties, especially for organisms from unconventional ecosystems (SORNPLANG; PIYADEATSOONTORN, 2016). This process comprises several stages, starting with *in vitro* experiments to evaluate functional criteria, technological properties, and safety aspects. These include assessing resistance to gastric acidity, enzymatic activity of bile salt hydrolase (BSH), tolerance to bile salts, adherence to mucus or human epithelial cells, and antimicrobial effects against potential pathogens. Following this, various animal models are used in subsequent *in vivo* experiments to investigate their efficacy in different conditions, such as metabolic disorders (LE BARZ *et al.*, 2019), cholesterol reduction (PEREIRA; GIBSON, 2002), antioxidant properties (LIN; CHANG, 2000), and cytotoxic effects on cancer cells (THIRABUNYANON; BOONPRASOM;

NIAMSUP, 2009). Finally, validation through clinical trials is essential (CASTRO-LÓPEZ *et al.*, 2021; DA SILVA *et al.*, 2024) (Figure 3).

Figure 3 – Main approaches used in the discovery, characterization, and production of a probiotic.



Source: DA SILVA *et al.* (2024).

The increasing demand for dairy products in Brazil has driven significant growth in the dairy industry, particularly in probiotic products. This expansion has resulted in a broader range of options for consumers, addressing the rising interest in functional foods, including fermented milk, yogurt, fresh cheese, dairy desserts, and infant formula (Table 1) (COLOMBO *et al.*, 2018).

Table 1 – Examples of Brazilian dairy products containing probiotic bacteria available for retail sale.

Dairy product	**Commercial name**	**Producer**	**Probiotic strain**
Fermented milk	Yakult®	Yakult®	*Lactobacillus casei* Shirota
	Chamyto®	Nestlé®	*L. paracasei*
	Ninho Soleil®	Nestlé®	*L. paracasei*
	Activia®	Danone®	*Bifidobacterium animalis* CN173010
	Actimel®	Danone®	*L. casei defensis*
	Danito®	Danone®	*L. casei*

	Parmalat®	Parmalat®	*L. acidophilius*, *L. casei*, *B. animalis*
	Batavito®	Batavo®	*L. casei*, *L. acidophilus*
	LC1 Active®	Danone®	*L. acidophilus*
Yogurt	Activia®	Danone®	*B. animalis* CN173010
	Lective®	Vigor®	*B. animalis* subsp. *animalis*
	Biofibras®	Batavo®	*B. lactis*, *L. acidophilus*
	Nesvita®	Nestlé®	*B. lactis*
	Plenus®	Itambé®	*B. lactis*, *L. acidophilus*
Cheese (fresh)	Sanbios®	Santa Clara®	*B. animalis* subsp. *lactis* Bi-07
	Stímula®	Balkis®	*L. acidophilus*
Dairy dessert	Sofyl®®	Yakult®	*L. casei* Shirota
	Activia®	Danone®	*B. animalis* CN173010
Infant formula	Nan Probio 2®	Nestlé®	*B. longum*, *L. rhamnosus*

Source: Adapted from COLOMBO *et al.*, 2018.

It is recommended to identify new potential probiotics using updated and validated methodologies that combine phenotypic and genotypic techniques, as phenotypic characteristics alone are unreliable, and molecular methods are strongly preferred (FAO *et al.*, 2002; MORELLI; CAPURSO, 2012). Genome sequencing of food-related bacteria, intestinal commensals, and probiotics has been extensively conducted, providing valuable insights into the diversity and evolution of these organisms, as well as revealing the molecular basis for their health-promoting activities, giving rise to the emerging field known as "probiogenomics" (VENTURA; TURRONI; SINDEREN, 2012).

2.4 PROBIOGENOMICS

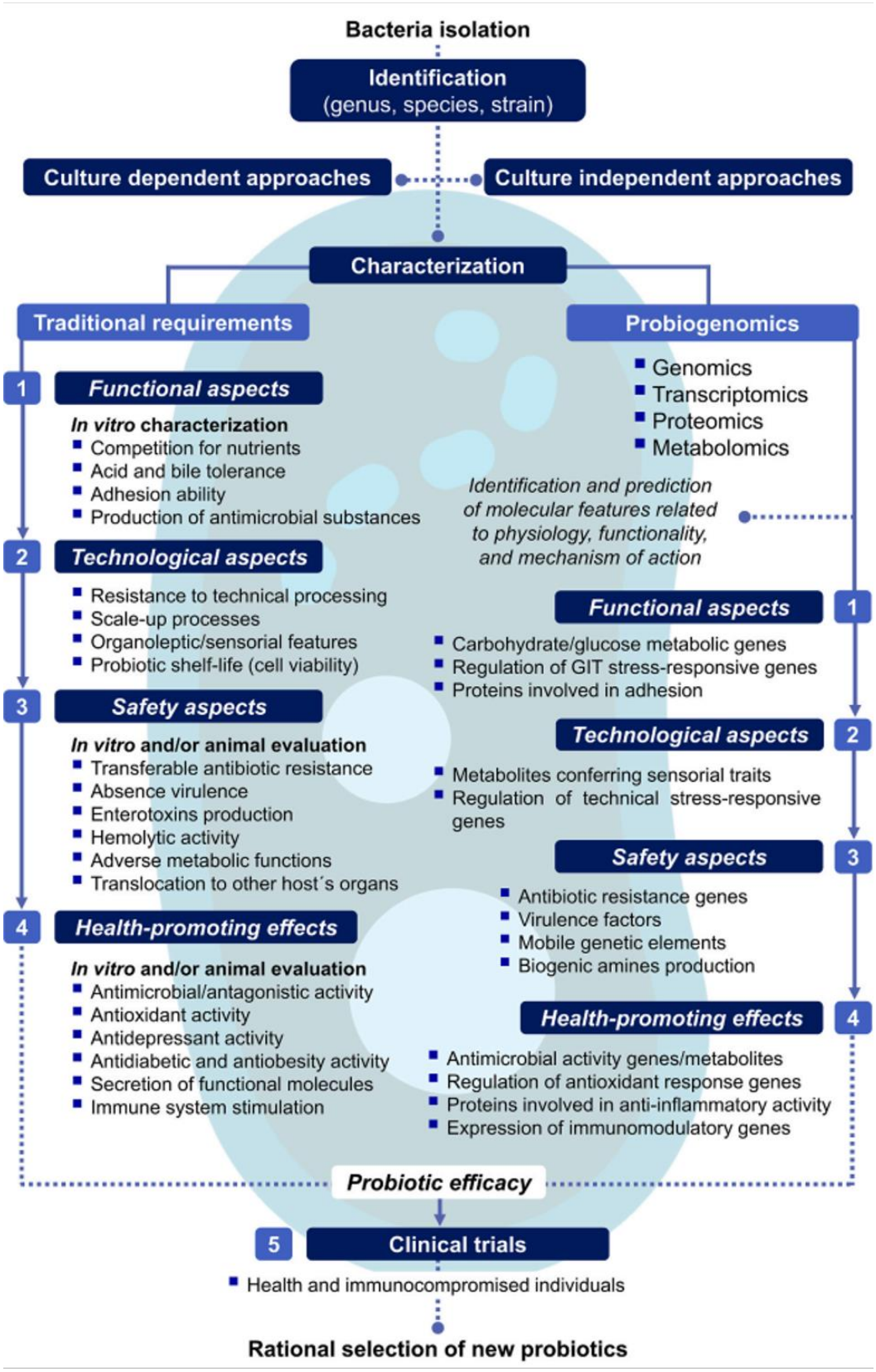
The global effort to characterize new probiotic strains is expanding, driven by the exponential increase in the consumption of products containing these microorganisms. This trend makes it a promising area for research investment, requiring thorough investigations to ensure the quality and safety of these products (LIN *et al.*, 2019). For this purpose, the validation and selection of new probiotics follow a series of traditional *in vitro* and *in vivo* assays, as previously mentioned. Moreover, the concept of strain-specificity in probiotic effects is well-established as a fundamental principle in this field of study (GUARNER; MALAGELADA, 2003). A major challenge in this process is the lack of standardization, which can lead to discrepancies and questionable results. It is also important to note that there are no universally essential attributes for all probiotics, and multiple mechanisms can be associated with specific clinical benefits (ACETI *et al.*, 2018).

Given the rapid advancements in genomic technologies, researchers are now able to explore deeper into the genetic makeup of probiotic strains, leading to a more precise

understanding of their functional capabilities. This genetic analysis allows for the identification of specific genes responsible for probiotic properties, such as adhesion to intestinal cells, production of antimicrobial substances, and modulation of the host immune system. Consequently, this molecular approach not only contributes to the characterization of known strains but also opens the door to discovering novel probiotics with unique and potentially superior health benefits (O'TOOLE; MARCHESI; HILL, 2017).

Probiogenomics is a research field that encompasses high-throughput techniques to evaluate the probiotic potential *in silico* suggested by phenotypic characteristics (ARIUTE *et al.*, 2023; VENTURA *et al.*, 2009). These capabilities enable the discovery of previously uncharacterized strains, thereby facilitating the development of predictive models for the rational selection of new probiotics (DE MELO PEREIRA *et al.*, 2018). These models incorporate detailed molecular insights into the organism's physiology, functionality, and mechanisms of action (YADAV *et al.*, 2017) (Figure 4).

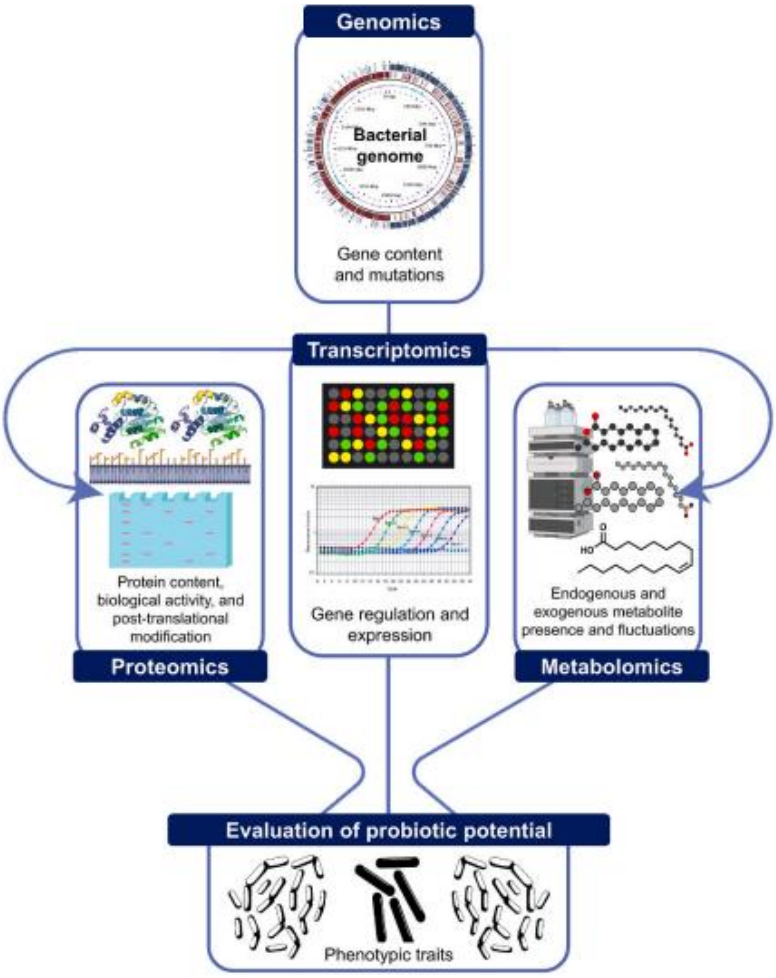
Figure 4 – Selection of new probiotics by traditional and probiogenomics approaches.



Source: CASTRO-LÓPEZ *et al.* (2021).

The “omics” approaches are required in probiogenomics studies and comprise genomics, transcriptomics, proteomics, and metabolomics. These methods enable comprehensive characterization of genetic sequences, RNA expression patterns, protein profiles, and metabolic pathways within probiotic strains (YADAV *et al.*, 2017). High-throughput whole genome sequencing has enabled the discovery of specific genes associated with probiotic characteristics, facilitating the characterization of probiotic bacterial strains. Moreover, functional genomic research has identified novel gene sets associated with probiotic functions by uncovering the molecular and functional mechanisms of probiotic strains, aiding in the assessment of their efficacy and potential applications in health and disease contexts (DOUILLARD; VOS, 2019) (Figure 5).

Figure 5 – Illustration of the probiogenomic cascade and the information derived from each "omics".



Source: CASTRO-LÓPEZ *et al.*, 2021.

Genomics encompasses the study of an organism's entire DNA sequence, referred to as the genome. This field involves sequencing, assembling, annotating, and analyzing the structure, function, and expression of genes within the genome. Advances in next-generation sequencing (NGS) technologies have revolutionized genomics by enabling the parallel sequencing of millions of small DNA fragments, surpassing the limitations of traditional Sanger sequencing methods (XU, 2017). These technologies, such as pyrosequencing, sequencing-by-synthesis, sequencing-by-ligation, semiconductor sequencing, and nanoball sequencing allow researchers to comprehensively explore genetic variations and understand genomic dynamics in various organisms (GOODWIN; MCPHERSON; MCCOMBIE, 2016). Genomic insights have broad applications in diverse fields, from evolutionary biology to personalized medicine, providing crucial information for understanding genetic diseases, drug development, and environmental adaptation (BRON *et al.*, 2019; WYSZYŃSKA *et al.*, 2015).

Transcriptomics involves the study of all RNA transcripts generated by an organism's genome, encompassing messenger RNA (mRNA) and non-coding RNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA), long non-coding RNA (lncRNA), and microRNA (miRNA) (BEDIA, 2018). Techniques like microarrays and RNA sequencing (RNA-Seq) are essential in transcriptomic research, offering efficient ways to measure and analyze gene expression patterns under diverse conditions (WANG *et al.*, 2019). In probiogenomics, transcriptomic analysis plays a crucial role in understanding how probiotic microorganisms respond to environmental stresses, interact with host systems, and regulate biological pathways (GARRIDO *et al.*, 2015; LV *et al.*, 2016; TERPOU *et al.*, 2019). This approach provides critical insights into the molecular mechanisms underlying probiotic functions (KLEEREBEZEM *et al.*, 2019), thereby contributing to their potential therapeutic applications in health and disease contexts.

Proteomics refers to the systematic investigation of the proteome, defined as the complete set of proteins expressed by the genome of an organism at a specific time and under particular physiological conditions. It involves studying protein composition, structural characteristics, abundance levels, and distinctive functional behaviors within biological systems (LARANCE; LAMOND, 2015). It encompasses the analysis of protein quantities, composition, structure, levels, and physiological functions in response to internal and external stimuli (CASTRO-LÓPEZ *et al.*, 2021). Various advanced techniques such as two-dimensional gel electrophoresis (2-DE), antibody-based methods like ELISA and western blotting, mass spectrometry (MS), and protein microarrays are employed to separate, quantify, identify, and characterize proteins within samples (TOSCANO; DE GRANDI; DRAGO, 2017; VAN GOOL

et al., 2020). Proteomics plays a crucial role in probiogenomics by mapping reference profiles of probiotic strains, identifying surface-exposed proteins involved in interactions with host cells, and assessing protein responses under diverse technological stresses (CALDERINI *et al.*, 2017; DA SILVA *et al.*, 2019; RUIZ *et al.*, 2016; SAVIJOKI *et al.*, 2019; SICILIANO; LIPPOLIS; MAZZEO, 2019).

Metabolomics is the scientific exploration of the metabolome, which encompasses all low-molecular-weight compounds, typically less than 1500 Da in size, as final products of cellular metabolic processes and create the unique chemical signature of an organism at a given moment (MISRA, 2020). It involves the systematic identification and quantification of metabolites across different physiological states and environmental conditions, indicating the complex interactions between genetics, nutrition, and the surrounding environment, thus reflecting the integrated outcome of genomic, transcriptomic, and proteomic activities (CAMBIAGHI; FERRARIO; MASSEROLI, 2017). Techniques in metabolomic analysis can be categorized into targeted and untargeted approaches, each offering distinct advantages in detecting specific known metabolites *versus* comprehensive profiling of the entire metabolite spectrum (CAMBIAGHI; FERRARIO; MASSEROLI, 2017; XU, 2017). Advanced technologies such as mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), and Raman spectroscopy are pivotal in achieving high sensitivity and resolution in metabolomics, enabling researchers to unravel metabolic pathways, characterize biomarkers, and explore the dynamic metabolic responses of microbial communities and probiotic strains (GODZIEN *et al.*, 2018).

The comprehensive knowledge gained through "omics" approaches aims to establish robust genetic typing and detailed characterization of bacterial strains used in probiotic applications. In the context of probiotic research, rigorous and detailed analyses are necessary to verify the safety and beneficial effects of these microorganisms.

2.4.1 Comprehensive *in silico* Analysis of Probiotic Safety Factors

Before probiotic strains can be considered for application, it is crucial to rigorously assess their safety attributes. This evaluation involves examining intrinsic characteristics such as pathogenicity, toxin production, and antibiotic resistance to mitigate potential adverse effects on host health. It is well established the specific criteria for determining probiotic safety, highlighting the necessity for comprehensive analysis before implementation (FAO *et al.*, 2002; MORELLI; CAPURSO, 2012). This evaluation is crucial due to potential adverse effects that

probiotics might have on host health, including allergic reactions, infections, and metabolic disturbances (LI *et al.*, 2020). Maintaining probiotic safety involves integrating probiogenomic approaches to ensure thorough analysis and understanding of the strains' genetic characteristics and interactions.

The overuse of antibiotics for the treatment of human and veterinary diseases led to widespread antibiotic resistance among pathogenic bacteria (ROZMAN *et al.*, 2020). Probiotics, although beneficial, can potentially harbor antibiotic-resistant genes, raising concerns about their role as reservoirs for resistance genes that could transfer to opportunistic pathogens. Understanding the mechanisms and genetic traits underlying resistance is essential to mitigate these risks (LI *et al.*, 2020). Advanced genomic techniques such as whole-genome sequencing (WGS) and *in silico* analysis have identified specific resistance markers in probiotic strains, with some strains proposed as potential probiotics despite containing genes associated with antibiotic resistance (ABRIOUEL *et al.*, 2017; ROZMAN *et al.*, 2020; TOROPOV *et al.*, 2020).

The identification of potential virulence factors in microbial genomes aims to evaluate the safety and functionality of probiotic strains for therapeutic or health-promoting purposes, encompassing enzymes, toxins, secreted effectors, and surface-associated proteins (CASTRO-LÓPEZ *et al.*, 2021). Recent studies have employed *in silico* methods to assess the safety of *Weissella* strains. Analysis revealed several virulence factors associated with genes encoding proteins involved in collagen adhesion, hemolysin production, and mucosal binding in *W. confusa* LBAE C39-2, while *W. cibaria* KACC 11862 harbored hemolysin-related virulence factors (ABRIOUEL *et al.*, 2015). The role of these virulence genes in the pathogenicity of *Weissella* species remains unclear and further investigation is necessary. A genomic analysis of the potential probiotic *Enterococcus durans* KLDS6.0930, isolated from traditional fermented products, has identified numerous putative virulence factors primarily linked to surface adhesion and biofilm formation, however, additional studies have predicted the strain to be non-pathogenic (LI *et al.*, 2018).

Characterizing mobile genetic elements (MGEs) is crucial for assessing the probiotic potential of bacterial strains, as MGEs can contribute to antibiotic resistance (PARTRIDGE *et al.*, 2018) and horizontal gene transfer (RANKIN; ROCHA; BROWN, 2011). Plasmids, transposons, and bacteriophages are key MGEs. Highlighting the importance of identifying MGEs in probiogenomic research, a study revealed the presence of four plasmids in *Lactiplantibacillus plantarum* BM4. Notably, pBM2 was found to encode replication-related

proteins, with all plasmids containing ORFs that could potentially exert bacteriostatic effects, thereby suggesting the safety profile of BM4 (JIE *et al.*, 2017).

In contrast, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas systems and bacteriocins act as adaptive immune mechanisms, providing protection against harmful effects from MGEs and competing bacteria (COTTER; HILL; ROSS, 2005; KLAENHAMMER, 1993; PETERS *et al.*, 2017). Probiotic safety has traditionally been assumed based on the historical use and genera classification such as *Lactobacillus* and *Bifidobacterium*. Probiogenomics emerges as a valuable approach to evaluate safety risks by identifying crucial genes or metabolites linked to potentially harmful activities, which are frequently missed in conventional laboratory assays. This genomic approach complements traditional methods, offering insights into the safety profiles and opportunistic pathogenic potential of probiotic strains (JAROCKI *et al.*, 2018; PLAVEC; BERLEC, 2020; VALERIANO *et al.*, 2019; ZHENG *et al.*, 2016).

2.4.2 Probiotic Trait Identification

As previously mentioned, for a microorganism to be considered probiotic, it must confer health-promoting effects, requiring elucidation of the mechanism responsible for a specific health benefit, which can often be found in taxonomic groups beyond the strain level (SANDERS *et al.*, 2018). For this purpose, probiosis-related genes are investigated, encompassing traits essential for strain survival post-consumption, such as resistance to stress conditions (acidic, bile, osmotic, and oxidative) and adhesion ability, along with activities with potential health benefits, including antimicrobial and antagonistic activities, antioxidant capacity, bacteriocins, and vitamin production (CASTRO-LÓPEZ *et al.*, 2021). Many studies have taken these attributes into consideration, providing valuable insights into the potential of various bacterial species, such as *Lactobacillus crispatus* (FONTANA *et al.*, 2021), *Lactobacillus helveticus* (TOROPOV *et al.*, 2020), *Lactiplantibacillus pentosus* (ABRIOUEL *et al.*, 2017), *Lactobacillus delbrueckii* (HAO *et al.*, 2011), *Lactiplantibacillus plantarum* (HAMON *et al.*, 2011; LIU *et al.*, 2024; OH *et al.*, 2022; ZHANG *et al.*, 2023), *Bifidobacterium breve* (VALDEZ-BAEZ *et al.*, 2022), *Bifidobacterium longum* (DA SILVA *et al.*, 2021), *Weissella confusa* (YUAN *et al.*, 2021), *Weissella cibaria* (SURACHAT *et al.*, 2022), and *Weissella paramesenteroides* (YADAV; SUNITA; SHUKLA, 2022).

Whole-genome analyses provide valuable insights into the genetic basis of microbial strains' abilities to adapt to specific ecological niches and perform specialized functions

(TATUSOVA *et al.*, 2016). This approach not only elucidates physiological traits crucial for industrial applications, such as the cooperative interaction in yogurt production between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (HAO *et al.*, 2011), but also facilitates targeted strain improvements and comprehensive safety assessments. In 2005, the concept of pan-genomics emerged, focusing on the collective genome of a species derived from multiple strain sequences and underscores the significance of comparative genomic analyses in understanding microbial diversity and functionality (TETTELIN *et al.*, 2005). Genes shared by all or most members of the pan-genome are called core genes, while those present in only a few members are termed accessory or dispensable genes, and those unique to a specific genome (strain) are known as unique genes (MIRA *et al.*, 2010). Therefore, the main benefit of the pan-genome method relies on its ability to create an effective framework for comparing extensive collections of closely related organisms, utilizing ribosomal protein markers to detect subtle evolutionary distinctions (CICCARELLI *et al.*, 2006; MENDE *et al.*, 2013).

2.5 *Weissella paramesenteroides* GENOMICS

Weissella species are Lactic Acid Bacteria (LAB) characterized by their Gram-positive coccoid or rod-shaped morphology (FUSCO *et al.*, 2015), distinguished by their lack of catalase activity and intrinsic resistance to vancomycin (PABARI *et al.*, 2020). Originally classified and then separated within the genus *Leuconostoc* (COLLINS *et al.*, 1993), they are commonly found in the fermentation of vegetables, meat, and fish (FESSARD; REMIZE, 2017), as well as in many traditional fermented foods such as Indian idli, dahi, kimchi, sauerkraut, and various dairy products (AHMED *et al.*, 2022; PATEL *et al.*, 2013; SHARMA *et al.*, 2018). During fermentation processes, *Weissella* spp. contribute not only to the development of desirable sensory attributes but also to the production of extracellular metabolites and exopolysaccharides (PATEL *et al.*, 2012). Thus, *Weissella* species have increasingly become the focus of studies and are being suggested as strains suitable for use in the food and pharmaceutical industries due to their probiotic, biotechnological, and bacteriocinogenic potential (YADAV; SUNITA; SHUKLA, 2022).

Weissella paramesenteroides is known for producing lactic acid, which contributes to fermented foods' preservation and flavor development (TEIXEIRA *et al.*, 2021). Additionally, it demonstrates key probiotic characteristics, including the ability to adhere to intestinal cells, modulate the host immune system, and produce antimicrobial substances that inhibit pathogenic

bacteria (WAN *et al.*, 2023). This can be observed in *Weissella paramesenteroides* WpK4, which demonstrated significant therapeutic effects by enhancing intestinal barrier integrity and ameliorating anxiety-like and depressive-like behaviors in chronically stressed mices (SANDES *et al.*, 2020). This strain also strengthened the mucosal barrier of the cecum through upregulation of the mucin MUC-2 expression, crucial for maintaining mucosal integrity and promoting epithelial regeneration (PRADO *et al.*, 2020). Additionally, WpK4 mitigated injuries induced by *Salmonella typhimurium* infection in murine models, effectively reducing bacterial translocation and moderating excessive inflammatory responses (ALVIM *et al.*, 2016). Another significant characteristic reported in *W. paramesenteroides* is its ability to produce antimicrobial peptides, with few bacteriocins characterized. Notably, the bacteriocin weissellin A, belonging to the pediocin-like class IIa, has shown inhibition activity against *Listeria* (PAPAGIANNI; PAPAMICHAEL, 2011). This capability underscores the potential of *W. paramesenteroides* as a source of bioactive compounds with antimicrobial properties.

With the advancement of sequencing technologies, the substantial increase in fully sequenced genomes deposited in databases has provided valuable sources of information for comparative genomics studies of various groups of microorganisms, including LAB (CASTRO-LÓPEZ *et al.*, 2021). LAB are extensively studied in probiogenomics, with *W. paramesenteroides* emerging as particularly promising for its probiotic potential, as mentioned (YADAV; SUNITA; SHUKLA, 2022).

2.6 BIOTECHNOLOGICAL APPLICATIONS OF LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) have garnered significant attention in biotechnology due to their versatility and beneficial properties. One of the primary applications of LAB is in the food industry, where they are extensively used for the fermentation of dairy, vegetable, and meat products (KORCZ; VARGA, 2021; LINARES *et al.*, 2017; STEFAŃSKA *et al.*, 2016). Due to the absence of a functional respiratory system, LAB generate energy through substrate-level phosphorylation, employing two primary metabolic pathways for hexose fermentation: homofermentative and heterofermentative, which enable them to efficiently convert sugars into lactic acid, a crucial metabolite in these fermentation processes (MORA-VILLALOBOS *et al.*, 2020). Fermentation by LAB not only enhances the safety and shelf-life of foods through acidification but also improves organoleptic properties and nutritional value by producing bioactive compounds such as vitamins and antimicrobial peptides (LINARES *et al.*, 2017; MADHUBASANI *et al.*, 2019).

Beyond food fermentation, LAB are recognized for their probiotic potential, as discussed previously. Therefore, certain strains can confer health benefits when consumed, such as supporting or reestablishing the gut microbiota, reducing the risk of various diseases, improving the metabolic functions of resident microbiota, enhancing intestinal immune responses, and contributing to other essential physiological functions (BRÜSSOW, 2019; DA SILVA *et al.*, 2020; VANGAY *et al.*, 2018; YADAV *et al.*, 2017).

Exopolysaccharides (EPS) produced by LAB have increasingly attracted the attention of researchers for their applications in the food industry due to their versatile functional properties (e.g., emulsification, texturization, sweetening, gelling, and water-binding capacity) as well as their bioactive attributes (JURÁŠKOVÁ; RIBEIRO; SILVA, 2022). Additionally, recent studies have further highlighted the health benefits of EPS, such as their immunomodulatory, prebiotic, anti-inflammatory, anti-biofilm, and antioxidant effects (AMIRI *et al.*, 2019; KŠONŽEKOVÁ *et al.*, 2016; LI *et al.*, 2014; SCHÖPPING *et al.*, 2022).

LAB are also being explored for their potential to develop recombinant bacteria for vaccine delivery. LAB can be administered through mucosal routes, which facilitates the induction of both mucosal and systemic immune responses, an important feature since many pathogens enter the human body via mucosal surfaces (ASHRAF; SHAH, 2014; MOJGANI; SHAHALI; DADAR, 2020; WELLS; MERCENIER, 2008). LAB are particularly well-suited as mucosal vaccine vectors due to their stability at room temperature, resistance to acidic and bile conditions, ability to activate both innate and adaptive immune responses, and their capacity for genomic modification using established molecular techniques (MANCHA-AGRESTI *et al.*, 2017; TAKAHASHI *et al.*, 2019). The development and advantages of LAB as vaccine vectors will be discussed in greater detail in Chapter 3 of this thesis.

This project is part of the research theme "Probiogenomics of Lactic Acid Bacteria: *in vitro* and *in silico* approaches for characterizing and selecting next-generation probiotics". LAB were isolated from unpasteurized milk from a farm herd in Uberaba, Minas Gerais. Among these isolates, four (*Weissella paramesenteroides*, *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, and *Ligilactobacillus agilis*) stood out for their potential probiotic traits, including resistance to acidic pH (3.5) and tolerance to 0.3% bile salts for four hours. They also demonstrated antagonistic activity against five indicator bacteria, including *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 (ROTTA *et al.*, 2020). These bacteria possess characteristics that qualify them as promising probiotics. In this context, the present work aimed to conduct a comprehensive study on the genomics of *Weissella paramesenteroides* UFTM 2.6.1 and its potential for probiosis and biotechnological applications.

3 GOALS

3.1 GENERAL GOAL

To conduct a comprehensive study on the genomics of *Weissella paramesenteroides* UFTM 2.6.1, exploring its probiotic potential through *in vitro* and *in silico* analyses, and investigating its biotechnological prospects.

3.2 SPECIFIC GOALS

(I) Perform 16S rDNA sequencing of *Weissella paramesenteroides* UFTM 2.6.1 to confirm its identity;

(II) Conduct complete genome sequencing of the *Weissella paramesenteroides* UFTM 2.6.1 to obtain the nucleotide sequence;

(III) Annotate the sequenced genome to identify genes, regulatory elements, and other genomic features;

(IV) Detect the presence of antibiotic resistance and virulence genes to assess the safety aspects;

(V) Identify and characterize mobile genetic elements and CRISPR-Cas systems present in the genome of *Weissella paramesenteroides* UFTM 2.6.1;

(VI) Investigate genes associated with probiotic properties, such as gastric acid resistance, adhesion to intestinal epithelium, and bacteriocin production;

(VII) Evaluate the inhibitory activity of *Weissella paramesenteroides* UFTM 2.6.1 against pathogenic bacteria *in vitro*;

(VIII) Conduct a comparative genomic analysis to identify conserved and strain-specific genes;

(IX) Select suitable plasmid vectors for cloning the RBD gene of SARS-CoV-2 in *Escherichia coli* and Lactic Acid Bacteria;

(X) Perform the transformation of the plasmid containing the RBD protein gene from SARS-CoV-2 into competent *Escherichia coli* cells;

(XI) Quantify the SARS-CoV-2 RBD protein in the supernatant and cell lysate of recombinant *E. coli*.

CHAPTER 1:
UNLOCKING PROBIOTIC POTENTIAL: GENOMIC INSIGHTS INTO *Weissella parameseteroides* UFTM 2.6.1

Unlocking Probiotic Potential: Genomic Insights into *Weissella paramesenteroides* UFTM 2.6.1

Submitted in Probiotics and Antimicrobial Proteins

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ABSTRACT

Weissella, a genus of lactic acid bacteria, has diverse beneficial attributes including probiotic activity and biotechnological applications. Therefore, the investigation of the *Weissella* genus has garnered growing interest. In this study, we sequenced the complete genome of *Weissella paramesenteroides* UFTM 2.6.1 isolated from unpasteurized cow's milk from the Triângulo Mineiro region and performed probiogenomic analyses. Taxonomic characterization confirmed the identity of *W. paramesenteroides*. The genome comprises 1,926 protein-coding genes, mainly related to cell metabolism, information storage and processing, and cellular processes and signaling. More than one hundred genes associated with probiotic functions were identified in the genome of *W. paramesenteroides* UFTM 2.6.1, including genes involved in stress response, bacterial persistence in the gastrointestinal tract, and biosynthesis of vitamins. *In silico* analysis of bacteriocin-related genes identified Pediocin, and subsequent *in vitro* testing confirmed that *W. paramesenteroides* UFTM 2.6.1 exhibits antimicrobial activity against *Listeria* spp. Genomic characterization revealed the presence of the replicon pLCK4 and four prophage regions, one of which was intact. Moreover, no CRISPR-Cas array or associated Cas proteins were found, along with an absence of resistance and virulence genes, suggesting a safety aspect of the evaluated strain. Pan-genome analysis unveiled 204 exclusive genes in the genome of *W. paramesenteroides* UFTM 2.6.1, which includes metabolism and stress-associated genes. In general, the results indicate probiotic potential of *W. paramesenteroides* UFTM 2.6.1. Further studies are required to ensure the safety and beneficial effects of this bacterium *in vivo*, aiming for future applications in food industry, animal and human medicine.

Keywords: probiotic, lactic acid bacteria, *Weissella*, whole-genome sequencing, probiogenomic, bioinformatic.

1 INTRODUCTION

Lactic Acid Bacteria (LAB) constitute a diverse group of Gram-positive bacteria, including cocci, coccobacilli, or bacilli. These bacteria are non-spore-forming, catalase negative, and characterized as microaerophilic or facultative anaerobes (ONWUAKOR *et al.*, 2014). The primary end product of carbohydrate fermentation is lactic acid (CHO; YIM; SEO, 2020; ONWUAKOR *et al.*, 2014). Lactic acid bacteria are extensively employed in the production of fermented foods because of the capacity of these microorganisms to impart desirable organoleptic characteristics and contribute to the preservation of such products (KLAENHAMMER *et al.*, 2005; ONWUAKOR *et al.*, 2014). The LAB group encompasses various genera, including *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Vagococcus*, *Tetragenococcus*, *Bifidobacterium* and *Weissella* (REUBEN *et al.*, 2019).

The genus *Weissella* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Leuconostocaceae* (COLLINS *et al.*, 1993; ZHENG *et al.*, 2020). These bacteria are typically found in diverse ecological niches, including fermented foods, plants, and the gastrointestinal tracts of humans and animals (APOSTOLAKOS; PARAMITHIOTIS; MATARAGAS, 2022; FUSCO *et al.*, 2015). Existing data indicate health-promoting properties such as anti-toxicity, immunomodulatory, and antitumor activity (HONG *et al.*, 2016; KWAK *et al.*, 2014; OJEKUNLE; BANWO; SANNI, 2017; YU *et al.*, 2019). *Weissella* sp. has also shown the capacity to produce antimicrobial compounds, protecting the host against bacterial and fungal infections (LI *et al.*, 2017; WANG *et al.*, 2017), and have the ability to produce exopolysaccharides (EPS), which has been associated with promising biotechnological potential (VALERIO *et al.*, 2020). Due to its diverse functionalities, probiotic attributes, biotechnological applications, and bacteriocinogenic potential, the investigation of the *Weissella* genus has garnered growing interest.

The development of high-throughput sequencing technology has allowed an expressive increase in information based on genomic analyses of different *Weissella* species. As the number of available genomes increases, probiogenomics emerges as a valuable tool for studying potential probiotic strains through the analysis of their genetic structure (FELIS *et al.*, 2017). Specifically, *Weissella paramesenteroides* has been the focus of studies on probiogenomics due to its ability to produce antimicrobial compounds and exopolysaccharides, as well as its probiotic properties (PABARI *et al.*, 2020; YADAV; SUNITA; SHUKLA, 2022). A potential probiotic bacteria must survive the passage through the gastrointestinal tract, adhere

to intestinal epithelial cells, produce antimicrobial compounds and modulate the immune response of the host. Additionally, aspects related to safety including the absence of antimicrobial resistance genes, virulence genes and genomic stability should be evaluated (MORELLI; CAPURSO, 2012; PACHECO-MARTÍNEZ *et al.*, 2023).

Weissella paramesenteroides UFTM 2.6.1, isolated from unpasteurized cow's milk from the Triângulo Mineiro region, exhibited resistance to acidic pH and bile salts, as well as antagonistic activity against *Enterococcus faecalis* 29212, *Staphylococcus saprophyticus* 35552, *Staphylococcus aureus* 25923, and *Escherichia coli* 35218. Furthermore, this strain was susceptible to commonly used antimicrobials in clinical practice such as ampicillin, clindamycin, chloramphenicol, erythromycin, penicillin, and tetracycline (ROTTA *et al.*, 2020). Therefore, in this work, we aimed to further investigate the potential probiotic profile of *Weissella paramesenteroides* UFTM 2.6.1 using probiogenomic and *in vitro* analysis.

2 MATERIALS AND METHODS

2.1 MICROORGANISM AND GENOMES

Weissella paramesenteroides UFTM 2.6.1, isolated from unpasteurized cow's milk from the Triângulo Mineiro region (ROTTA *et al.*, 2020), was maintained in De Man, Rogosa, and Sharpe (MRS; Difco, Sparks, NV, USA) broth supplemented with 2% glucose (w/v) and 0.1% Tween 80 (v/v). The culture was incubated for 24 hours under a microaerophilic atmosphere, at 37°C.

Genomes of *Weissella* sp. and other bacteria used in this study for phylogenetic and pan-genome analysis were retrieved from the GenBank database (October/2023 to April/2024), and are listed in Table S1.

2.2 DNA EXTRACTION

The genomic DNA extraction was performed following an adapted phenol-chloroform-isoamyl alcohol extraction protocol (ANAHTAR; BOWMAN; KWON, 2016). Bacterial cells were lysed in a buffer containing lysozyme and RNAse A, followed by incubation at 37 °C. The lysate was treated with SDS, tris-saturated phenol (pH 8.0) and chloroform-isoamyl alcohol to separate the aqueous phase containing DNA. The DNA was precipitated using potassium acetate (5M) and ice-cold 100% ethanol, washed with 70% ethanol, and dissolved in TE buffer (pH 8.0). DNA integrity was verified via agarose gel electrophoresis and quantified using NanoDrop™ (Thermo Scientific, Wilmington – DE, USA).

2.3 GENOME SEQUENCING OF *Weissella paramesenteroides* UFTM 2.6.1 AND TAXONOMIC IDENTIFICATION

The genomic DNA was sequenced using the paired-end (2 x 150 pb) sequencing strategy and Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Quality filtering was carried out using Trimmomatic software v0.39 with the following parameters: trailing:10, leading:10, slidingwindow:4:20, minlen:50 (BOLGER; LOHSE; USADEL, 2014). The reads were assembled using SPAdes v3.9.1 (BANKEVICH *et al.*, 2012) using default parameters and k-mer sizes of 21, 33, 55, 77, 99 and 127. The complete genome sequence of *Weissella paramesenteroides* FDAARGOS_414 (CP023501.1) was downloaded in nucleotide FASTA

format from the GenBank database. Subsequently, scaffolding was performed using AlignGraph (BAO; JIANG; GIRKE, 2014) with the reference genome, using a minimum distance of 150 bp and a maximum distance of 1150 bp between paired reads, with the fast mapping mode enabled. The quality of the assembled genome was assessed with QUAST (GUREVICH *et al.*, 2013). Contigs with lengths shorter than 500 bp were removed.

The genome was annotated using Prokka (SEEMANN, 2014) via the public Galaxy server (JALILI *et al.*, 2022), using default parameters. In addition, functional annotation was performed using the evolutionary genealogy of genes: Non-supervised Orthologous Groups (EggNOG 5.0) (HUERTA-CEPAS *et al.*, 2019).

For taxonomic identification, the average nucleotide identity (ANI) using BLASTN best hits (one-way ANI) and the correlation indexes of tetra-nucleotide signatures (Tetra), were calculated using JSpecies (RICHTER; ROSSELLÓ-MÓRA, 2009). A dendrogram was constructed by calculating the ANI for all *Weissella* spp. genomes and outgroups using the Python module pyani v0.2.12 (PRITCHARD *et al.*, 2015).

2.4 IDENTIFICATION OF ANTIBIOTIC RESISTANCE GENES, VIRULENCE GENES, MOBILE GENETIC ELEMENTS, AND CRISPR-CAS SYSTEMS

The detection of antibiotic resistance genes (ARGs) and virulence genes (VGs) was carried out using the default parameters of Resistance Gene Identifier (RGI) (ALCOCK *et al.*, 2020) and VirulenceFinder (JOENSEN *et al.*, 2014) tools, respectively. Additionally, we applied ABRicate v1.0.1 (SEEMAN, 2020) with default settings to screen for ARGs, VGs, and mobile genetic elements (MGEs). The identification of clustered regularly interspaced short palindromic repeats (CRISPRs) was assessed using CRISPRCasFinder (COUVIN *et al.*, 2018), whereas genes related to integrated prophages were identified using PHASTER (ARNDT *et al.*, 2018). If no hits higher than 80% of identity were found during the BLAST searches, the result was considered negative.

2.5 IDENTIFICATION OF PROBIOTIC MARKER GENES

In this study, an *in-house* database consisting of 270 genes related to probiotic features was constructed based on data obtained from the literature (CARPI *et al.*, 2022; KHULLAR *et al.*, 2022; LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2008; NGUYEN; KIM, 2018; SCHÖPPING *et al.*, 2022; WONGLAPSUWAN *et al.*, 2024). The database includes

genes implicated in stress response (n=180), adhesion (n=37), exopolysaccharide biosynthesis (n=16), biofilm formation (n=8), antimicrobial activity (n=2), gut persistence (n=3), immunomodulation (n=4), starvation (n=6) and biosynthesis of vitamins (n=14). A manual curation to identify these genes was performed based on genome annotation. The results were assessed based on the presence or absence of matches in the database.

2.6 *In silico* AND *in vitro* IDENTIFICATION OF ANTIMICROBIAL COMPOUNDS

The identification of putative genes encoding antimicrobial compounds in the genome of *W. paramesenteroides* UFTM 2.6.1, as well as in other *Weissella* species available on NCBI (Table S1), was performed using BAGEL4 (VAN HEEL *et al.*, 2018).

The antagonistic activity *in vitro* was assessed using the spot-on-low technique as described by Booth *et al.* (1977). *Listeria monocytogenes* ATCC 19112, *Listeria monocytogenes* ATCC 7644 and *Listeria innocua* ATCC 33090 were used as indicator bacteria. Antimicrobial activity was determined by the presence of inhibition zones (> 6 mm in diameter) around the colonies of *W. paramesenteroides* UFTM 2.6.1, indicating the inhibition of indicator microorganism growth.

2.7 PAN-GENOME ANALYSIS

Forty-five genomes of *W. paramesenteroides* available on NCBI (Table S1) were used for the pan-genome analysis. The analysis was performed using Roary (PAGE *et al.*, 2015) via the public Galaxy server (JALILI *et al.*, 2022), using the default parameters. Additionally, the function of unique proteins identified in the genome of *W. paramesenteroides* UFTM 2.6.1, which were annotated as hypothetical proteins, was predicted using the UniProt database (APWEILER *et al.*, 2004). The parameters applied included a minimum query coverage of 50%, sequence similarity above 40%, and an e-value <1e-10.

2.8 DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available in the article and/or its supplementary files. The assembled genome of *Weissella paramesenteroides* UFTM 2.6.1 was deposited at European Nucleotide Archive (ENA) under the accession number ERZ24772468.

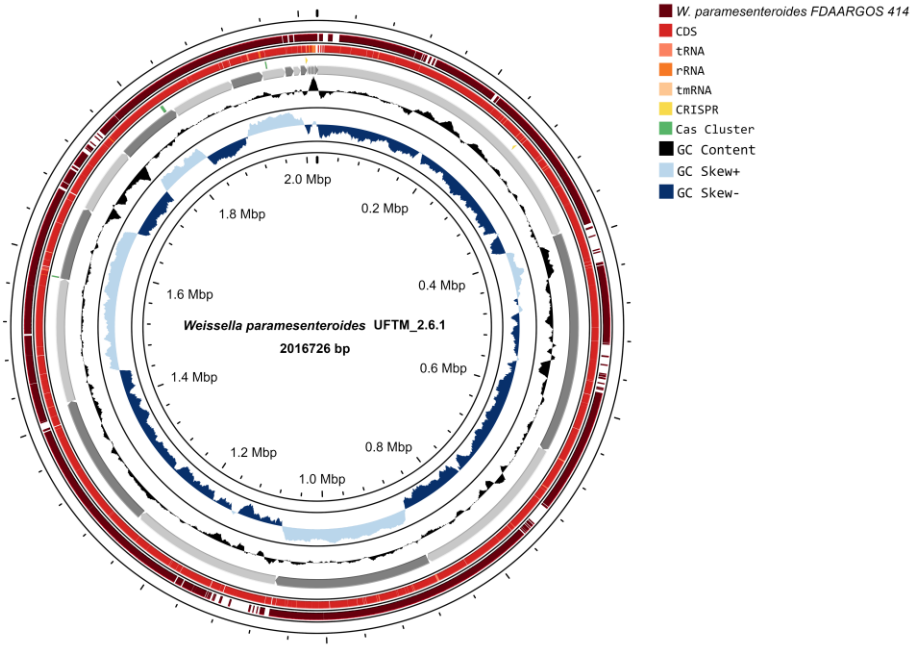
3 RESULTS

3.1 WHOLE-GENOME CHARACTERIZATION OF *Weissella paramesenteroides* UFTM 2.6.1 AND TAXONOMY IDENTIFICATION

The genome assembly of *W. paramesenteroides* UFTM 2.6.1 generated 26 contigs with a total size of 2,019,488 bp, GC content of 38.01%, and N50 value of 195.511 bp. The genome annotation resulted in a total of 1,926 protein-coding sequences (CDSs), including 725 hypothetical proteins, 68 tRNA, 11 rRNA, and 1 tmRNA (Fig. 6).

Taxonomic characterization using similarity comparison by ANI estimated 97.17% and a Tetra index of 0.99835 in comparison to the reference genome of *W. paramesenteroides* FDAARGOS 414 (Accession number: NZ_CP023501.1), thereby identifying the bacteria as a strain of *W. paramesenteroides* species (Fig. 6). Comparisons of *W. paramesenteroides* UFTM 2.6.1 with other *W. paramesenteroides* strains and other species of *Weissella* showed ANI values between 97.81 and 68.51 (Fig. 7).

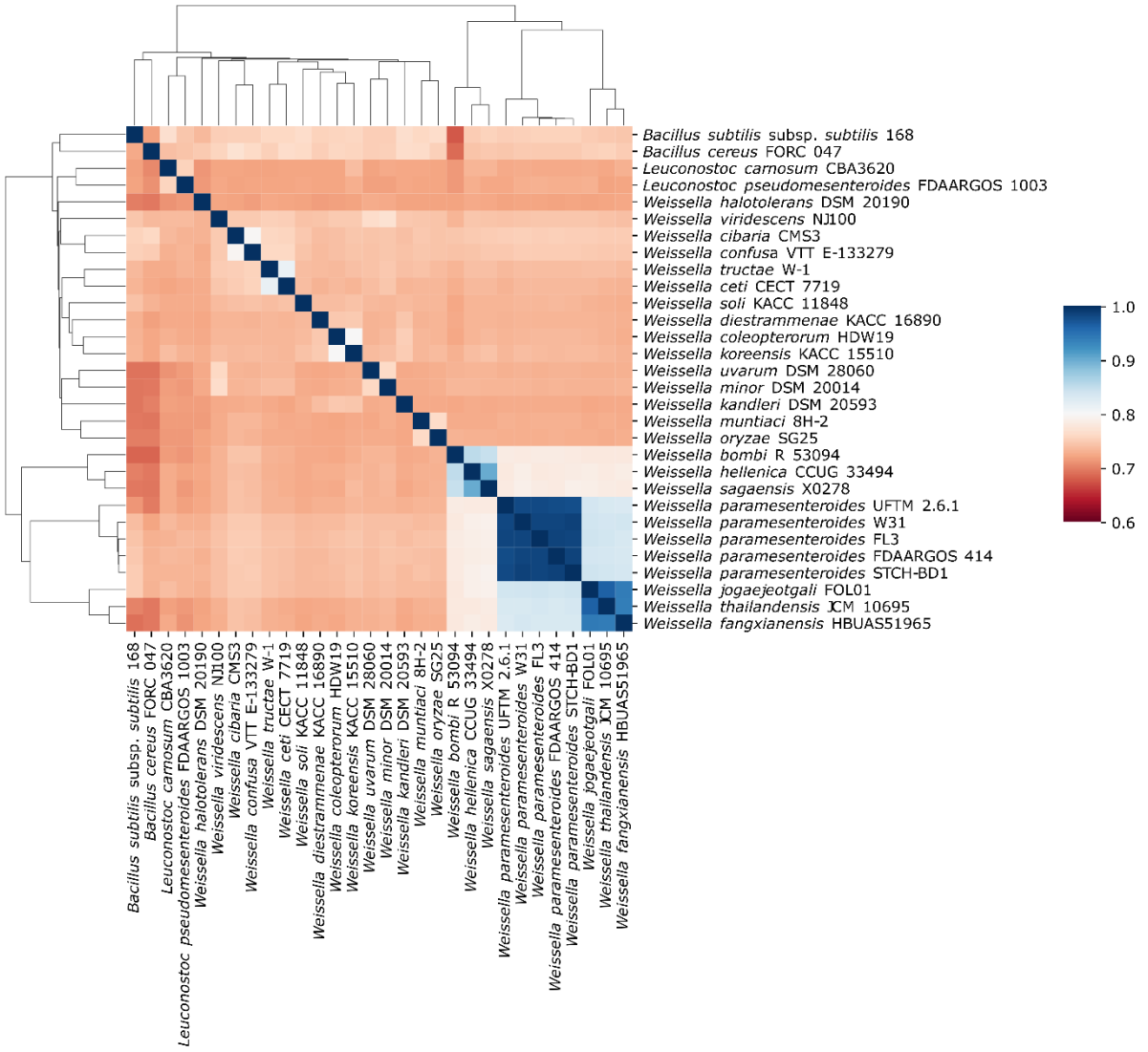
Figure 6 – Genome circular view of *Weissella paramesenteroides* UFTM 2.6.1.



Source: Own authorship (2024).

Note: From the outermost to the innermost ring, the first ring represents the map of the reference genome (*W. paramesenteroides* FDAARGOS 414); the second ring shows the coding sequences (CDS) of the analyzed genome according to Prokka annotation (both strands combined), in addition to tRNA, rRNA, and tmRNA; the third ring shows CRISPRCasFinder annotation, CRISPRs are marked (yellow arrow) and Cas Cluster (green color), the fourth ring corresponds to the 26 contigs identified; the fifth ring displays the G+C content, and the sixth ring shows the G/C skew information on the (+) strand (light blue color) and (–) strand (dark blue color). The figure was generated using ProkSee Web Server (https://proksee.ca).

Figure 7 – Heatmap representing the Average Nucleotide Identity (ANI) between genomes of *Weissella* spp.



Source: Own authorship (2024).

Note: *Leuconostoc carnosum* CBA3620, *Leuconostoc pseudomesenteroides* FDAARGOS 1003, *Bacillus cereus* FORC 047, and *Bacillus subtilis* subsp. *subtilis* 168 were used as the outgroup. The heatmap color indicates the ANI values as shown in the figure scale.

Among the total proteins predicted, 1,681 matched EggNOG DB proteins (1,601 single EggNOG and 80 multi-EggNOG proteins) and 245 proteins presented no hit (Table 1). The functional annotation of *W. paramesenteroides* UFTM 2.6.1 genome revealed diverse protein profiles. Proteins were classified into categories crucial for energy production and conversion (73 proteins), amino acid transport and metabolism (125 proteins), carbohydrate transport and metabolism (130 proteins), coenzyme metabolism (61 proteins), nucleotide metabolism (106), and cell envelope biogenesis (88 proteins). Notably, the analysis indicated the presence of proteins crucial for genomic stability and regulation, including proteins involved in replication (137), transcription (128), and translation (156). Additionally, proteins associated with defense mechanisms (34) and signal transduction pathways (35) were observed. However, some proteins remain uncharacterized (333).

Table 2 - EggNOG category distribution of proteins predicted in the genome *Weissella paramesenteroides* UFTM 2.6.1.

EggNOG Categories		Description	Count
Metabolism (673)	C	Energy production and conversion	73
	E	Amino acid transport and metabolism	125
	F	Nucleotide transport and metabolism	106
	G	Carbohydrate transport and metabolism	130
	H	Coenzyme transport and metabolism	61
	I	Lipid transport and metabolism	51
	P	Inorganic ion transport and metabolism	104
	Q	Secondary metabolites biosynthesis, transport and catabolism	23
Cellular processes and signaling (274)	D	Cell cycle control, cell division, chromosome partitioning	31
	M	Cell wall/membrane/envelope biogenesis	88
	N	Cell motility	4
	O	Post Translational modification, protein turnover, chaperones	43
	T	Signal transduction mechanisms	35
	U	Intracellular trafficking, secretion, and vesicular transport	39
	V	Defense mechanisms	34

Information storage and processing (421)	A	RNA processing and modification	1
	J	Translation, ribosomal structure, and biogenesis	156
	K	Transcription	128
	L	Replication, recombination, and repair	137
Poorly characterized (333)	S	Function unknown	333

3.2 DETECTION OF ANTIMICROBIAL RESISTANCE GENES, VIRULENCE-ASSOCIATED GENES, MOBILE GENETIC ELEMENTS AND CRISPR-CAS SYSTEMS

In our study, the analysis performed with ABRicate did not identify antimicrobial resistance genes and other virulence genes in the genome of *W. paramesenteroides* UFTM 2.6.1. The replicon pLCK4 was identified (Accession Number: DQ489739.1) with 84.70% identity. The software PHASTER identified four phages, of which one was considered intact, with a length of 47.1 kb and 37.66% GC content (Table S2). Lastly, the analysis performed using CRISPRCasFinder did not identify the presence of CRISPR array followed by Cas proteins.

3.3 DETECTION OF PROBIOTIC MARKER GENES

A total of 103 genes linked to probiotic functions were identified in the genome of *W. paramesenteroides* UFTM 2.6.1 (Table 2). These genes are mostly involved in stress response, encompassing resistance to various stressors, such as acid (n=24), bile (n=17), cold (n=3), heat (n=7), organic solvent (n=1), osmotic (n=4) and oxidative stress (n=7), or other (n=23). Additionally, genes associated with bacterial persistence in the gastrointestinal tract were identified, such as those involved in adhesion (n=5), biofilm formation (n=2) and exopolysaccharides biosynthesis (n=3). Biosynthetic genes of vitamins were also detected in the genome of *W. paramesenteroides* UFTM 2.6.1 (n=7), predominantly linked to vitamin B12, folate and riboflavin production.

Table 3 – Putative probiotic marker genes identified in *W. paramesenteroides* UFTM 2.6.1 genome.

Gene	Protein	Probiotic feature
exoA	Exodeoxyribonuclease III	Adhesion
gpr	L-glyceraldehyde 3-phosphate reductase	Adhesion
gtfA	glucosyltransferase	Adhesion
pycA	Pyruvate carboxylase	Adhesion
tuf	Elongation factor Tu	Adhesion
cpdA	3',5'-cyclic-AMP phosphodiesterase	Biofilm formation
crr	Sugar PTS system EIIA component	Biofilm formation
btuD	Vitamin B12 import ATP-binding protein BtuD	Biosynthesis of vitamins
cobB	NAD-dependent protein deacetylase	Biosynthesis of vitamins
cobC	Adenosylcobalamin/alpha-ribazole phosphatase	Biosynthesis of vitamins
folT	Folate transporter FolT	Biosynthesis of vitamins
ribF	Bifunctional riboflavin kinase/FMN adenylyltransferase	Biosynthesis of vitamins
ribU	Riboflavin transporter RibU	Biosynthesis of vitamins
ribZ	Riboflavin transporter RibZ	Biosynthesis of vitamins
galE	UDP-glucose 4-epimerase	Exopolysaccharides biosynthesis
gatB	UTP--glucose-1-phosphate uridylyltransferase	Exopolysaccharides biosynthesis
glmU	Bifunctional UDP-N-acetylglucosamine pyrophosphorylase	Exopolysaccharides biosynthesis
ansA	Asparaginase	Acid stress
atpA	ATP synthase subunit alpha	Acid stress
atpB	ATP synthase subunit a	Acid stress
atpC	ATP synthase epsilon chain	Acid stress
atpD	ATP synthase subunit beta	Acid stress
atpF	ATP synthase subunit b	Acid stress
atpG	ATP synthase gamma chain	Acid stress
atpH	ATP synthase subunit delta	Acid stress

clpE	ATP-dependent Clp protease, ATP-binding subunit ClpE	Acid stress
clpX	ATP-dependent ClpX protease	Acid stress
ddl	D-alanine--D-alanine ligase	Acid stress
ffh	Signal recognition particle protein	Acid stress
gap	Glyceraldehyde-3-phosphate dehydrogenase	Acid stress
guaA	GMP synthase [glutamine-hydrolyzing]	Acid stress
pgi	Glucose-6-phosphate isomerase	Acid stress
plsC	1-acylglycerol-3-phosphate O-acyltransferase	Acid stress
ppk	Polyphosphate kinase	Acid stress
pyk	Pyruvate kinase	Acid stress
recA	Protein RecA	Acid stress
relA	GTP pyrophosphokinase	Acid stress
tig	Trigger factor	Acid stress
tpiA	Triosephosphate isomerase	Acid stress
uvrA	UvrABC system protein A	Acid stress
yjbM	GTP diphosphokinase	Acid stress
argS	Arginine--tRNA ligase	Bile tolerance
glf	UDP-galactopyranose mutase	Bile tolerance
glnA	Glutamine synthetase	Bile tolerance
ltaS1	Lipoteichoic acid synthase 1	Bile tolerance
nagB	Glucosamine-6-phosphate deaminase	Bile tolerance
oppA	Oligopeptide–binding protein OppA	Bile tolerance
pdhd/lpdA	Dihydrolipoyl dehydrogenase	Bile tolerance
pepF	Oligoendopeptidase F	Bile tolerance
pepO	Endopeptidase PepO	Bile tolerance
ponA/pbp1A	DD-transpeptidase	Bile tolerance
ppaC	Putative manganese-dependent inorganic pyrophosphatase	Bile tolerance
pyrG	CTP synthase	Bile tolerance
rplD	50S ribosomal protein L4	Bile tolerance
rplE	50S ribosomal protein L5	Bile tolerance

rplF	50S ribosomal protein L6	Bile tolerance
rpsC	30S ribosomal protein S3	Bile tolerance
rpsE	30S ribosomal protein S5	Bile tolerance
cspB	Cold shock protein	Cold stress
cspC		Cold stress
trxA	Thioredoxin	Cold stress
ctsR	Transcriptional regulator CtsR	Heat stress
hcrA	Heat-inducible transcription repressor HrcA	Heat stress
hslO	Molecular chaperone Hsp33	Heat stress
lexA	transcriptional repressor LexA	Heat stress
recX	regulatory protein RecX	Heat stress
ruvA	Holliday junction ATP-dependent DNA helicase RuvA	Heat stress
secA	Preprotein translocase subunit SecA	Heat stress
sph	Oleate hydratase	Organic solvent stress
gbuA	Glycine betaine/carnitine transport ATP-binding protein GbuA	Osmotic stress
glpF	Aquaporin family protein	Osmotic stress
iolS	General stress protein 69	Osmotic stress
osmV	Osmoprotectant import ATP-binding protein OsmV	Osmotic stress
cydB	cytochrome d ubiquinol oxidade subunit II	Oxidative stress
cydD	ABD-transporter ATP-binding protein/permease	Oxidative stress
msrB	Peptide methionine sulfoxide reductase MsrB	Oxidative stress
pyrK	dihydroorotate dehydrogenase electron transfer unit	Oxidative stress
pox5	Pyruvate oxidase	Oxidative stress
trxB	Thioredoxin reductase	Oxidative stress
yaaA	peroxide stress protein YaaA	Oxidative stress
copA	Copper transporting ATPase	Acid stress, Bile tolerance
dnaK	Chaperone protein DnaK / HSP70	Acid stress, Bile tolerance
eno2	Enolase 2	Acid stress, Bile tolerance

gpmA	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	Acid stress, Bile tolerance
pgk	Phosphoglycerate kinase	Acid stress, Bile tolerance
dnaJ	Chaperone protein DnaJ	Acid stress, Bile tolerance, Thermic stress
grpE	Protein GrpE / HSP-70 cofactor	Acid stress, Bile tolerance, Thermic stress
htrA	Serine protease HtrA	Acid stress, Bile tolerance, Thermic stress
luxS	S-ribosylhomocysteine lyase	Acid stress, Bile tolerance, Thermic stress, Production of antimicrobial compounds
ftsY	Signal recognition particle receptor FtsY	Acid stress, Osmotic stress
nhaC	Na+/H+ antiporter NhaC	Acid stress, Osmotic stress
strA	Sortase A	Adhesion, Bile resistance
groL	60 kDa chaperonin groEL	Environmental stress resistance
groS	10 kDa chaperonin groES protein	Environmental stress resistance
clpP	Clp protease ClpP	Stress resistance
clpC	ATP-dependent Clp protease ATP-binding subunit ClpC	Stress resistance
msrA	Peptide methionine sulfoxide reductase MsrA	Stress resistance
msrC	Free methionine-R-sulfoxide reductase	Stress resistance
mutY	A/G-specific adenine DNA glycosylase	Stress resistance
nhaK	Monovalent cation/hydrogen antiporter	Stress resistance
nox	NADH oxidase	Stress resistance
rpoE	RNA polymerase sigma factor	Stress resistance
sigA	RNA polymerase sigma factor SigA	Stress resistance

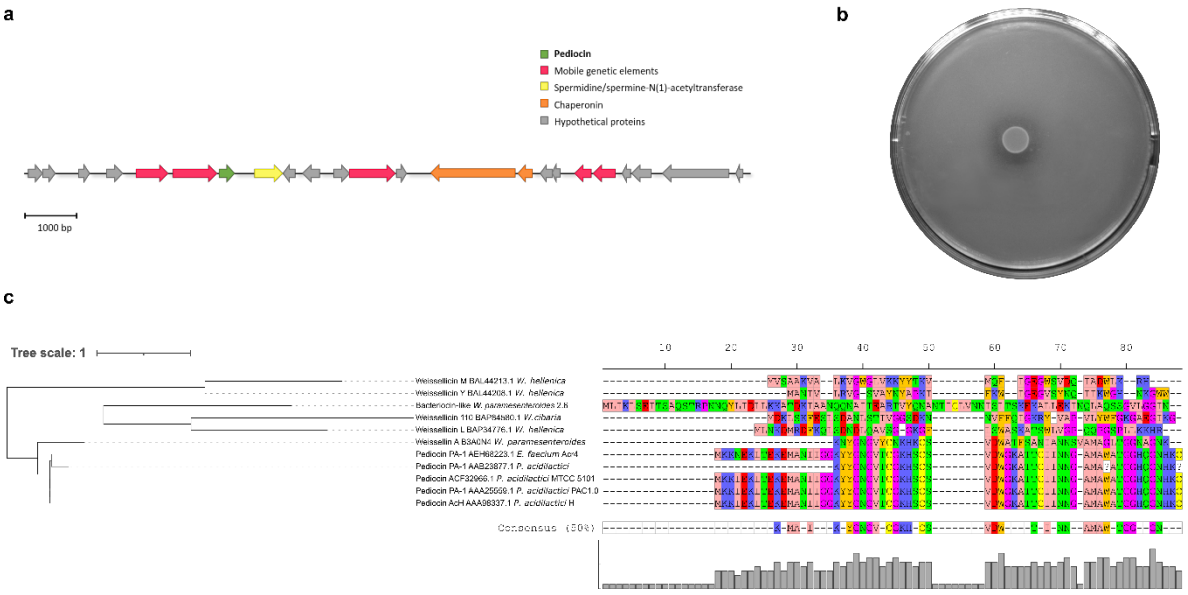
3.4 *In silico* AND *in vitro* DETECTION OF ANTIMICROBIAL COMPOUNDS

In silico identification of genes encoding antimicrobial compounds using BAGEL4 revealed the presence of Pediocin (Bit Score = 47.3654) in the genome of *W. paramesenteroides* UFTM 2.6.1 (Fig. 8a). In addition to the strain analyzed in this study, BAGEL4 analysis of all complete genomes of *Weissella* species available on NCBI (n = 362) revealed hits only in strains of *Weissella cibaria*, *Weissella confusa*, *Weissella diestrammenae*, *Weissella hellenica*, *Weissella minor*, *Weissella muntiaci*, *Weissella sagaensis*, and *Weissella uvarum*. However,

none of the identified genes corresponded to Pediocin, as observed in the strain analyzed in this study (Table S3).

W. paramesenteroides UFTM 2.6.1 exhibited antimicrobial activity *in vitro* against *Listeria monocytogenes* ATCC 19112, *L. monocytogenes* ATCC 7644, and *L. innocua* ATCC 33090, as shown in Fig. 8b. Although the antimicrobial peptide gene identified in the genome of *W. paramesenteroides* UFTM 2.6.1 was associated with Pediocin, the alignment of the peptide identified with sequences of Pediocin, Weissellicin, and Weissellin (the last two synthesized by *Weissella*), revealed the clustering of the detected peptide with bacteriocins produced by other strains of the same genus (Fig. 8c).

Figure 8 – *In silico* and *in vitro* assessment of antimicrobial activity in *W. paramesenteroides* UFTM 2.6.1.



Source: Own authorship (2024).

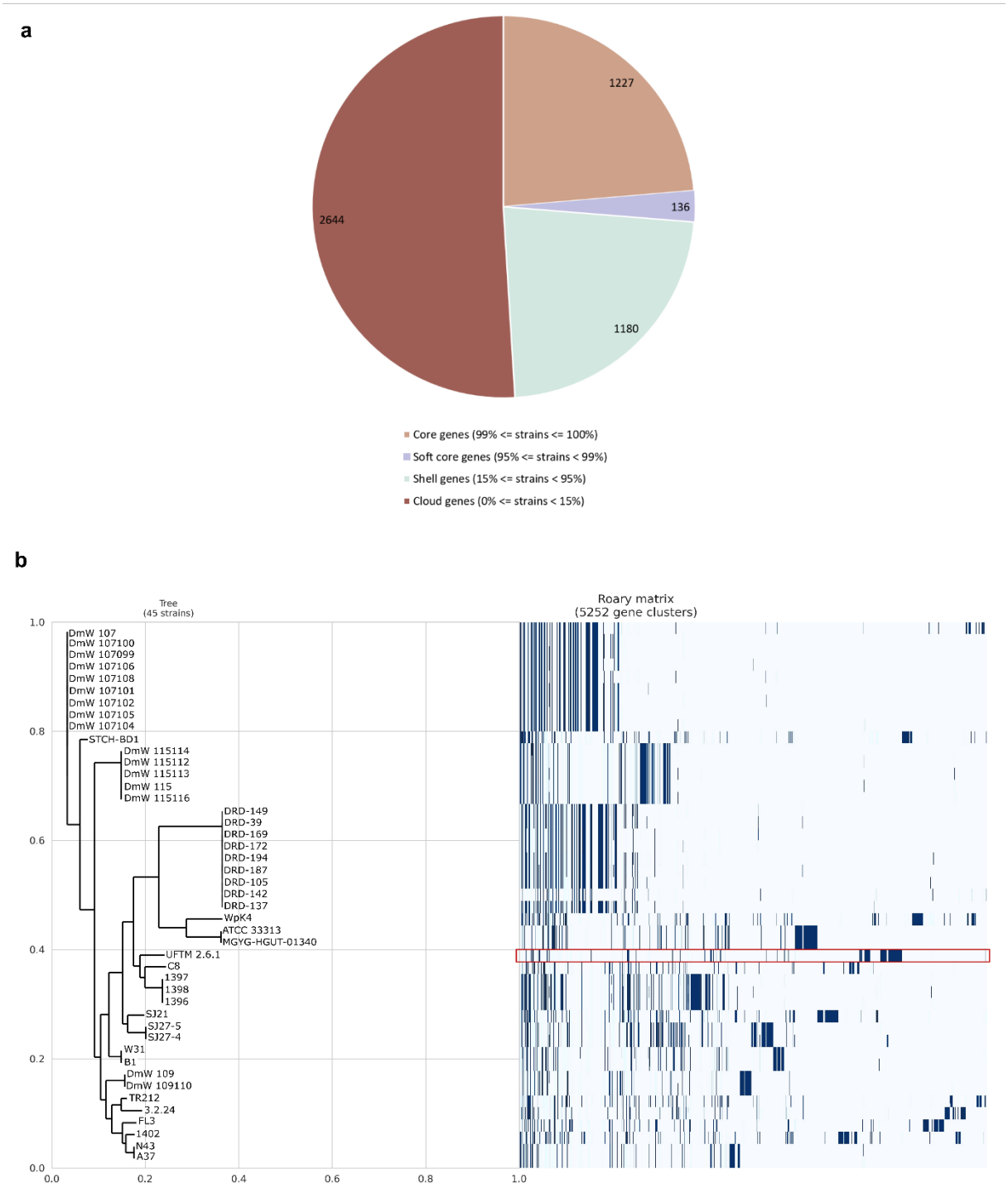
Note: **a** Schematic representation of the Pediocin gene cluster present in *W. paramesenteroides* UFTM 2.6.1 genome, predicted by BAGEL4. **b** *In vitro* inhibition assay of *W. paramesenteroides* UFTM 2.6.1 against *Listeria innocua* ATCC 33090. **c** Phylogenetic reconstruction of Pedicion, Weiseillin, and Weissellicin amino acid sequences. The alignment of the antimicrobial peptide sequences, including the core peptide identified in this study (Bacteriocin-like *W. paramesenteroides* 2.6) is also shown.

3.5 PAN-GENOME ANALYSIS

A pan-genome analysis was performed to investigate the complete genetic

characteristics of *W. paramesenteroides*, revealing the core, accessory, and unique genes among the 45 strains of *W. paramesenteroides* used in this analysis. A total of 1,227 genes are shared by all *W. paramesenteroides* strains, constituting the core genome of this species, along with 136 soft-core genes, 2,644 cloud genes, and 1,180 shell genes (Fig. 9a, Table S4). A distinct gene pattern was observed in *W. paramesenteroides* UFTM 2.6.1, consistent with the data annotation conducted by Prokka (Fig. 9b). This analysis revealed 204 exclusive genes in the genome of *W. paramesenteroides* UFTM 2.6.1. Among these, are genes encoding proteins responsible for carbohydrate (oligosaccharides, arabinose, and lactose) and micronutrient (zinc) transport such as *msmX*, *frmB*, *araQ*, *lacF*, and *zucC*. In addition, two loci (KBJPMBOP_00533 and KBJPMBOP_01678) annotated as hypothetical proteins were predicted by UniProt as Qx99_01798 and HMPREF0877_1597, respectively, and correspond to stress response-related genes (Table S5).

Figure 9 – Pan-genome analysis of *Weissella paramesenteroides* strains.



Source: Own authorship (2024).

Note: **a** Pie chart representing the distribution of genes within the core, soft core, shell, and cloud categories of the *W. paramesenteroides* strains. **b** Comparative analysis of gene content among the 45

W. paramesenteroides genomes presented in a matrix. The matrix delineates strain-specific genes (light blue) and those universally present in all strains (dark blue). A distinct pattern of genes in *W. paramesenteroides* UFTM 2.6.1 is emphasized in red.

4 DISCUSSION

The NCBI repository encompasses 362 publicly available draft and complete genome sequences of *Weissella* species, accompanied by an additional 64 plasmids (source: http://www.ncbi.nlm.nih.gov/genome/browse/ using '*Weissella* [orgn]' as the search query, as of February 2024). In the context of *W. paramesenteroides*, the database houses 47 complete genome sequences and eight plasmids. In this study, we sequenced the genome of *W. paramesenteroides* UFTM 2.6.1 isolated from unpasteurized cow's milk from the Triângulo Mineiro region, a strain that has previously demonstrated probiotic potential *in vitro* (ROTTA *et al.*, 2020). In the genome of *Weissella paramesenteroides* UFTM 2.6.1, 1,681 genes were annotated in the COG database, predominantly linked to the metabolism category, especially in carbohydrate transport and metabolism. These findings are consistent with the literature on comparative genomics of *Weissella paramesenteroides*, which identifies that the majority of functional genes in the core genome are involved in amino acid and nucleotide metabolism and transport, suggesting the species' potential for utilizing a variety of these compounds and indicating capabilities in heterofermentation (WAN *et al.*, 2023).

Bacterial taxonomy predominantly relies on the sequence of the 16S rRNA gene. However, some species belonging to the same genus may share highly similar 16S rRNA gene sequences, leading to challenges in effectively distinguishing some bacteria (CASE *et al.*, 2007). In the NCBI server, taxonomic classification is commonly established based on ANI (Average Nucleotide Identity) values (CIUFO *et al.*, 2018). When the ANI value of a particular species in comparison to a reference strain surpasses 95%, the taxonomic classification permits assignment of the species to the same taxonomic category as the reference strain (GORIS *et al.*, 2007). The ANI taxonomic characterization of the isolate sequenced in this study estimated 97.17% identity and 0.99835 of Tetra to *W. paramesenteroides* FDAARGOS 414 (Accession number: NZ_CP023501.1), confirming its high nucleotide identity with this specie. The comparison of the genomes of all *W. paramesenteroides* strains, including *W. paramesenteroides* UFTM 2.6.1, reveals ANI values ranging from 97.06% to 98%, exceeding the threshold for species demarcation. Notably, the *W. paramesenteroides* strains DmW118 and DmW118119 emerge as outliers in the group of this species, exhibiting ANI values of 77.31% and 77.32%, respectively, when compared to the *W. paramesenteroides* UFTM 2.6.1 strain, corroborating with the observations described in a previous study that compared *W. paramesenteroides* genomes (WAN *et al.*, 2023).

Genomic analysis of the *W. paramesenteroides* UFTM 2.6.1 revealed a distinctive genome composition associated with probiotic features. The proteins identified in the genome are mainly associated with stress response, adhesion and biosynthesis of vitamins, A total of 86 stress-related genes were identified, encompassing thermic, osmotic, oxidative, acid, organic solvent and bile tolerance. These attributes not only elucidate the intrinsic ability of this strain to tolerate diverse environmental stressors but also contribute to its resilience for probiotic applications. Genes encoding chaperones (*dnaJ*, *groL*, *groES*) were detected, contributing to a general stress response by protecting and removing damaged proteins, similar to the potentially probiotic strain *Bifidobacterium breve* 110 1A (VALDEZ-BAEZ *et al.*, 2022). The presence of the *glfP* and *gbuA* genes, known for its osmoprotective role, enhances the bacterial ability to survive in diverse environments, including the gastrointestinal tract (CARPI *et al.*, 2022). Its presence suggests the capacity of *W. paramesenteroides* UFTM 2.6.1 to promote host health by bolstering stress tolerance and survivability, making it a promising candidate for probiotic applications.

The application of newly isolated LAB strains as probiotics depends on the capacity to maintain viability following gastrointestinal transit and to promote health benefits by influencing the composition of the gut microbiota, for example (BOESTEN; DE VOS, 2008; MOROEANU *et al.*, 2015). To achieve successful colonization and prolonged persistence in the gastrointestinal tract (GIT), probiotic bacteria must also demonstrate the capacity to adhere to mucosal surfaces (HAMON *et al.*, 2011; NATIONAL INSTITUTES OF HEALTH (NIH), 2023). *W. paramesenteroides* UFTM 2.6.1 has genes linked to this property, including *tuf*, *gpr* and *galE*, which contribute to the specific adhesion of the bacteria by promoting exopolysaccharide production and interaction between host cells and extracellular matrix components (WONGLAPSUWAN *et al.*, 2024).

The genomic analysis also revealed the presence of genes linked to the production of riboflavin (B2) and cobalamin (B12), such as *ribZ*, *ribU*, *ribF*, and *btuD*. These vitamins are involved in crucial metabolic reactions and pathways but are not stored in the body, thus requiring a daily intake of animal-derived foods (HANNA *et al.*, 2022). In addition, essential B-complex vitamins are not naturally synthesized by humans but are found in certain microorganisms like *Lacticaseibacillus casei* and *Lactiplantibacillus plantarum* (YOSHII *et al.*, 2019), playing a vital role in human health.

Besides the probiotic features highlighted above, lactic acid bacteria, including *W. paramesenteroides*, may ferment sugars such as cellobiose, fructose, galactose, lactose, maltose, melibiose, raffinose, ribose, sucrose, trehalose, and xylose to produce lactic acid and

other metabolites (DE MELO PEREIRA *et al.*, 2018; FUSCO *et al.*, 2015). In the genome of *W. paramesenteroides* UFTM 2.6.1, a rich genetic repertoire involved in bacterial metabolism was detected, suggesting that this bacterium might be capable of fermenting a range of carbohydrates. The versatility of heterofermentative organisms in metabolizing various substrates is essential for the adaptation to diverse environments (FILANNINO *et al.*, 2016; RYALL; EYDALLIN; FERENCI, 2012), and extensively explored in industrial processes like food and beverage fermentation (INTELLIGENCE MORDOR, 2023).

Another important feature of probiotic bacteria is the synthesis of compounds that protect the host against pathogenic microorganisms. Previous data indicate that bioactive secondary metabolites from probiotic cultures, including *W. paramesenteroides*, influence bacterial community interactions, reducing the negative impacts or characteristics such as virulence factors expression, biofilm formation, pathogen colonization ability, and improving protection of the intestinal mucosa against damage by pathogenic microorganisms (FONSECA *et al.*, 2019; NORDESTE *et al.*, 2017). For instance, lactic acids produced by LAB inhibit pathogen survival and inactivate the human immunodeficiency virus by increasing acidity (ALAKOMI *et al.*, 2000; TYSSEN *et al.*, 2018).

Among the secondary metabolites produced by bacteria, bacteriocins are ribosomally synthesized antibacterial peptides or proteins that show bactericidal or bacteriostatic activity against the target cell (DE VUYST; LEROY, 2007). Numerous investigations have explored the antimicrobial potential of *Weissella* strains, including *W. paramesenteroides* (PAPAGIANNI; PAPAMICHAEL, 2011, 2012; PAPAGIANNI; SERGELIDIS, 2013; YOSHIYAMA *et al.*, 2013). For instance, *W. paramesenteroides* W31 has demonstrated bactericidal efficacy against other Gram-positive bacteria, similar to the *W. paramesenteroides* DX strain, in which the bacteriocin “weissellicin A” was identified and characterized (PAPAGIANNI; PAPAMICHAEL, 2011; WAN *et al.*, 2023). *In silico* investigation of bacteriocin-related genes identified Pediocin in the genome of *W. paramesenteroides* UFTM 2.6.1.

An *in vitro* inhibition assay was conducted to evaluate the production of antimicrobial compounds by *W. paramesenteroides* UFTM 2.6.1. As Pediocin (Class II bacteriocin) demonstrates activity against *Listeria* spp. (PÉREZ-RAMOS *et al.*, 2021), we used this bacterium as an indicator microorganism. *W. paramesenteroides* UFTM 2.6.1 was capable of inhibiting *Listeria monocytogenes* ATCC 19112, *L. monocytogenes* ATCC 7644, and *L. innocua* ATCC 33090, thereby showing the advantageous characteristic of inhibiting pathogenic bacteria. The unique sequence of the bacteriocin-encoding gene identified in this

study in *W. paramesenteroides* genome, in addition to the observed presence of several mobile genetic elements flanking the Pediocin biosynthetic gene cluster (data not shown), we suggest that this gene might have been acquired by horizontal gene transfer. However, further studies need to be conducted to evaluate this hypothesis.

With the progress in sequencing methodologies, genomes from diverse lactic acid bacterial groups have become accessible for analysis. This increased accessibility to genomic data not only facilitates comparative genomics research but also exposes the richness of genomic variation within this dataset. The results of the pan-genome analysis revealed the presence of strain-specific and shell genes in *W. paramesenteroides*, indicating both strain-specific adaptations and shared genomic features, which is associated with substantial genetic diversity and potential variations in biological properties and adaptive strategies among different strains. A distinct cluster of genes was observed in the genome of *W. paramesenteroides* UFTM 2.6.1, including 204 exclusive genes. Among the unique amino acid sequences found, thioredoxin reductase and F0F1 ATP synthase subunit A are associated with oxidative stress and proton pump functions, respectively. Under low pH conditions, it is commonly observed that F0F1 ATP synthase subunits exhibit high levels of expression and act as proton pumps in Gram-positive bacteria as already observed in potentially probiotic strains of *Lactiplantibacillus plantarum* (OH *et al.*, 2022; ŠEME *et al.*, 2015) and in *Bifidobacterium* species (HAMON *et al.*, 2011; KULLEN; KLAENHAMMER, 1999; VALDEZ-BAEZ *et al.*, 2022). In addition, the unique proteins found in *W. paramesenteroides* UFTM 2.6.1 related to carbohydrates and micronutrient assimilation, reinforce the capacity of this bacteria to grow using distinct substrates, which is an advantageous property for future industrial applications.

5 CONCLUSION

The results reported in our study indicate that the bacterium *W. paramesenteroides* UFTM 2.6.1 exhibits probiotic potential, as evidenced by the identification of genes related to probiotic properties and the absence of antimicrobial and other virulence genes, revealing a promising safety profile, a crucial aspect that align with the desirable traits for probiotic microorganisms. Our findings are supported by previous *in vitro* studies demonstrating resistance to acid stress and bile salts, as well as the ability of this bacteria to inhibit the growth of potentially pathogenic bacteria. The synthesis of a potential novel bacteriocin by *W. paramesenteroides* 2.6.1 further underscores the probiotic potential of this strain and highlights the importance of its investigation for biotechnological purposes. Further research is warranted to analyze the beneficial properties and safety of using this bacterium as a probiotic *in vivo*.

CHAPTER 2:
RECOMBINANT LACTIC ACID BACTERIA: AN INTEGRATIVE REVIEW

Recombinant lactic acid bacteria: an integrative review

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ABSTRACT:

Lactic Acid Bacteria (LAB) form a heterogeneous group of non-pathogenic, Gram-positive bacteria, which produce lactic acid as the main final product of carbohydrate fermentation. Some LABs have the status of "generally recognized as safe" (GRAS) being safe for human consumption. These microorganisms stand out as a promising candidate for mucosal recombinant vaccines due to ease on construction of the recombinant vector and capacity of inducing mucosal and systemic immunity. The present integrative review aimed to discuss the most recent data on the development of vectors using LAB for recombinant vaccines. Searches were carried out in the PubMed and Scopus electronic databases, and original articles in English, published between 2016 and 2021 were selected. The "lactic bacteria", "recombinant vaccine" and "bacterial vector" descriptors were used. Sixty-seven articles were initially identified and 9 were eligible for the review. Most of the recombinant bacteria vaccines presented immunogenic capability, inducing the production of immunoglobulins, cytokines and reducing death rates in animal models for different pathogens and toxins, demonstrating the promising activity of this technology approach for future vaccines development. Moreover, because of many advantages, such as, induction of mucosal immunity, ease of administration and cost-effectiveness of these immunizers, it should be emphasized that these vaccines can add more value to the available and constantly evolving biotechnology tools. Thus, this review presents data that confirm the efficacy of LABs as vaccine vectors in animal models, which can stimulate mucosal and systemic immunities. However, further clinical trials are needed to confirm its safety and investigate if these results translate to humans.

Keywords: Lactic acid bacteria, Recombinant vaccine, Bacterial vector.

1 INTRODUCTION

Lactic Acid Bacteria (LAB) form a heterogeneous group of non-pathogenic, Grampositive bacteria that do not form endospores, negative catalase, microaerophilic or facultative anaerobes, which produce lactic acid as the main final product of carbohydrate fermentation (ONWUAKOR *et al.*, 2014). LABs are widely used in the production of fermented foods due to their ability to confer desirable organoleptic characteristics and act in the preservation of these products (KLAENHAMMER *et al.*, 2005; ONWUAKOR *et al.*, 2014), and due to the status of "generally recognized as safe" (GRAS) by the American Food and Drug Administration (FDA), when safe for human consumption (MARKOWIAK; ŚLIZEWSKA, 2017).

In addition, some LABs are considered probiotic because they have beneficial effects on the host and because they occupy important niches in the gastrointestinal tract (GIT) of humans (SORNPLANG; PIYADEATSOONTORN, 2016). By definition, probiotics are "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (BIELECKA, 2006; FAO *et al.*, 2002).

The use of microorganisms, specifically live bacteria, as vectors for recombinant vaccines, has been widely studied and, thanks to technological advances achieved in the areas of molecular biology and genetic engineering, these vectors are capable of presenting heterologous antigens through introduction of genes encoding the antigen of interest (NASCIMENTO; LEITE, 2012).

Still regarding the recombinant vaccines, a group of bacteria that stands out strongly as a promising candidate is the LAB group, due to its specific characteristics, such as the lipoteichoic acid of Gram-positive bacteria (which are capable of generating cellular activation), the use of the mucosa as an alternative route to induce immunity, besides inducing humoral and/or cellular immunity (YURINA, 2018a).

Thus, the present review aimed to present and discuss the most recent data on the development of recombinant vaccines using lactic acid bacteria as vectors.

2 METHODOLOGY

The present study is an integrative review. Searches were carried out in the PubMed and Scopus electronic databases, and original articles were selected in English. The following descriptors were used: “lactic bacteria”, “recombinant vaccine” and “bacterial vector”. Only articles published between 2016 and 2021 were considered. Nine (9) articles were selected to conduct this review after completed analysis.

3 RESULTS AND DISCUSSION

Study Selection Process Sixty-seven (67) articles were identified in the database search. Three independent investigators carried out the entire analysis of inclusion and eligibility for the study. Duplicates were removed and the remaining titles / abstracts were analyzed, leading to the elimination of thirty-six (36) articles according to the selection criteria (Table 3).

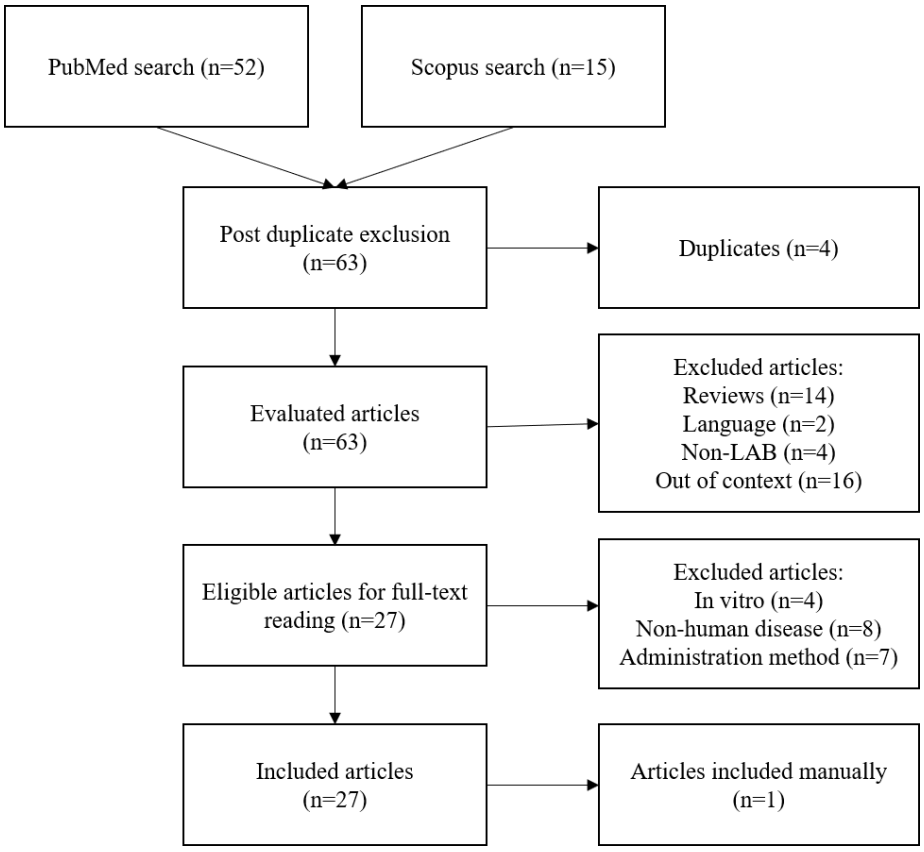
Table 4 – Inclusion and exclusion criteria for the study.

INCLUSION CRITERIA
Original articles / Animal studies / English articles / Published between 2016-2021 / Related to the use of LAB as vectors for recombinant vaccines.
EXCLUSION CRITERIA
Vaccine adjuvant analysis only / Analysis of non-human diseases.

Source: Own authorship (2021).

After analysis of full-texts, 20 articles were excluded. An extra article was added for meeting the eligibility criteria although it was not identified in the search using the descriptors. Finally, nine (9) articles were selected as eligible to be addressed in the present study (Figure 10).

Figure 10 – Study flow diagram.



Source: Own authorship (2021).

3.1 CHARACTERISTICS OF THE EXPERIMENTS

The predominant countries where studies were carried out were Iran (n = 4) and China (n = 3), and the two remaining studies were performed in Malaysia and India. The animal model chosen in the studies was female BALB/c mice (n = 7), except for one study that used female C57BL/6 mice (TAGHINEZHAD-S *et al.*, 2019). The age of the animals varied from 4 to 8 weeks and the animals per experiment group ranged from 6 to 40 (Table 4).

Table 5 – Experiments' characteristics.

Reference	Mice ♀	Age	Groups	n / Group
ALIMOLAEI, M. *et al.*, 2018	BALB/c	6 – 8 weeks	3	10
ZHANG, R. *et al.*, 2016	BALB/c	6 weeks	3	6

SONG, B. *et al.*, 2018	BALB/c	7 weeks	4	40
MOHAMMADI, E. and GOLCHIN, M., 2020	BALB/c	6 - 8 weeks	3	12
SREEROHINI, S. *et al.*, 2019	BALB/c	4 - 6 weeks	2	6
JOAN, S. S. X. *et al.*, 2016	BALB/c	6 - 8 weeks	3	6
ALIMOLAEI, *et al.*, 2017	BALB/c	6 - 8 weeks	3	10
TAGHINEZHAD-S, S. *et al.*, 2019	C57BL/6	6 - 8 weeks	4	10

Source: Own authorship (2021).

3.2 CHARACTERISTICS OF THE INTERVENTIONS

The studies interventions evaluated lactic acid bacteria strains as vaccine vectors. Half of the studies used *Lactococcus lactis* NZ9000, and the other half used *Lactobacillus casei* ATCC 393. The oral vehicle for vaccination and booster doses were common among the studies. However, the colony-forming unit (CFU) varied, such as the number of doses and the interval between them (Table 5). Regarding the target pathogen, three studies tested vaccines against two types of *Clostridium perfringens* toxins (n = 3), and the other targets were *Helicobacter pylori*, *Brucella abortus*, *Escherichia coli* O157:H7 and *Shigella dysenteriae*, H1N1 influenza and HPV-16. In addition, most studies aimed to develop an alternative to the conventional vaccines already used against *C. perfringens*, *B. abortus*, H1N1 influenza and HPV-16 (n = 6), while the other studies approaches are the development of recombinant vaccines for non-preventable pathogens by immunization until this moment (Table 5).

Table 6 – Intervention characteristics.

Reference	**Pathogen**	**Pre-existing vaccines**	**LAB strain**	**CFU**	**Dose - dosage**	**Interval between doses**
ZHANG, R. *et al.*, 2016	*Helicobacter pylori*	No	*Lactococcus lactis* NZ3900	5×10^11	4 - 0.2 mL	14 d
SONG, B. *et al.*, 2018	*Clostridium perfringens*	Yes	*Lactobacillus casei* ATCC 393	2×10^9	12 - 0.2 mL	14 d

MOHAMMADI, E. and GOLCHIN, M., 2020	*Brucella abortus*	Yes*	*Lactobacillus casei* ATCC 393	10^9	9 - 0.2 mL	14 d
SREEROHINI, S. *et al.*, 2019	*Escherichia coli* O157:H7; *Shigella dysenteriae*	No	*Lactococcus lactis* NZ3900	2×10^9	12 - 0.1 mL	14 d
JOAN, S. S. X. *et al.*, 2016	H1N1 influenza	Yes	*Lactococcus lactis* NZ3900	~10^10	9 - ?	14 d
ALIMOLAEI, M. *et al.*, 2017	*Clostridium perfringens*	Yes	*Lactobacillus casei* ATCC 393	10^9	9 - 0.2 mL	16 d
TAGHINEZHAD-S, S. *et al.*, 2019	HPV-16	Yes	*Lactococcus lactis* NZ3900	10^9	9 - 0.1 mL	14 d

Source: Own authorship (2021).

Note: “d” - days; “?” - information not found; “*” - only for animals.

3.3 CHARACTERISTICS OF THE VECTOR DEVELOPMENT

Regarding the methodological approach for the construction of the recombinant bacteria, the procedures were very similar among the studies (Table 6). Except for one study that used a constructed bacteria from a previous paper of the same group (SONG *et al.*, 2018, 2014a), all the other studies described the construction of their own vectors. Moreover, most studies intended to express their chosen protein in the cell surface (n = 6), except for SREEROHINI *et al.* (2019) that doesn’t specify this information and for ZHANG, *et al.* (2016) that constructed a plasmid without any modifications for cell wall anchoring. Regarding the development of anchored proteins, only JOAN, *et al.* (2016) failed to do so. In order to enhance immunogenicity, eliminate toxicity or optimize protein production, four studies made modifications in the genes used for immunogenicity (Table 6).

Table 7 – Cloning Vector Characteristics.

Reference	Antigen Gene	Cloning Vectors	Additional gene modification	Site of expression
ALIMOLAEI, *et al*., 2018	Alpha-toxin - DQ202275.1	pT1NX	Dendritic cell-targeting peptide sequence	Cell surface
ZHANG, R. *et al.*, 2016	omp22; HpaA	pNZ8110	?	Cytoplasm
SONG, B. *et al.*, 2018	Alpha-toxin	pGBHCupp; pUC57–A1821	?	Cell surface
MOHAMMADI, E. and GOLCHIN, M., 2020	omp19	pT1NX	Dendritic cell-targeting peptide sequence	Cell surface
SREEROHINI, S. *et al.*, 2019	stx2A - NC_002695.1	pNZ8148	Removal of toxicity by restricting amplification of Stx2a1	Do not specify
JOAN, S. S. X. *et al.*, 2016	HA1	pNZ8048	?	Cell surface
ALIMOLAEI, *et al.*, 2017	β-toxin - L13198.1	pT1NX	Elimination of toxicity of βtoxin by specific mutation; Dendritic cell-targeting peptide sequence	Cell surface
TAGHINEZHAD-S, S. *et al.*, 2019	E6; optiE6	pNZ8123	Optimization for enhancement of protein production	Cell surface

Source: Own authorship (2021).

Note: When specified, the GenBank ID of the antigen gene sequence can be found in the “Antigen gene” column after a ‘-’. When more than one information in each cell is provided it is separated by ‘;’. When the information was not found it is indicated by “?”.

3.4 RESPONSES TO VACCINES SAFETY AND EFFICACY

In order to evaluate the safety and efficacy of the developed vaccines some studies performed additional tests after immunization. Six studies performed a challenge after immunization and additional analysis were conducted by five of them (Table 7).

Table 8 – Vaccine safety and efficacy.

Reference	Follow-up	Results	Additional analysis	Additional analysis results
ZHANG, R. *et al.*, 2016	ø	ø	ø	ø
SONG, B. *et al.*, 2018	10 days	100% survival (1.5 MLD); 67% survival (2 MLD)	Splenic lymphocyte proliferation test	Increased cell proliferation.
MOHAMMADI, E.; GOLCHIN, M., 2020	14 days	Higher degrees of protection (OMP19)	Gastrointestinal histopathology	No histopathological damage in the small intestine.
SREEROHINI, S. *et al.*, 2019	21 days	100% survival (*S. dysenteriae*); 84% survival (*E. coli* O157)	Kidney and gastrointestinal histopathology	Minimal tubular dilation.
JOAN, S. S. X. *et al.*, 2016	ø	ø	ø	ø
ALIMOLAEI, M. *et al*., 2017	10 days	100% survival (1 and 5 MLD); 50% survival (10 MLD)	Gastrointestinal histopathology	No important histopathological changes, except two mice with mild lymphocytic enteritis.
TAGHINEZHAD-S, S. *et al.*, 2019	60 days	60% survival (*L. lactis*optiE6); 40% survival (*L. lactis*-E6)	Tumor growth (follow-up: 15 days)	*L. lactis*-optiE6 showed better therapeutic antitumor effects compared to *L. lactis*E6.

Source: Own authorship (2021).

Note: “MLD”: minimum lethal dosage; “ø”: did not perform challenge and / or additional analysis.

3.4.1 Antibody response

Data analysis demonstrated that immunization was effective inducing specific IgG (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020; SONG *et al.*, 2018; SREEROHINI; BALAKRISHNA; PARIDA, 2019; TAGHINEZHAD-S *et al.*, 2019; ZHANG *et al.*, 2016) and IgA (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; SREEROHINI; BALAKRISHNA; PARIDA, 2019) in treated animal sera compared to the control groups. The same result was observed in mucosal specific IgA levels (JOAN *et al.*, 2016; MOHAMMADI; GOLCHIN, 2020; SONG *et al.*, 2018; SREEROHINI; BALAKRISHNA; PARIDA, 2019; TAGHINEZHAD-S *et al.*,

2019). However, in another study, only in fecal suspensions these differences were observed, but not in stomach washes (JOAN *et al.*, 2016). No statistical difference was observed in serum IgA (JOAN *et al.*, 2016), intestinal sIgA (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; ZHANG *et al.*, 2016) and stomach sIgA (JOAN *et al.*, 2016) after immunization. Booster doses were necessary to induce significant specific antibody response in three of the studies (SONG *et al.*, 2018; SREEROHINI; BALAKRISHNA; PARIDA, 2019; TAGHINEZHAD-S *et al.*, 2019). Moreover, MOHAMMADI and GOLCHIN (2020) demonstrated that the bacterial vector vaccine developed was able to induce high levels of specific antibodies (IgA and IgG) as the traditional vaccine already used to prevent *Brucella abortus* disease.

3.4.2 Cytokine response

In regard to cytokine production and profile, only four studies performed this analysis and the methods varied between accessed cytokines, statistical approach, and measurement tests (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020; SONG *et al.*, 2018; TAGHINEZHAD-S *et al.*, 2019). Three studies analyzed IL-2, IL-4, IL-10, IFNγ (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020; SONG *et al.*, 2018), two calculated the concentrations of these molecules in the serum (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020) and SONG *et al.* (2018) used flow cytometry of splenocytes for measuring intracellular levels of cytokines. MOHAMMADI and GOLCHIN (2020) found significant increase in the level of all investigated cytokines compared to both controls (PBS and empty vector). The same cytokines enhancement was observed for SONG *et al.* (2018) in immunized groups. ALIMOLAEI *et al.* (2017) only observed a significant increase in IFNγ level. Moreover, TAGHINEZHAD-S *et al.* (2019) investigated two recombinant bacteria and observed increase in the level of IL-2 and IFNγ in intestinal mucosal lymphocytes, splenocytes, and vaginal lymphocytes of immunized mice, although the recombinant bacteria with optimized antigen led to a major effect than the wild-type version.

4 DISCUSSION

The development of recombinant vaccines using live bacterial vectors has been intensively studied, as they are able to effectively stimulate the immune system, including long term adaptive immunity and provide an alternative delivery to invasive or potential routes of contamination to mimic the entry of the pathogen (YURINA, 2018a). Therefore, this vaccine technology approach is innovative and an advantageous alternative for classic vaccines.

In the present review the majority of the studies were performed in Asian countries (Iran, China, Malaysia and India). The interest of these countries in the development of recombinant vaccines is expected, since in a context of less developed countries, whose epidemiological transition is difficult, research into infectious diseases is widely investigated and, therefore, investing in a technology known to be effective for a range of diseases is advantageous (SONG *et al.*, 2018).

Lactic acid bacteria are a potential group of bacteria to serve as a safe and effective vehicle for delivering antigens as they are non-pathogenic and non-invasive, act as natural adjuvants, do not colonize the host's mucosal surfaces for a long period and can survive the gastrointestinal tract (YURINA, 2018a). *Lactococcus lactis* genome was first sequenced and it is easily manipulated justifying this strain as the main LAB investigated, and *Lactobacillus casei* is increasing in importance as a promisor bacterial vector for mucosal immunization (YURINA, 2018a). Accordingly, *L. casei* ATCC 393 and *L. lactis* NZ3900 were the LAB strains selected by the studies reviewed in the present work, since their oral administration enhanced local and / or systemic immunities, including humoral and / or cellular immune responses (SONG *et al.*, 2018). Additionally, the strains selection was based on LAB characteristics already described as probiotic properties, potential immune-modulatory capacities (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; JOAN *et al.*, 2016) and safety advantage in comparison to attenuated vaccines (ZHANG *et al.*, 2016). The administration of multiple booster doses was necessary to ensure better immune response in the tested vaccines (Table 5). Despite this, the use of LAB as recombinant vaccines presents several advantages justifying its development even when current vaccines against these diseases are already being commercialized. Some of these advantages involve low hypersensitivity responses, simple production, good cost-effectiveness, home self-administration and no need for injections or cold transport (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; TAGHINEZHAD-S *et al.*, 2019).

In order to develop functional recombinant vaccines, it is necessary to construct vectors which are effective in the expression of the target proteins to ensure cloning success by the

microorganism of choice (SONG *et al.*, 2014a). This process usually begins with screening for specific immunogenic proteins followed, when necessary, by removal of toxicity-related sequences or addition of genes as adjuvants, stimulating antigen presentation, dendritic cell attraction or anchorage of proteins in the bacterial cell wall (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017). Both strategies (gene addition and removal) were used by the reviewed studies, in order to ensure benefits in the safety and improved efficacy of vaccines (Table 6). After selection of gene sequences and construction of plasmids, antigen production must be analyzed for successful protein expression (SONG *et al.*, 2014a). In this review, only one study failed to express the antigen as planned, which aimed to express the HA1 epitope on the cell wall. However, the vaccine produced was able to induce the production of anti-HA sIgA, but only in feces, confirming the occurrence of mucosal immunity (JOAN *et al.*, 2016).

Although recombinant bacterial vaccines are usually constructed by insertion of target genes in the plasmidial DNA, an important possible limitation is the lack of essential genes in the plasmids, which can lead to its elimination after a few generations if there is no induction by their promoters (SONG *et al.*, 2014a). On that way, the insertion of the target gene in bacterial chromosomal DNA was the alternative used by SONG, B. *et al.* (2018) to avoid this limitation providing gene stability, eliminating selection requirements and ensuring genes transmission to other generations.

Following the successful construction of the recombinant bacterial vector, confirmation of the safety and efficacy of the vaccines was demonstrated (Table 7). Interestingly, ALIMOLAEI *et al.* (2017) and SONG *et al.* (2018) produced vaccines against the alpha-toxin of *Clostridium perfringens* and different mortality rates were observed, illustrating the importance of further investigation in this vaccine approach. Furthermore, ZHANG *et al.* (2016) demonstrated systemic immunization by specific IgG induction in sera against *H. pylori*, while SONG *et al.* (2018) was only able to confirm mucosal immunization against H1N1 influenza with higher IgA levels in fecal suspensions, although the absence of the challenge and additional analysis could diminish the accuracy of these results. It is noteworthy that *H. pylori* vaccine remains inexistent, thus, the immune response demonstrated by ZHANG *et al.* (2016) could represent an initial step in the development of this vaccine.

It is expected that LAB vaccines orally administered elicit a strong mucosal immunity as it is the first barrier to prevent infection through the gastrointestinal, respiratory and urogenital tract (NASCIMENTO; LEITE, 2012; SONG *et al.*, 2018). As expected, higher mucosal specific IgA levels were observed in the studies (MOHAMMADI; GOLCHIN, 2020;

SONG *et al.*, 2018; SREEROHINI; BALAKRISHNA; PARIDA, 2019; TAGHINEZHAD-S *et al.*, 2019). However, JOAN, *et al.* (2016) found no significant levels of serum and stomachal (specific IgA after immunization, whereas showed significantly higher fecal IgA, suggesting restricted induction of mucosal immunity to the intestinal lumen. ALIMOLAEI, GOLCHIN and EZATKHAH (2017), ALIMOLAEI, *et al.* (2017) and ZHANG, *et al.* (2016) also demonstrated that sIgA did not present any difference after immunization, therefore demonstrating inefficiency in the induction of mucosal immunity. Despite these results, it is important to consider that unsuccessful anchorage antigen to the vector cell surface and / or small sample size of the study could justify this data.

Recombinant bacteria as live vectors for mucosal vaccination, especially lactic acid bacteria, are a strategic use of their ability to not only induce local mucosal response against their own proteins, but also to stimulate adaptive and humoral immunity against expressed heterologous antigens in them, since most commercialized vaccines are only successful for activating B cells and consequently generating specific antibodies against pathogens mainly combated through humoral immunity (NASCIMENTO; LEITE, 2012). In this regard, the successful induction of systemic immunity by secreting specific IgG (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020; SONG *et al.*, 2018; SREEROHINI; BALAKRISHNA; PARIDA, 2019; TAGHINEZHAD-S *et al.*, 2019; ZHANG *et al.*, 2016) and IgA (ALIMOLAEI *et al.*, 2017; ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; SREEROHINI; BALAKRISHNA; PARIDA, 2019) in immunized animals, compared to control groups, confirm this vaccination approach efficacy.

MOHAMMADI and GOLCHIN (2020) provides evidence that the bacterial vaccine reached high levels of immunoglobulins (IgA in the intestinal content and IgG in the serum) in the same way as the traditional vaccine already in use to prevent Brucella abortus disease in animals. This result provides a new perspective on the prevention of brucellosis, since there is no human vaccine available and the vaccine used in animals is partially accepted, due to serious side effects, such as abortion in pregnant female.

Along with immunoglobulin production, another way to evaluate immunogenicity of candidate vaccines is detecting cytokine production in the serum or in cultured splenocytes. Cytokines such as IL-2, IL-4, IFN-γ and IL-10 are notably produced by Th1 (IL2 and IFN-γ) and Th2 (IL-4 and IL10) lymphocytes, and their measurement can indicate the induction of these immune profiles (ALIMOLAEI; GOLCHIN; EZATKHAH, 2017). In the analyzed studies, the cytokines increase (IL-2 and IFN-γ or IL-4 and IL-10) were attributed to an indication of cellular immune response or humoral immune response, respectively

(ALIMOLAEI; GOLCHIN; EZATKHAH, 2017; MOHAMMADI; GOLCHIN, 2020). Moreover, the production of IL-2 and IFN-γ has also been attributed to a clearance enhancement of intracellular pathogens and the class shifting from IgM to IgG (TAGHINEZHAD-S *et al.*, 2019), which is consistent with the highest levels of IgG found in the studies.

Along with the effect over the induction of systemic and mucosal immunity, recombinant bacterial vaccines can contribute for neoplasia treatment. In that sense, it is known that two oncoproteins, E6 and E7, are involved in the transformation of HPV-infected cells into neoplasia, therefore, TAGHINEZHAD-S *et al.* (2019) engineered a *L. lactis* bacteria expressing the E6 oncogene and it proficiently stimulated the mucosal and systemic immunities, as already described. However, minor tumor growth rate, lower progression of tumor size and better survival rate were observed in immunized mice with *L. lactis* harboring codon-optimized E6 in comparison to immunized mice with *L. lactis* harboring native E6.

In summary, these data show the importance of investigating the development of new technologies and their optimization for the construction of more effective and safer vaccines that are easier to administer and more cost-effective, adding even more value to the available and constantly evolving biotechnology tools.

5 CONCLUSION

Recombinant vaccines using lactic acid bacteria have been extensively studied due to their diverse intrinsic beneficial characteristics and, consequently, optimization in the effectiveness of this vaccine approach. This review presents data that confirm the efficacy of LABs as vaccine vectors in animal models, which can stimulate mucosal and systemic immunities. However, due to the limitations of the studies, further clinical trials are needed to investigate if these results translate to humans in all groups to be immunized.

CHAPTER 3:
EXPRESSION AND QUANTIFICATION OF THE SARS-CoV-2 RECEPTOR BINDING DOMAIN (RBD) PROTEIN BY RECOMBINANT BACTERIA: EXPLORING BIOTECHNOLOGICAL POTENTIAL FOR VACCINE VECTOR DEVELOPMENT

1 INTRODUCTION

1.1 COVID-19 PANDEMIC

In December 2019, an outbreak of viral pneumonia affected the population of Wuhan, China. The cases were epidemiologically linked to the Huanan seafood market in Hubei province (WENJIE *et al.*, 2020; ZHU *et al.*, 2020). Through genetic sequencing and bioinformatics tools, a new human-infecting coronavirus was identified, initially named "novel coronavirus 2019" or "2019-nCoV" (LU *et al.*, 2020). Evidence from the sequencing of the SARS-CoV genome suggested that mutations likely enabled a "spill-over," making it a pathogen capable of infecting humans (HARRISON; LIN; WANG, 2020; RABALSKI *et al.*, 2022). Consequently, the virus was renamed "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-CoV-2), responsible for causing "Coronavirus Disease" (COVID-19) (LU; STRATTON; TANG, 2020). Initially, this outbreak triggered pneumonia cases in China, rapidly spreading worldwide (MOHAMED; EL-AZIZ; STOCKAND, 2020). In January 2020, the World Health Organization (WHO) declared COVID-19 a public health emergency of international concern, and on March 11, 2020, it was characterized as a pandemic (WHITWORTH, 2020; WHO, 2020).

By June 9, 2024, there were 775,615,736 reported SARS-CoV-2 infections and 7,051,323 related deaths in over 100 countries worldwide (WORLD HEALTH ORGANIZATION, 2024). Before the widespread availability of COVID-19 vaccinations, governments employed contact tracing as a key measure to limit the virus's transmission (WHITWORTH, 2020). Given the devastating impact of COVID-19, a global effort began to achieve "herd immunity" or "community immunity," where immunity initially develops at the individual level and then extends to the population level (RANDOLPH; BARREIRO, 2020). Active immunization is capable of generating herd immunity, highlighting the urgent need for vaccines, as the rapid spread of SARS-CoV-2 threatened to overwhelm healthcare systems, resulting in increased mortality not only from COVID-19 but also from other causes due to resource depletion (MISTRY *et al.*, 2022; RANDOLPH; BARREIRO, 2020). The remarkable research efforts in developing an effective COVID-19 vaccine have led to the availability of several safe and effective options (ABBASI, 2022; ALTMANN; BOYTON, 2022; DUBÉ; MACDONALD, 2022). Consequently, over 17.26 billion vaccine doses have been administered worldwide as of June 2024 (OUR WORLD IN DATA, 2024).

Although most studies suggest that current vaccines effectively prevent severe COVID-19 progression (MISTRY *et al.*, 2022), there remains uncertainty regarding the most efficient vaccine approach. Additionally, the long-term side effects of the currently approved COVID-19 vaccines remain unknown (MOHAMED *et al.*, 2022), requiring long-term follow-up investigations. Ongoing genomic research and global surveillance of SARS-CoV-2 variants, combined with available therapies, allow scientists to assess the need for periodic vaccine updates and other treatments.

1.2 SARS-CoV-2

Human coronaviruses (HCoVs) are members of the *Coronaviridae* family of coronaviruses (CoVs) responsible for various respiratory tract diseases, from common colds to bronchiolitis and pneumonia (PENE *et al.*, 2003). They can also cause potential enteric and neurological diseases (ARBOUR *et al.*, 2000; RESTA *et al.*, 1985; VIJGEN *et al.*, 2005). Studies indicate that the rapid evolution of HCoVs is linked to high rates of nucleotide substitution and recombination (VIJGEN *et al.*, 2005). The periodic emergence of HCoVs around the world has led to major outbreaks of fatal pneumonia since the early 21st century (WU *et al.*, 2021). The first outbreak was caused by the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) in November 2002 in Foshan, China (GE; HU; SHI, 2015), leading to a global infection in 2003 with a 10% mortality rate (LEE *et al.*, 2003). The second pandemic was caused by the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in June 2012 in Jeddah, Saudi Arabia (GE; HU; SHI, 2015), with a 35% mortality rate (DE GROOT *et al.*, 2013). Finally, SARS-CoV-2 was responsible for the third major outbreak, resulting in the COVID-19 pandemic (ZHU *et al.*, 2020).

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a single-stranded positive-sense RNA virus, enveloped, belonging to the *Betacoronavirus* genus (LU *et al.*, 2020). SARS-CoV-2 causes a spectrum of symptoms from mild respiratory and acute respiratory distress syndrome to systemic manifestations, potentially leading to death. Beyond clinical concerns, the infection exhibits high transmissibility, which is facilitated by subclinical cases, contributing to its widespread incidence and prevalence (ROTHAN; BYRAREDDY, 2020).

The first complete genome of SARS-CoV-2 was described on January 3, 2020, by scientists at the National Institute for Viral Disease Control and Prevention (IVDC) in China. They used a combination of Sanger sequencing, Illumina sequencing, and Nanopore sequencing

from a bronchoalveolar lavage fluid (BALF) sample of a patient from Wuhan (WENJIE *et al.*, 2020). This sequencing effort enabled the detection of viral variations, or 'variants,' which arise naturally through nucleotide changes in the viral genome during replication (LI *et al.*, 2021).

Viral variations, or "variants," occur through nucleotide changes in the viral genome that naturally arise during replication. According to the WHO, SARS-CoV-2 variants are classified into two categories: variants of concern (VOC) and variants of interest (VOI). VOCs are characterized by higher transmissibility, increased virulence, resistance to vaccines or immunity from prior infections, and the ability to evade diagnostic detection (MISTRY *et al.*, 2022). According to the latest update, the VOC includes several lineages descended from Omicron (WORLD HEALTH ORGANIZATION, 2024). The VOI comprises specific lineages such as JN.1 and KP.2, which have distinct genetic characteristics and have been documented in recently collected samples (WORLD HEALTH ORGANIZATION, 2024).

The emergence of SARS-CoV-2 variants challenges the effectiveness of vaccines and natural immunity. Viral immunity is established after a primary immune response to a viral antigen, leading to immunological memory. For SARS-CoV-2, natural immunity can develop from a prior infection. However, the existence and duration of specific immunological memory cells that confer enhanced protection in previously infected individuals are poorly understood (MELLET; PEPPER, 2021). Understanding the molecular mechanisms essential for SARS-CoV-2 infection in human cells provides insights into viral transmissibility and pathogenesis and identifies targets for control strategies.

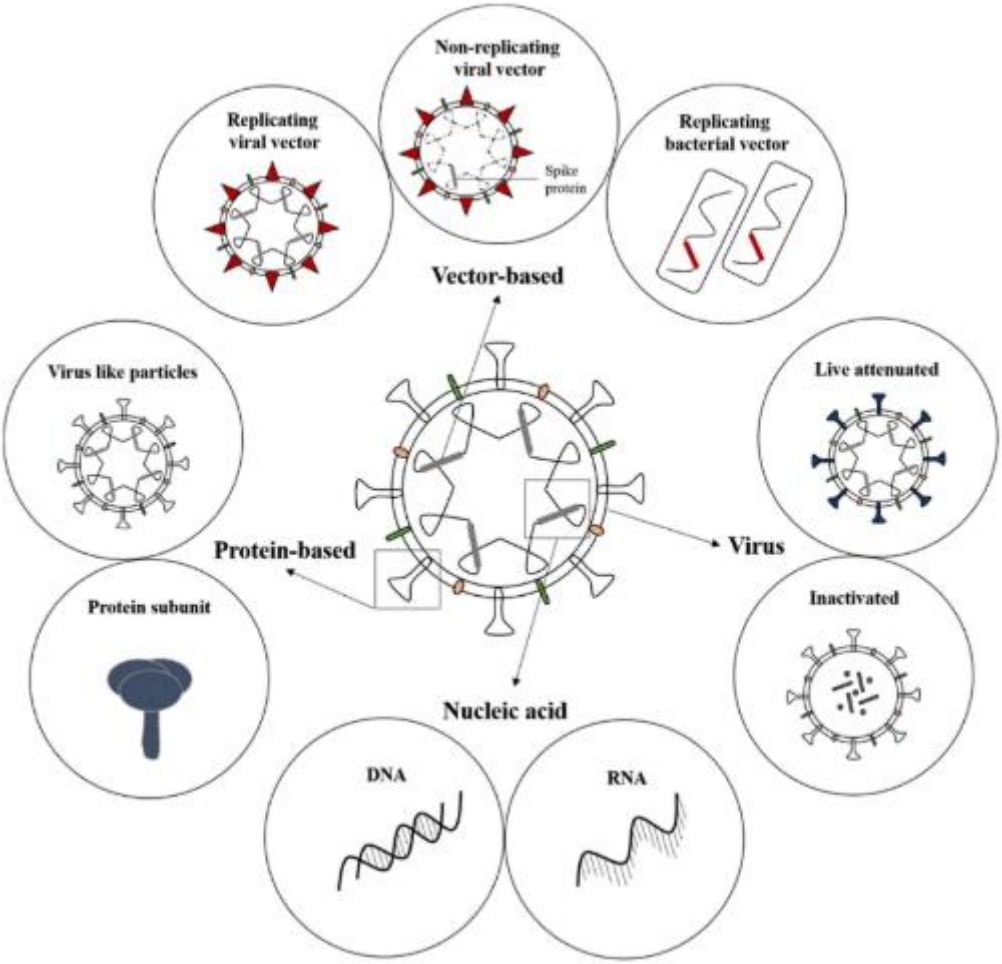
In this context, coronaviruses have four main structural viral genes: nucleocapsid (N), membrane (M), envelope (E), and spike (S). The spike (S) protein, integrated into the viral envelope, aids the virus's entry into target cells and has two subunits, S1 and S2 (JACKSON *et al.*, 2022; MAIER; BICKERTON; BRITTON, 2015). The receptor-binding domain (RBD) is located in the S1 subunit, where mutations are of most concern, as this domain interacts with the angiotensin-converting enzyme 2 (ACE2) receptor to enter host cells. Most fitness-enhancing amino acid changes in circulating variants are found in the RBD (JACKSON *et al.*, 2022; ZHOU *et al.*, 2021). The transmembrane S2 subunit facilitates the fusion of the viral and cellular membranes (HOFFMANN; KLEINE-WEBER; PÖHLMANN, 2020). Understanding variant emergence and its effect on vaccine strategies targeting the S protein can lead to improved, updated vaccines and appropriate treatment protocols to control virus spread (MISTRY *et al.*, 2022).

Research in these areas is crucial for addressing the challenge of establishing durable immunity against SARS-CoV-2 and managing COVID-19 outcomes effectively.

1.3 DEVELOPMENT OF VACCINE STRATEGIES AGAINST COVID-19

Vaccines are among the most effective tools for protecting individuals against infectious diseases that can be prevented through immunization, including COVID-19, and their associated morbidity and mortality (WORLD HEALTH ORGANIZATION, 2020). COVID-19 vaccine candidates have been developed using different vaccine strategies, including non-replicating or replicating viral vectors, protein subunits, inactivated or attenuated viruses, mRNA, and DNA (Figure 11).

Figure 11 – Types of SARS-CoV-2 vaccines.



Source: MELLET; PEPPER, 2021.

Among the COVID-19 vaccines formulated and approved by the United States Food and Drug Administration (FDA), the following technology platforms and their respective representatives are: mRNA-based vaccines (Pfizer-BioNTech Comirnaty – BNT162b2, Moderna – mRNA-1273), protein-based vaccines (Novavax – NVX-CoV2373), viral vector-based vaccines (Johnson & Johnson Janssen – Ad26.COV2.S, Oxford-AstraZeneca – AZD1222/ChAdOx1, Sputnik V – Gam-COVID-Vac-rAd26/rAd5), and traditional inactivated vaccines (Sinopharm – BBIBP-CorV, Sinovac – CoronaVac, and Covaxin – BBV152) (BADEN *et al.*, 2021; ELLA *et al.*, 2021; LOGUNOV *et al.*, 2020; MELLET; PEPPER, 2021; VAN DOREMALEN *et al.*, 2021; WU *et al.*, 2021; ZOU *et al.*, 2021).

The emergence and spread of SARS-CoV-2 variants globally represent a significant threat to public health, as they compromise the efficacy of currently used vaccines (WIBMER *et al.*, 2021; ZHOU *et al.*, 2021). These variants possess genomic alterations that may increase the viral fitness, including improved immune escape mechanisms, which enhance their survival compared to earlier circulating strains (HADJ HASSINE, 2022; MISTRY *et al.*, 2022).

Other vaccination strategies against COVID-19 are under investigation, including bacterial vector-based vaccines. The use of microorganisms, particularly live bacteria, as vaccine vectors has been extensively studied (CHAU *et al.*, 2023; TAGHINEZHAD-S *et al.*, 2021). Advances in molecular biology and genetic engineering have enabled these vectors to present heterologous antigens by introducing genes that encode the antigen of interest (NASCIMENTO; LEITE, 2012). In this type of vaccine system, genetically modified recombinant bacteria have most of their virulence genes deleted, making the organism non-virulent and ensuring the safety of the host (DA SILVA *et al.*, 2014). The ability to easily incorporate large target sequences using plasmid or phage systems, along with the lower costs and reduced labor involved, makes live bacterial vectors a favorable choice for genetically modified bacterial production (LIN; VAN; SMOOKER, 2015).

The immune response induced by specific bacterial characteristics, such as lipopolysaccharide (LPS) in Gram-negative bacteria, lipoteichoic acid (LTA) in Gram-positive bacteria, or Pathogen-Associated Molecular Patterns (PAMPs) recognized by Pattern Recognition Receptors (PRRs), effectively activates the immune system and stimulates the long-term adaptive immune response, making these organisms suitable vectors for use in vaccination strategies (BECKER; NOERDER; GUZMÁN, 2008; YURINA, 2018a). Therefore, live bacterial vectors can serve as effective vaccine platforms by activating innate cells, thereby inducing cellular and humoral immune responses against the delivered target antigens (DA SILVA *et al.*, 2014; DETMER; GLENTING, 2006). Furthermore, they can enhance vaccine

efficacy by inducing trained immunity, which involves long-term functional reprogramming innate immune cells (COVIÁN *et al.*, 2021; GOODRIDGE *et al.*, 2016; NETEA *et al.*, 2020). Additionally, vaccination with live bacterial vectors can be administered via mucosal routes (intranasal, oral, or intravaginal), offering a promising alternative to invasive methods (LEE *et al.*, 2006; LI; ZHANG; ZHOU, 2008). Oral administration, in particular, is straightforward and does not require specialized skills (YURINA, 2018a, 2018b), while also being the primary point of pathogen entry.

The scientific community's ongoing efforts to develop an effective and safe vaccine require continuous evaluation of all vaccine platforms and their respective advantages, which should be thoroughly investigated and subsequently proposed.

1.3.1 Use Of Lactic Acid Bacteria Recombinant Vectors In Vaccination Strategies

A major challenge in developing countries is the increasing population coupled with rising infectious disease incidence and challenges in vaccine delivery and coverage, significantly impacting morbidity and mortality. Additionally, the high cost of vaccines adds to this challenging scenario. Asian countries like Iran, China, Malaysia, and India lead in the research and development of recombinant vaccines, particularly because they are regions commonly affected by epidemiological transitions, where less developed nations encounter significant challenges and extensive research on infectious diseases is crucial (ROCHA *et al.*, 2021). In this context, investing in technology proven effective against multiple diseases is advantageous (ROCHA *et al.*, 2021; SONG *et al.*, 2014b). Thus, exploring more accessible and effective vaccine technologies for long-term immunity is crucial.

Probiotic Lactic Acid Bacteria (LAB) have garnered considerable interest as promising candidates for safe and effective vaccines. In addition to benefiting human health and typically holding GRAS status, LABs used in vaccination strategies can be administered via mucosal routes, inducing both mucosal and systemic immune responses (WELLS; MERCENIER, 2008). This is crucial as many pathogens utilize mucosal surfaces for entry into the human body (ASHRAF; SHAH, 2014; MOJGANI; SHAHALI; DADAR, 2020).

LAB possess essential characteristics that make them robust candidates as mucosal vaccine vectors, including stability at room temperature, resistance to acids and bile, capability to activate innate and adaptive immune responses endogenously, and their ability to undergo genomic modification using well-established molecular techniques (MANCHA-AGRESTI *et al.*, 2017; TAKAHASHI *et al.*, 2019). In the context of COVID-19, an *in vitro* study indicated

that recombinant *Lactiplantibacillus plantarum* expressing the optimized spike gene from SARS-CoV-2 can induce mucosal immune responses against the pathogen. However, further *in vivo* studies are necessary to validate these findings (LEE; KIM, 2022; WANG *et al.*, 2020).

While recombinant bacterial vaccines are usually constructed by inserting the target gene into plasmid DNA, a significant potential limitation is the lack of essential genes on plasmids and the high energetic cost associated with plasmids maintenance on the cell, which can lead to their elimination after several generations (SONG *et al.*, 2018). Plasmid integration into the chromosome ensures stable maintenance and consistent expression of the gene of interest, eliminating the requirement for selective pressure to retain the plasmid, as demonstrated by the integration of the plasmid-encoded alpha toxin gene of *Clostridium perfringens* into the genome of *Lacticaseibacillus casei* (SONG *et al.*, 2014a). This is particularly advantageous in industrial processes where large-scale and prolonged production is required. Additionally, the chromosomal integration of genes reduces the metabolic burden on the host cells, leading to higher cellular fitness and potentially increased production of the target protein (GAO *et al.*, 2014).

For plasmid integration into the genome, insertion sequences (ISs) can be utilized, as they are compact genetic structures typically ranging in size from 800 to 2,500 base pairs. They encode transposition functions and are widely distributed in both the chromosomes and plasmids of LAB (MAHILLON; CHANDLER, 1998). For example, the IS1223 is an insertion element isolated in *Lactobacillus* spp., demonstrating functionality as an integration vector in LAB (WALKER; KLAENHAMMER, 1994). IS1223 facilitates stable integration of the gene of interest into the host's chromosomal DNA via homologous recombination (JANG *et al.*, 2003).

These genetic tools are particularly advantageous in industrial applications where stable genetic modifications are crucial for ensuring consistent and reliable production (YONGPING XIN *et al.*, 2019). Advances in genomic sequencing techniques have significantly increased the number of fully sequenced genomes for probiotic bacterial strains, including LAB, enabling a deeper understanding of metabolic pathways through the use of genomic computational tools (YADAV *et al.*, 2017). These advancements allow for the evaluation and proposal of novel, promising probiotic LAB strains as recombinant vaccine vectors. Therefore, the present study aimed to design and select suitable plasmid vectors for cloning the RBD gene of SARS-CoV-2 in Lactic Acid Bacteria to explore its biotechnological potential for the development of vaccine vectors.

2 MATERIAL AND METHODS

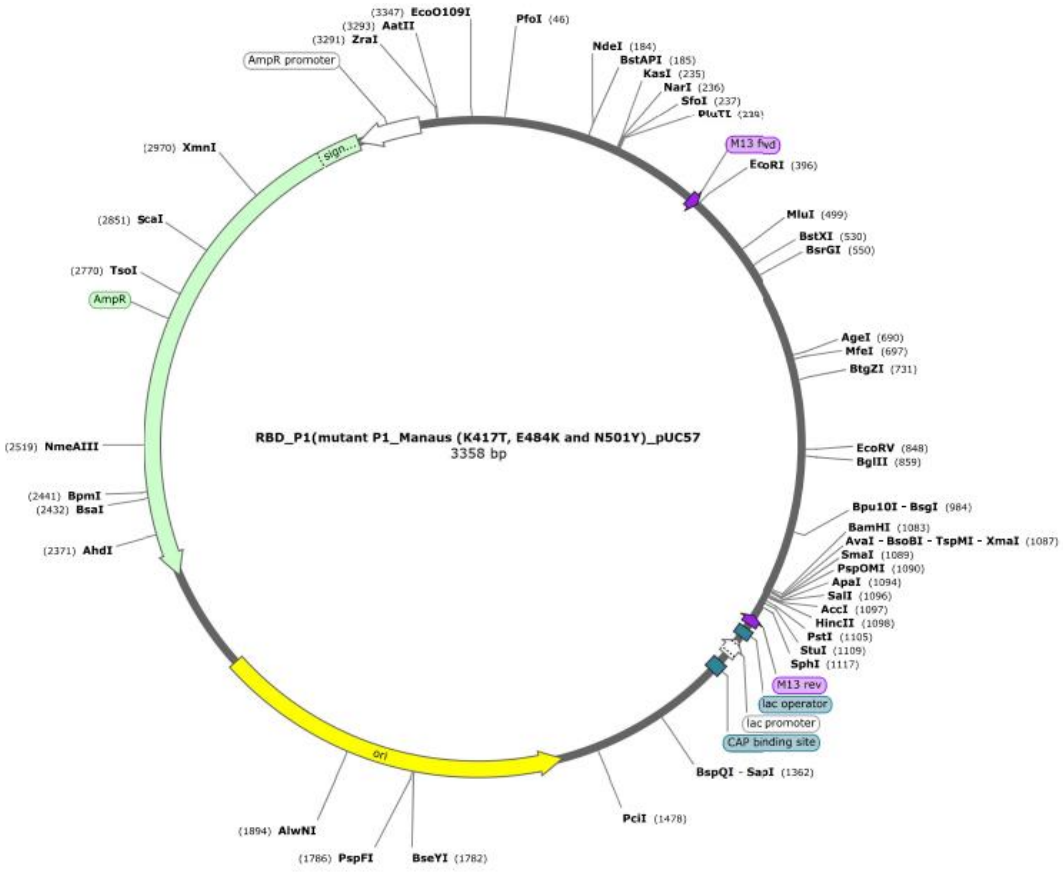
2.1 PLASMID DESIGN FOR BACTERIAL INTEGRATION IN BIOTECHNOLOGICAL APPLICATIONS

The design of the synthesized plasmids for *Escherichia coli* and Lactic Acid Bacteria was performed. The *E. coli* competent bacterial strain cloning followed the transformation protocol and subsequent analyses described below.

2.2 *Escherichia coli* STUDY DESIGN AND BACTERIAL CLONING

The cloning vector synthesis for *Escherichia coli* was performed by ACTGene (*Análises Moleculares* Ltda., Brazil) in collaboration with GenScript Biotech Corporation. The gene encoding the RBD region of the SARS-CoV-2 spike protein from the P1 variant (also known as Gamma or GR/501Y.V3), identified in December 2020 in Manaus, Brazil, was inserted into the pUC57 plasmid, along with the Ampicillin resistance gene (*Amp*), resulting in a 3358 bp vector (Figure 12).

Figure 12 – Map of the synthesized cloning vector for *Escherichia coli*.



Source: Own authorship using SnapGene software.

Competent *Escherichia coli* BL21 (DE3) cells (Novagen) were used for the transformation process. Initially, Luria Bertani (LB) agar plates supplemented with ampicillin (50 µg/mL) were incubated at 37°C for 1 hour to achieve thermal equilibration. BL21 (DE3) competent cells were kept on ice until ready for use. Subsequently, 1–5 µL containing 5 to 10 ng of the target DNA construction was added to the competent cells. The mixture of competent cells and DNA was incubated on ice for 30 minutes. Following this incubation, the tubes underwent a brief heat shock at 42°C for 30 seconds. Subsequently, the tubes were immediately returned to ice for 2 minutes to promote cell recovery. After the recovery phase, 250 µL of pre-warmed super optimal broth with catabolite repression (SOC) medium at 37°C was added to each tube. The tubes were then incubated at 37°C with shaking at 225 rpm for 1 hour. Post-incubation, 100 µL from each transformation reaction was spread onto LB agar plates supplemented with ampicillin (50 µg/mL). The agar plates were left to dry under sterile conditions for 15 minutes before incubating at 37°C for approximately 16 hours to allow the

growth of successfully transformed cells. Subsequently, a single colony was inoculated into 2 mL of LB + Amp (50 μg/mL) medium and incubated again at 37°C with shaking at 250 rpm for 12 hours (0.6 to 0.8 OD_{600}). For long-term storage, 840 μL of the culture was transferred to a new microtube and mixed with 160 μL of 50% (v/v) glycerol. The aliquots were frozen in liquid nitrogen (N2) and stored at -80°C (SAMBROOK; FRITSCH; MANIATIS, 1989).

2.2.1 Plasmid DNA Extraction

For plasmid DNA extraction the transformant bacteria were cultured in LB broth supplemented with ampicillin (50 μg/mL) for 24 hours at 37°C. Approximately 1.5 mL of the culture was used for plasmid DNA extraction. The culture was centrifuged at 14,000 rpm for 20 seconds at room temperature to pellet the cells, and the supernatant was discarded. Subsequently, the pellet was resuspended in 100 μL of lysis buffer [2M glucose, 1M Tris (pH 8.0), 0.5M EDTA (pH 8.0), lysozyme, ddH2O], and incubated on ice for 5 minutes. After incubation, 200 μL of SDS alkaline solution was added, immediately mixing the tube by inversion, and incubating it again on ice for 5 minutes. Subsequently, 150 μL of cooled sodium acetate solution was added, the tube was repeatedly inverted to mix, and then incubated on ice for 15-30 minutes. After incubation, the tubes were centrifuged at 35,800 g for 5-10 minutes at 4°C. The supernatant was then transferred to a new tube, to which 300 μL of saturated phenol-tris and 300 μL of chloroform were added and vigorously mixed by shaking. This was followed by centrifugation at 14,000 rpm for 10 minutes at room temperature. The supernatant was transferred to new tubes, followed by adding 1.0 mL of cooled 95% ethanol and incubating at -20°C for 30 minutes. After incubation, centrifugation was performed at 14,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 100 μL of sodium acetate. Then, 200 μL of ice-cold 95% ethanol was added, the tube was incubated at -20°C for 15 minutes, and then centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed with 1 mL of chilled 70% ethanol. The excess ethanol was drained from the tubes by inversion, and they air-dried at room temperature for approximately 10 minutes. After drying, the pellet was resuspended in 40 μL of TE buffer pH 8.0 containing 50 μg/mL of RNase A (or RNase ONE). The solution was incubated at room temperature for 10 minutes, and stored at -20°C until use.

2.2.2 Integrity of Plasmid DNA

To analyze the integrity of the plasmid DNA, aliquots of the samples were loaded onto a 0.8% agarose gel (Kasvi®) prepared using TBE buffer (Tris-Borate-EDTA), and electrophoresed for approximately 1 hour and 40 minutes at 100 volts and 400 milliamps. A 1 Kb Super Ladder molecular weight standard (New England Biolabs, UK Ltd) was used. Subsequently, the gel was stained with Ethidium Bromide, and visualized using a UV transilluminator (GE Healthcare, UK).

2.2.3 Quantification of SARS-CoV-2 RBD protein

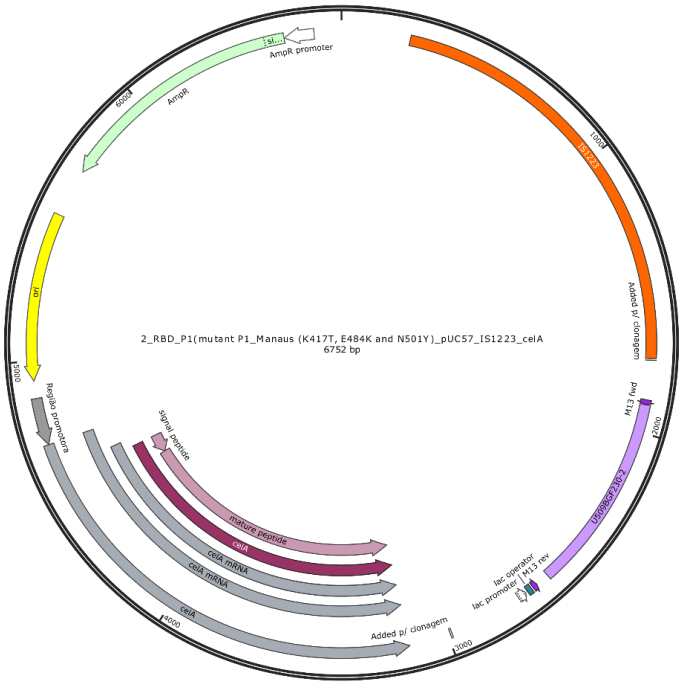
The protein concentration of the SARS-CoV-2 Spike RBD region in the supernatant and cell lysate of *Escherichia coli* BL21 (DE3) cultures transformed with the pUC57 plasmid containing the gene of interest was determined using the Enzyme-Linked Immunosorbent Assay (ELISA), specifically the "Human SARS-CoV-2 RBD ELISA Kit" from Thermo Fisher. The transformed bacteria were cultured in 5 mL of LB broth containing ampicillin (50 µg/mL) for 24 hours at 37°C. After this period, a subculture was performed by transferring 4 mL of the bacterial growth broth into 20 mL of fresh medium under the same conditions and incubated for an additional 48 hours at 37°C. The supernatant was separated by centrifugation at 12,000 rpm, at 4°C for 15 minutes (Heraeus Megafuge 16S, Thermo Scientific). The pellet was resuspended in 5 mL of saline solution, and sonicated five times for 1 minute each, with intervals of twenty seconds to disrupt the cells without protein degradation. The ELISA assays were conducted following the manufacturer's recommendations. Optical density readings were taken using a microplate reader (SPECTRAMAX 190, Molecular Devices) at 450 nm wavelength using the Softmax Pro program. The protein quantification was calculated from standard curves generated by serial dilution of the respective recombinants. Results were expressed as mean ± standard error of the mean (SEM).

2.3 LACTIC ACID BACTERIA STUDY DESIGN

The cloning vector synthesis for Lactic Acid Bacteria was performed by GenOne (*Soluções em Biotecnologia*, Brazil). The plasmid designed for transformation into LAB was based on the pUC57 vector from GenScript Biotech Corporation, which includes the *Amp* cassette.

Three specific sequences were synthesized for this purpose. First, the U509BGF230-2 cassette was designed to facilitate the synthesis of the RBD protein from SARS-CoV-2, positioned between *Eco*RI and *Bam*HI restriction sites. This cassette was optimized for LAB use and controlled by the Lac promoter. Secondly, a sequence was incorporated to insert IS1223, strategically placed following a *Kas*I restriction site. An additional *Kas*I site was introduced downstream of the cloning sequence, intended for genomic insertion into LAB (bacteria of interest). This segment also includes two ORFs facilitating recombinase synthesis within the sequence. Lastly, a cassette was developed for the expression of *cel*A, adapted from GenBank K03088.1 by removing an unidentified protein sequence. The choice of the *cel*A gene as a selection marker replaces traditional antimicrobial resistance genes, allowing transformants to be easily identified by forming clear halos around colonies on cellulase screening plates. This cassette was positioned after a *Pci*I enzyme site, with another *Pci*I site added following the cloning sequence to allow for additional modifications as necessary (Figure 13). These designs aimed to enable efficient transformation and expression of specific genetic material in Lactic Acid Bacteria, customized for specific research applications.

Figure 13 – Map of the synthesized cloning vector for Lactic Acid Bacteria.



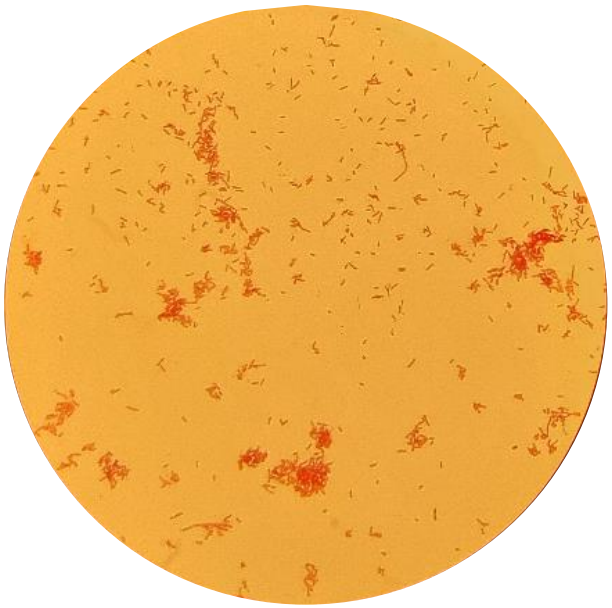
Source: Own authorship using SnapGene software.

3 RESULTS

3.1 CLONING AND PLASMID DNA

After completing the heat shock cloning protocol, the confirmation of plasmid incorporation into *Escherichia coli* BL21 was initially carried out by observing growth in LB medium containing ampicillin. Only the transformed BL21 cells were able to grow in the medium with the antibiotic. Subcultures were plated onto LB agar plates supplemented with ampicillin, resulting in the formation of seven transformant colonies. Purity was verified using Gram staining and light microscopy to observe cellular morphology, confirming the presence of Gram-negative bacilli (Figure 14).

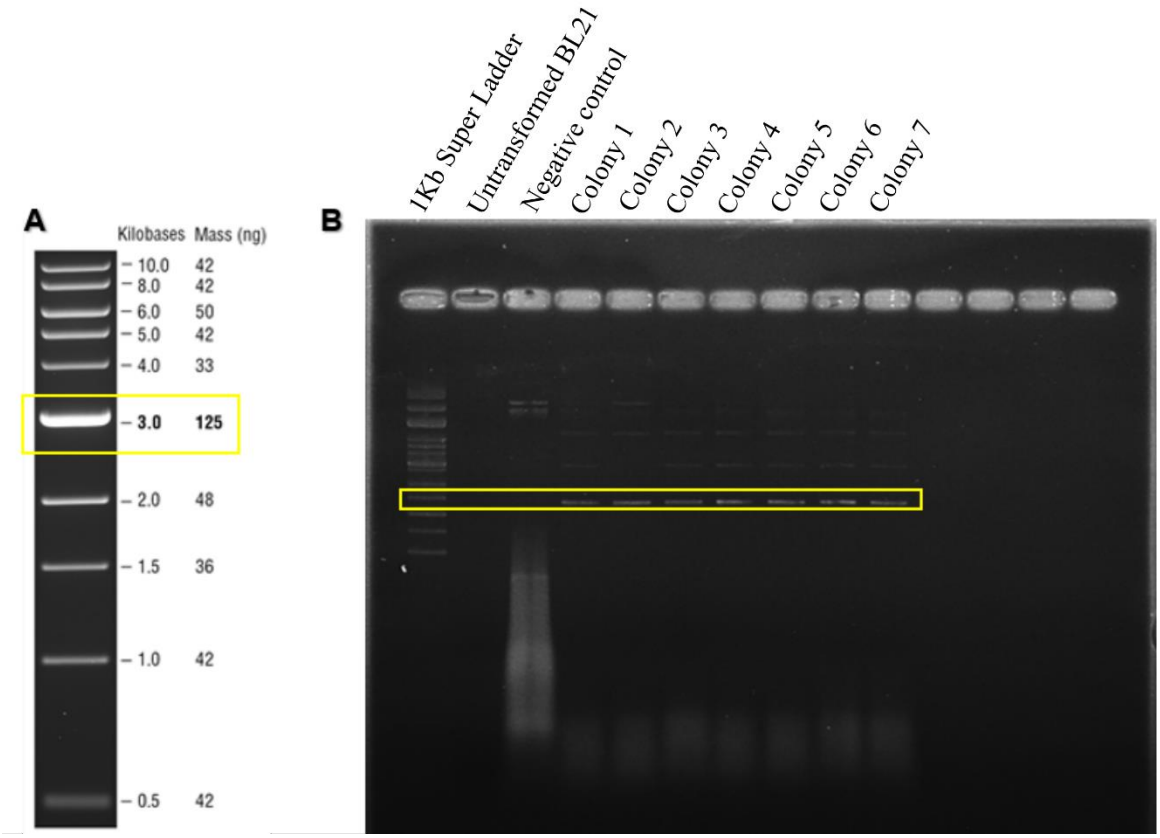
Figure 14 – Cell morphology of the transformants by Gram staining.



Source: Own authorship.
Note: Cell morphology of the transformants was assessed following Gram staining and examined using oil immersion microscopy (100x magnification), confirming the presence of Gram-negative bacilli.

Finally, to confirm the presence of the plasmid, plasmid DNA extraction was performed from the seven transformants, followed by an evaluation of integrity on an agarose gel. Each sample exhibited a band corresponding to the expected molecular weight (3358 bp) of the synthesized plasmid (Figure 15).

Figure 15 – Evaluation of plasmid DNA integrity of the transformants on agarose gel.



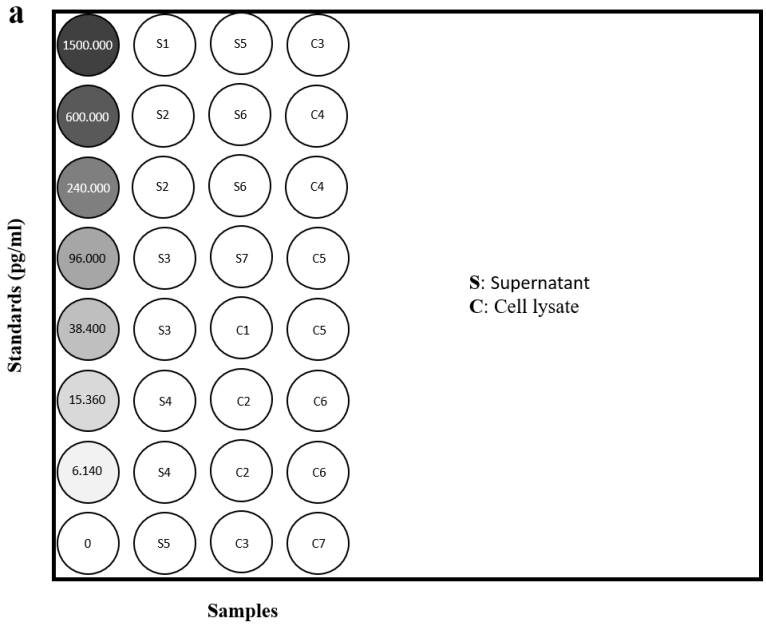
Source: Own authorship.
Note: 0.8% agarose gel for confirming plasmid incorporation in the seven transformants. A – 1 kb DNA Super Ladder. B – Gel showing plasmid DNA integrity extracted from the seven transformant colonies.

3.2 QUANTIFICATION OF SARS-CoV-2 RBD PROTEIN BY ELISA

To quantify the RBD protein synthesized by the seven transformants, assays were performed on their respective supernatants and cell lysates. The analysis revealed that only colony 5, indicated by red rectangles, displayed absorbance values (0.100 and 0.110) within the reading range (Figure 16).

Figure 16 – Absorbance readings from the supernatants and cell lysates of the transformants.

a



b

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.012	-0.001	0.110	-0.000								
B	1.483	-0.003	-0.003	-0.001								
C	0.949	-0.002	-0.004	0.001								
D	0.517	-0.001	-0.001	0.033								
E	0.231	-0.002	0.000	0.030								
F	0.097	-0.001	0.002	0.001								
G	0.040	0.003	0.005	0.001								
H	0.000	0.100	0.003	0.000								

Endpoint
L1 450
Off
Calibrate On
Plate Last Read:
17:10 24/06/2022

Formula: L1
Data Mode: Absorbance
Plate Blank Used L1 = 0.057

Standards (pg/ml)

Sample	Concentration	BackCalcConc	Wells	Values	MeanValue	Std.Dev.	CV%
St01	1500.000	1508.342	A1	2.012	2.012	0.000	0.0
St02	600.000	592.254	B1	1.483	1.483	0.000	0.0
St03	240.000	242.752	C1	0.949	0.949	0.000	0.0
St04	96.000	97.691	D1	0.517	0.517	0.000	0.0
St05	38.400	36.279	E1	0.231	0.231	0.000	0.0
St06	15.360	14.800	F1	0.097	0.097	0.000	0.0
St07	6.140	7.124	G1	0.040	0.040	0.000	0.0

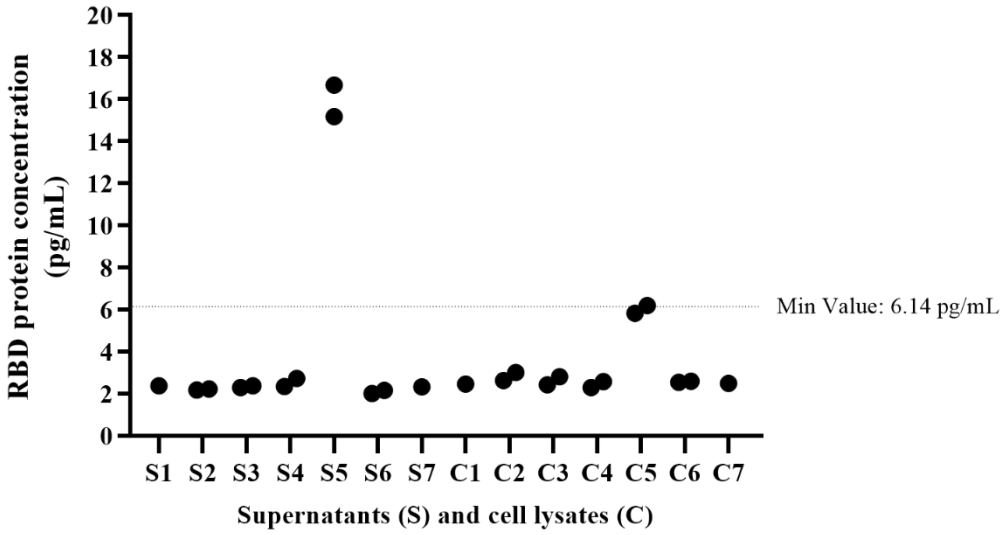
Smallest standard value: 0.040
Largest standard value: 2.012

Source: Own authorship. Table generated by the Softmax Pro software and adapted by the author.

Note: **a.** ELISA plate map showing the layout of samples. **b.** Absorbance readings from the supernatants (S) and cell lysates (C) of the transformants.

The analysis was conducted in duplicate to ensure accuracy and reliability. It was observed that only colony 5 exhibited protein synthesis values within the standard curve range (1,500.00 – 6.14 pg/mL) of the ELISA kit used. The supernatant from colony 5 (S5) demonstrated significant production levels, with results of 16,683 pg/mL and 15,173 pg/mL, respectively, while the cell lysate (L5) showed a value of 6,197 pg/mL (Figure 17).

Figure 17 – Quantitative analysis of RBD protein in pg/mL in the supernatants and cell lysates of the transformants.



Source: Own authorship. Graph generated using GraphPad 8.4.3 software.

4 DISCUSSION

Research on alternative COVID-19 vaccines is crucial due to the ongoing challenges posed by the pandemic and the evolving nature of SARS-CoV-2 variants (WIBMER *et al.*, 2021; ZHOU *et al.*, 2021). While effective vaccines have been developed and widely distributed (ABBASI, 2022; ALTMANN; BOYTON, 2022; DUBÉ; MACDONALD, 2022), the emergence of new viral strains raises concerns about vaccine effectiveness (MOHAMED *et al.*, 2022). These new variants often show increased transmissibility and adaptability to different environmental conditions, which can lead to vaccine resistance and evasion of the host immune system (BARAL *et al.*, 2021; CAMERONI *et al.*, 2022).

Given that the virus primarily spreads through the upper respiratory and gastrointestinal tracts, mucosal delivery of SARS-CoV-2 antigens represents a promising strategy to combat COVID-19 transmission, as evidenced by recent efforts focused on the delivery of SARS-CoV-2 antigens to mucosal surfaces (WANG *et al.*, 2020; ZHANG *et al.*, 2022), exemplified by the development of oral vaccines such as the *Chlamydomonas reinhardtii* microalgae vaccine (DEHGHANI *et al.*, 2022; KIEFER *et al.*, 2022). Recombinant bacterial vectors, such as Lactic Acid Bacteria, offer a promising approach as these microorganisms not only possess characteristics that facilitate genetic modification (MANCHA-AGRESTI *et al.*, 2017; TAKAHASHI *et al.*, 2019) but also can induce both mucosal and systemic immune responses (ASHRAF; SHAH, 2014; MOJGANI; SHAHALI; DADAR, 2020). Moreover, their cost-effectiveness makes them an appealing option for new vaccine strategies (ROCHA *et al.*, 2021). Research and development in these areas are essential to ensure durable and effective immune responses, critical for controlling COVID-19 and mitigating its global impact.

Escherichia coli stands out as one of the most utilized hosts for recombinant protein production, playing a crucial role in both research and industry (GOPAL; KUMAR, 2013; HUANG; LIN; YANG, 2012; JIA; JEON, 2016; LIU *et al.*, 2015; ROSANO; CECCARELLI, 2014). It serves as the host for over 30% of approved therapeutic proteins (BAESHEN *et al.*, 2015; HUANG; LIN; YANG, 2012), owing to its extensive genetic manipulation tools and ability to grow to high cell densities on inexpensive media, facilitating exceptionally high protein production (CHOI; KEUM; LEE, 2006; GOPAL; KUMAR, 2013; JIA; JEON, 2016; ROSANO; CECCARELLI, 2014). Among the strains, BL21(DE3) is preferred for its absence of *Lon* and *Omp*T proteases (RATELADE *et al.*, 2009; ROSANO; CECCARELLI, 2014), and when paired with the T7 expression system utilizing a highly efficient T7 promoter, it significantly enhances protein transcription rates compared to native *E. coli* systems (DURANI;

SULLIVAN; MAGLIERY, 2012; ROSANO; CECCARELLI, 2014; STUDIER; MOFFATT, 1986; TEGEL; OTTOSSON; HOBER, 2011).

The choice of BL21(DE3) for cloning the RBD region of the SARS-CoV-2 spike protein from the P1 variant was, therefore, based on its established utility in recombinant protein production (BAESHEN *et al.*, 2015). Successful plasmid incorporation was confirmed by colony growth in LB medium supplemented with *Amp* and the presence of the synthesized plasmid with the expected molecular weight of 3358 bp.

In bacterial systems, recombinant proteins can be directed to three different cellular locations during expression, each offering unique advantages and disadvantages depending on the experimental objectives. Secretion into the extracellular space allows for high production levels and native conformation but exposes the protein to degradation by proteases and dilution due to cell growth (SØRENSEN; MORTENSEN, 2005). In the bacterial periplasm, the oxidative environment supports proper protein folding, though this location typically results in lower quantities of protein (MAKRIDES, 1996). In the bacterial cytoplasm, proteins often form insoluble aggregates called inclusion bodies, converting these aggregates into soluble and functional proteins requires denaturing agents (CALDERINI *et al.*, 2017). The cellular localization of viral antigens in recombinant probiotics, whether expressed in the cytoplasm, anchored to the cell wall, or secreted, significantly influences their susceptibility to environmental factors and their recognition by the immune system (MOHSENI *et al.*, 2019), with cell wall expression generally eliciting more robust host immune responses compared to cytoplasmic or secreted expression (DIEYE *et al.*, 2001; MICHON *et al.*, 2016). Our results demonstrated significant RBD protein synthesis in both supernatant and cell lysate from BL21(DE3) transformants, highlighting its advantage for biotechnological production by enabling efficient protein recovery and scalability essential for industrial applications. These preliminary findings underscore BL21(DE3)'s viability as an initial platform for the expression and characterization of recombinant proteins.

Probiotic-based vaccines, particularly those utilizing LAB such as *L. plantarum*, *L. casei*, and *L. lactis*, have shown promising efficacy in both prophylactic and therapeutic approaches against respiratory and non-respiratory viral infections, including emerging viruses like SARS and influenza (SUNDARARAMAN *et al.*, 2020; WYPYCH; WICKRAMASINGHE; MARSLAND, 2019). Future investigations will focus on evaluating its transformation efficiency in LAB, confirming the stability of RBD protein expression, and assessing the functionality of integrated genetic elements. These studies are crucial for establishing the practical utility of this plasmid in biotechnological research, demonstrating its

versatility across various applications in the field. Our designed plasmid, developed specifically for LAB, serves as a sophisticated tool for genetic manipulation, enabling efficient transformation and precise gene expression control. Engineered with optimizations for Gram-positive bacteria, it features a cassette for synthesizing the SARS-CoV-2 RBD protein under the regulation of the *Lac* promoter, strategically positioned recombinase sequences, and customizable modification sites. A sequence was incorporated to insert IS1223, which was the first insertion element isolated in lactobacilli with its functionality demonstrated (WALKER; KLAENHAMMER, 1994), showing efficacy in its use as IS-based integration vectors in Lactic Acid Bacteria (JANG *et al.*, 2003). Furthermore, a cassette was developed for the expression of c*el*A as a screening marker for the integration vector, to avoid future antibiotic resistance issues after removal of the *Amp* cassette, and offering a non-toxic option for food-grade vectors (JANG *et al.*, 2003).

Given the significant efforts in advancing vaccine platforms like probiotic-based technologies, addressing widespread misinformation and promoting accurate information about vaccine safety and importance remains critical, particularly highlighted during the COVID-19 pandemic, which has posed significant challenges to vaccine acceptance (LOOMBA *et al.*, 2021; MACDONALD *et al.*, 2021). Therefore, research initiatives must continue to focus not only on advancing vaccine technologies but also on strengthening public communication strategies (WIE; JUNG; KIM, 2023). Educating the public about vaccine safety, efficacy, and importance, while actively combating misinformation and navigating political influences, will be crucial in promoting extensive vaccine uptake and ensuring an effective response to future public health emergencies (ADOLPH *et al.*, 2021).

5 CONCLUSION

The rapid evolution of the SARS-CoV-2 virus, highlighted by the emergence of variants, underscores the ongoing necessity for genomic surveillance and the exploration of innovative vaccine technologies to anticipate future strategies and improve accessibility. While current vaccines have played a critical role in reducing disease severity, uncertainties persist regarding the persistence of immunity and long-term safety. The present study has efficiently cloned the RBD protein in *Escherichia coli*, revealing insights into its expression localization and recognition by human antibodies. The next crucial phase involves expanding this research to Lactic Acid Bacteria, where further validation is required to evaluate its potential as a suitable vaccine platform. Future investigations should focus on exploring LAB metabolic pathways, optimizing its transformation efficiency, enhancing antigen expression, and evaluating vaccine efficacy in relevant models. These efforts aim to advance vaccine technologies against COVID-19 and potentially other infectious diseases, emphasizing the essential role of multidisciplinary research in microbiology, immunology, and epidemiology in addressing future health challenges.

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