

**APPLICATION OF PROBIOTIC STRAINS
IN FOOD AND BEVERAGE MATRICES**

Francesca Rizzi

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Francesca Rizzi



FACULTAT DE VETERINÀRIA
DEPARTAMENT DE CIÈNCIA ANIMAL I DELS ALIMENTS

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BEVERAGE MATRICES**

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The present thesis report is presented by Francesca Rizzi to obtain the title of
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Barcelona.

Thesis supervisors:
Dr. Marta Capellas Puig
Dr. Pol Huedo Moreno

Thesis supervisor and tutor:
Dr. Bibiana Juan Godoy



Industrial doctoral project partnering:



&

UAB
Universitat Autònoma
de Barcelona

Alla mia famiglia italo-catalana...

"Live as if you were to die tomorrow. Learn as if you were to live forever."

– Mahatma Gandhi

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ABSTRACT

The application of probiotics in food and beverage matrices represents an evolving area of research within the domain of functional foods, aiming to provide health benefits beyond basic nutrition. In recent years, this sector has witnessed rapid growth due to heightened consumer interest in enhancing physical well-being and fostering a balanced lifestyle. As a result, companies in the probiotic industry are increasingly motivated to explore new applications for their strains, enabling them to broaden their market opportunities and meet the demand for innovative health-oriented products.

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, have been extensively studied for their ability, among others, to improve digestive health, enhance immune response, and exert a potential systemic effect through the diverse gut-organ axes.

Dairy products such as yogurt and kefir have traditionally served as a source for isolation of novel probiotics, mostly belonging to lactic acid bacteria (LAB) group. Likewise, although to lesser extent, well-characterized probiotics have also been incorporated into dairy products to generate the first functional foods. However, the shift in consumer preferences towards plant-based diets, along with the growing demand for vegan and lactose-free products, has driven innovation in incorporating probiotics into non-dairy food matrices, including juices and other beverages, confectionery, snacks and bakery products.

Food and beverage matrices are dynamic, environments that encompass a wide range of variable parameters, including pH, water activity, temperature, and oxygen concentrations, playing key roles in determining the stability and survival rates of probiotic strains. The fundamental challenges in this area involve maintaining high counts of viable probiotic cells, around 10^8 - 10^9 CFU/g or per serving, throughout product self-life, to ensure health benefit efficacy.

This industrial Ph.D. project, promoted by the company AB-Biotics S.A. and the AGAUR scholarship in collaboration with the Universitat Autònoma de Barcelona, has investigated the impact of the incorporation of well-characterized probiotics from AB-Biotics collection into a wide range of food and beverage matrices, as alternative probiotic carriers.

For a number of matrices, not only introduction but also fermentation has been explored.

In agreement with previous research, our results show that probiotics applications must be tested case by case and that optimal matrix and strain selection, precise inoculation timing,

and controlled storage conditions are key parameters to maintaining probiotic viability to ensure consistent health benefits in end products. Special attention was given to understanding the biochemical transformations driven by fermentation, with particular interest in how these changes may contribute to enhancing the health-promoting features of products as well as the impact on the organoleptic profile. Thanks to this combined approach involving physicochemical analyses, deep metabolomics and sensory evaluations, different biochemical pathways have been recognized.

Across non-fermented matrices, *Bacillus coagulans* Unique IS-2 spores demonstrated high stability at ambient temperature in confectionery products, snacks, bakery products and beverages. This robustness is attributable to its physiology, which provides enhanced tolerance to heat, extreme pH, and oxidative stress, characteristics that generally limit the survival of bifidobacteria and lactic acid bacteria (LAB). In dairy products and frozen desserts fortified with the I3.1 blend (*Lactiplantibacillus plantarum* CECT 7484, *L. plantarum* CECT 7485, *Pediococcus acidilactici* CECT 7483), probiotic viability consistently remained above clinically relevant thresholds during storage, confirming the suitability of these matrices as efficient carriers. In non-fermented beverages, *B. coagulans* Unique IS-2 again exhibited excellent stability, maintaining high viability in isotonic drinks stored at ambient temperature and in orange juice stored under refrigeration, even after exposure to HTST processing. Additionally, *Lactiplantibacillus plantarum* and *Pediococcus acidilactici* strains maintained high stability in nonfermented orange juice stored under refrigeration, whereas *Bifidobacterium longum* CECT 7894 exhibited a rapid decline, losing more than 5 log CFU within 48 hours. Physicochemical characterisation across all matrices showed that probiotic inclusion did not substantially alter key quality parameters, including pH, water activity, overrun, melting behaviour, and colour stability. Sensory evaluations revealed generally no significant differences between probiotic and control formulations, confirming broad consumer acceptability. Exceptions identified in isolated products suggest that targeted formulation improvements could be necessary to further optimise sensory performance.

In fermented matrices, selected strains of *Lactiplantibacillus plantarum*, *Pediococcus acidilactici*, and *Lactiacaseibacillus paracasei* effectively supported co-fermentation with classical yogurt starters and remained stable during refrigerated storage in milk, soy, and oat systems. Acidification kinetics, gelation behaviour, and coagulation properties showed clear strain-dependent patterns, underscoring the ecological and technological diversity among

LAB. Single strain fermentations in tiger nut beverages and fruit juices further demonstrated the ability of these strains to modulate pH, texture, and biochemical profiles. Metabolomic analyses in fruit juices fermented with *L. plantarum* KABP051 revealed the synthesis of multiple bioactive metabolites including phenyllactic acid (PLA), hydroxyphenyllactic acid (HPLA), acetylcholine, short-chain fatty acids (SCFAs), and phenolic compound derivatives, highlighting the strain potential to enhance the functional value of fermented beverages.

Collectively, this work provides the first comprehensive cross matrix evaluation of AB-Biotics probiotic strains in real food systems, addressing the needs of an increasingly health-conscious and diverse consumer base.

The findings demonstrate that probiotic performance is shaped by a complex and dynamic interplay between strain-specific genomic traits, ecological specialization, and matrix-dependent physicochemical constraints. This integrated framework contributes to the identification of robust predictors of probiotic stability across diverse food matrices and supports the technological feasibility of formulating probiotic foods that preserve both biological functionality and sensory quality throughout processing and storage. Overall, these results provide robust scientific basis for industrial application and for guiding future innovation in probiotic-enriched functional foods and beverages.

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LIST OF ABBREVIATIONS

AC	Antioxidant activity
ACh	Acetylcholine
AGAUR	Agència de Gestió d'Ajuts Universitaris i de Recerca
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
aw	Water activity
B	Blank
BM	Bowel movement
BS	Backscattering (Turbiscan analysis)
BV	Bacterial vaginosis
CECT	Colección Española de Cultivos Tipo
cP	Centipoise
CFU	Colony forming units
CO ₂	Carbon dioxide
CSIC	Spanish National Research Council
DAD	Diode-array detector
DASS-42	Depression, anxiety, and stress scale
DBH	Dopamine Beta-Hydroxylase
DC1	Dark chocolate bars enriched with probiotic blend I3.1
DC2	Dark chocolate bars enriched with probiotic blend AB-KOLICARE
DC3	Dark chocolate chunks enriched with probiotic blend I3.1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSHEA	Dietary Supplement Health and Education Act (USA)
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
ERH	Equilibrium relative humidity
F ₀ F ₁ -ATPase	F ₀ F ₁ - adenosine triphosphatase

FGID	Functional gastrointestinal disorder
FXR	Farnesoid X receptor
F _x	Formulation x (x = 1, 2, 3, 4)
FDR	False discovery rate
FOS	Fructooligosaccharides
FOSHU	Foods for specified health use (Japan)
FSMP	Foods for special medical purposes
GABA	Gamma-aminobutyric acid
GAE	Gallic acid equivalent
GI	Gastrointestinal
GI	Gingival index
GOS	Galactooligosaccharides
GRAS	Generally recognized as safe (FDA)
HADS	Hospital anxiety and depression scale
HDL	High-density lipoprotein (cholesterol)
HMO	Human milk oligosaccharide
HPLA	Hydroxyphenyllactic acid
HTST	High-temperature short-time (pasteurization)
IAAs	Iso- α -acids
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-D	Irritable bowel syndrome, diarrhea-predominant
IBS-M	Irritable bowel syndrome, mixed/alternating
IBS-QoL	Irritable bowel syndrome quality of life questionnaire
IBS-SSS	Irritable bowel syndrome severity scoring system
ISAPP	International Scientific Association for Probiotics and Prebiotics
KCl	Potassium chloride
LAB	Lactic acid bacteria
LcS	<i>Lactocaseibacillus casei</i> strain Shirota
LHBT	Lactose hydrogen breath test
LGG	<i>Lactocaseibacillus rhamnosus</i> GG (ATCC 53103)
MCC	Milk chocolate cream
MC1	Milk chocolate bars enriched with probiotic blend I3.1

MC2	Milk chocolate bars enriched with probiotic blend AB-KOLICARE
MD	Mass dropped (ice cream)
MDA	Malondialdehyde
MI	Initial mass (ice cream)
MIC	Minimum Inhibitory Concentration
MRS	de Man, Rogosa, and Sharpe (culture medium)
MTCC	Microbial Type Culture Collection and Gene Bank
NAG	N-acetyl glutamine
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NDA	Non-disclosure agreement
NHPs	Natural Health Products (Canada)
NK-cell	Natural-killer cell
NTU	Nephelometric turbidity units
P	Probiotic
PCA	Principal component analysis
PCA	Plate Count Agar
PF	Probiotic-fermented
PLA	Phenyllactic acid
QoL	Quality of Life
QPS	Qualified Presumption of Safety (EFSA)
QTOF	Quadrupole time-of-flight (mass spectrometry)
RH	Relative humidity
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S.A.	Sociedad Anónima
SCFA	Short-chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIBO	Small intestinal bacterial overgrowth
SMLS	Static Multiple Light Scattering
TA	Titrateable acidity
TDO	Tryptophan 2,3-Dioxygenase

TGR5	Takeda G-protein–coupled Receptor 5
TE	Trolox equivalent
TMAO	Trimethylamine N-oxide
TMP	1,1,3,3-tetramethoxypropane
TPC	Total phenolic content
TPH2	Tryptophan Hydroxylase 2
Tregs	Regulatory T cells
TSI	Turbiscan Stability Index
TSS	Total soluble solids
Tx	Time point (x = 0, 1, 2 etc. days/months)
UAB	Universitat Autònoma de Barcelona
UC	Unità Cento (unit to ferment 100 L)
UHT	Ultra-high temperature
UPLC	Ultra-performance liquid chromatography
URTI	Upper respiratory tract infections
VBNC	Viable-but-non-culturable
VDCC	Vegan dark chocolate cream
VHCC	Vegan hazelnut chocolate cream
VS.I	Visceral Sensitivity Index
Wc	Weight of a predefined volume of the mix after churning (ice cream)
WGS	Whole genome sequencing
Wmix	weight of a predefined volume of the mix before churning (ice cream)
WSN	Water-soluble nitrogen

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Peer-reviewed research article: *Lactiplantibacillus plantarum* KABP051: Stability in Fruit Juices and Production of Bioactive Compounds During Their Fermentation

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1 INTRODUCTION

1.1 MICROBIOTA

The term microbiota encompasses the complex and dynamic community of microorganisms: bacteria, fungi, archaea, algae, viruses, and protozoa, that inhabit a particular ecological niche. These microbial communities are found across diverse environments, including soil, water, plants, animals, as well as the human body (Berg et al., 2020; Simon et al., 2019), and play fundamental roles in their ecosystems, contributing to nutrient cycling, organic matter decomposition, and the overall bionetwork balance (Fierer, 2017). It is now widely recognized that nearly every habitat and organism on Earth hosts its own unique microbiota. When inhabiting a host, microorganisms can act in a commensal, mutualistic, or pathogenic manner, directly affecting the homeostasis of the environment in which they reside. The term "microbiota" is often used interchangeably with "microbiome". However, they have distinct definitions which are important to distinguish. "Microbiota" specifically refers to the consortia of microorganisms inhabiting a particular environment, examples include the oral microbiota or gut microbiota in humans (Berg et al., 2020). In contrast, "microbiome" encompasses not only these microorganisms but also their collective genetic material, metabolic products, structural components, and the environmental factors that influence them (Marchesi & Ravel, 2015). By connecting microbial communities and their ecological context, the broader "microbiome" concept provides a holistic framework for understanding microbial roles and interactions within complex ecosystems (Berg et al., 2020; Proctor et al., 2019; Ursell et al., 2012).

1.2 HUMAN MICROBIOTA

The human microbiota, also known as "hidden organ" (Hou et al., 2022), is mainly constituted by bacteria that primarily resides in the gastrointestinal (GI), but it is also found on the skin, in the mouth, and other body sites. In the GI, the bacterial number reaches an average of around 100 trillion, equating to approximately 10^{14} cells (Sender et al., 2016). It has been estimated that the GI microbiota contributes over 150 times more genetic information than that of the entire human genome (Grice & Segre, 2012).

For instance, a prominent study by Qin et al., (2010) established a comprehensive human gut microbial gene catalogue, revealing the extensive diversity of microbial genes present in

the human gut. The human microbiota composition varies depending on the specific site within the body (Figure 1). Among the different microbiotas that inhabit the human body, gut microbiota is the major reservoir, with more than 2.000 species, and regarded as the most crucial for supporting human health (Ahlawat et al., 2021).

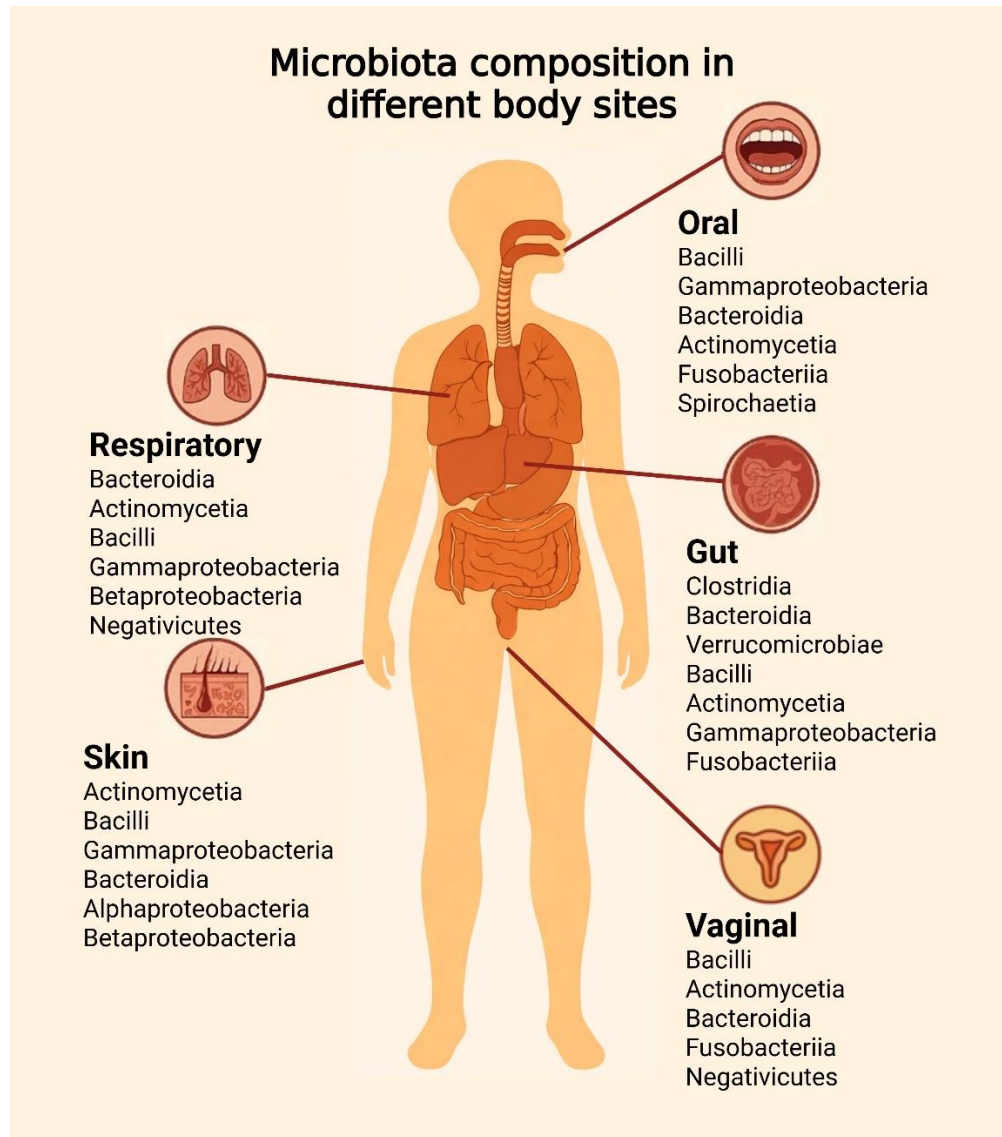


Figure 1. Human microbiota composition across body sites. Predominant bacterial classes are shown for the gut, vaginal tract, skin, respiratory tract, and oral cavity. The figure was created in <https://BioRender.com>.

Taxonomically, bacteria are classified according to phyla, classes, orders, families, genera, species and strains. Generally, a healthy gut microbiota is mainly composed of 6 phyla including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Rinninella et al., 2019). Among them, Firmicutes and Bacteroidetes are the predominant phyla in the human gut microbiota, collectively constituting approximately

90% of the microbial population. They play significant roles in energy harvesting and metabolic and immune functions, while Actinobacteria and Proteobacteria mainly contribute to the synthesis of essential nutrients and immune modulation. Research has highlighted how the balance among microbiota phyla is crucial for health (Hou et al., 2022).

The composition and function of these bacterial communities vary along the GI tract and are influenced by a range of factors, including host characteristics such as age, sex, and ethnic background; anatomical and physiological conditions such as pH, bile acid concentration, digestive enzyme activity, and mucus composition; external elements like diet, medications, and environmental exposures; as well as intrinsic bacterial traits such as metabolic activity and adhesion capacity (Hul et al., 2024; Zheng et al., 2017).

To analyse and characterize the complexity of microbial diversity, scientists rely on two key metrics. Alpha-diversity measures the species richness and evenness within a single sample. Commonly used alpha diversity indices include the Shannon and Simpson indices, which assess both species abundance and distribution. Beta-diversity, in contrast, compares microbial composition across different samples or environments, highlighting differences in community membership and structure. These inter-sample comparisons are essential for understanding variations in microbiota composition across populations, environmental conditions, and disease states (Knight et al., 2018; Lozupone et al., 2012).

In recent years, high-throughput sequencing of 16S ribosomal RNA (rRNA) genes has become a widely used method for the phylogenetic identification and quantification of bacterial communities within the gut microbiota. By amplifying the 16S rRNA gene, which contains both conserved and variable regions, this method enables distinction of bacterial taxa down to the genus and species level. This approach has revolutionized microbiome research by providing comprehensive microbial community profiles, offering critical insights into the microbiota complexity, diversity, and role in human health and disease (Huttenhower et al., 2012; Janda & Abbott, 2007). Additionally, metagenomics and DNA sequencing techniques have refined bacterial population analyses, bypassing the need for traditional culturing methods (Athanasopoulou et al., 2023). Advanced methods, such as shotgun metagenomic sequencing, have substantially improved the taxonomic and functional resolution of microbiome analyses.

Shotgun metagenomics is a culture-independent approach in which total DNA extracted from a sample is randomly fragmented and sequenced without prior amplification of specific marker genes. This strategy enables high-resolution taxonomic profiling at the species and,

potentially, strain level, as well as comprehensive functional characterization of microbial communities based on their complete genetic repertoire (Almeida et al., 2019; Qin et al., 2010).

A healthy gut microbiota is typically characterized by high taxonomic diversity, richness of microbial genes, and a stable core microbiota (Fan & Pedersen, 2021). These attributes contribute to a robust and resilient ecosystem able to adapt and to support host health and immune functions, maintaining balance despite environmental, physiological, and dietary changes (Hul et al., 2024; Zheng et al., 2020a).

1.3 HUMAN GUT MICROBIOTA

The gut microbiota plays a pivotal role in host physiology, with wide-ranging effects on metabolic, immune, and neuroendocrine homeostasis. Metabolically, intestinal microbial communities expand the host's enzymatic repertoire, enabling efficient digestion and absorption by fermenting otherwise indigestible polysaccharides and processing proteins and lipids, functions that complement, rather than duplicate, human metabolic capacity (Fan & Pedersen, 2021). In particular, Rowland et al. (2018) detailed how microbial fermentation yields short-chain fatty acids (SCFAs), acetate, propionate, and butyrate, which nourish colonocytes, reinforce epithelial barrier integrity, and modulate systemic energy balance and immunity. SCFA-driven signalling through G protein-coupled receptors and epigenetic regulation further explains their influence on both local and peripheral tissues (Mann et al., 2024). Additionally, gut microbes synthesize and remodel B-group vitamins and vitamin K, contributing to coagulation, energy metabolism, and intestinal integrity (Kau et al., 2011; Lai et al., 2022).

Beyond the gastrointestinal tract, the microbiota communicates along multiple bidirectional gut–organ axes (Figure 2). Through neural (vagal and enteric) signals, endocrine, immune, and metabolic pathways, the gut–brain axis links microbial metabolites, including tryptophan-derived kynurenines and SCFAs, to central nervous system regulation, influencing cognition, behaviour, stress responses, and neuroinflammation (Cryan & Dinan, 2012; Mulder et al., 2023; Vuong et al., 2017).

Along the gut–lung axis, gut derived metabolites such as SCFAs enhance pulmonary immune tolerance, strengthen epithelial barriers, and reduce excessive airway inflammation,

protective effects that complement their involvement in asthma and acute lung injury susceptibility (Druszczyńska et al., 2024; Eladham et al., 2024; J. Liu et al., 2026).

The gut–liver axis, anatomically connected through the portal vein, exposes the liver to microbial ligands (e.g., LPS) and metabolites (e.g., bile acids), which regulate steatosis, fibrosis, and detoxification via Farnesoid X Receptor/ Takeda G-protein–coupled Receptor 5 (FXR/TGR5) signalling (Hsu & Schnabl, 2023; Lee et al., 2024; Song et al., 2025).

The gut–skin axis links intestinal dysbiosis to inflammatory dermatoses, including psoriasis and atopic dermatitis, through systemic immune activation and barrier-related metabolites (Jimenez-Sanchez et al., 2025; Munteanu et al., 2025).

Within the gut–kidney axis, dysbiotic microbiota has been associated with chronic kidney disease through links to impaired epithelial barrier function and the accumulation of microbial uremic toxins such as indoxyl sulphate and p-cresyl sulphate. In contrast, a well-balanced gut–kidney axis is characterized by microbial communities that generate protective metabolites, including short chain fatty acids (SCFAs) and indole derived compounds, which help maintain epithelial integrity, modulate renal inflammation, and support metabolic homeostasis (Stavropoulou et al., 2021; Tsuji et al., 2025).

Finally, the gut–heart axis integrates microbial metabolites into cardiovascular physiology. While TMAO (trimethylamine N-oxide) has been used as a biomarker of cardiometabolic risk, SCFAs exert cardioprotective effects, being associated with reduced vascular inflammation, improved blood pressure regulation, and lower risk of hypertension and atherosclerosis (Bui et al., 2023; Sagmeister et al., 2024).

Across axes, SCFAs and bile acid receptors such as FXR/TGR5 serve as key molecular mediators facilitating organ-to-organ communication (Lee et al., 2024; Mann et al., 2024).

Beyond these interorgan relationships, the microbiota is also essential for immune system development and regulation.

Commensal microorganisms promote immune maturation through the induction of regulatory T cells (Tregs), modulation of effector pathways, and maintenance of oral tolerance mechanisms that prevent inappropriate inflammation (Belkaid & Hand, 2014; Thursby & Juge, 2017). Colonization resistance, achieved through nutrient competition, niche exclusion, and production of antimicrobial compounds such as bacteriocins, protects against pathogenic overgrowth (Huang et al., 2021; Khan et al., 2021; Panwar et al., 2021). These foundational interactions highlight the need for mechanistic understanding to guide microbiome-targeted therapeutics (Fan & Pedersen, 2021).

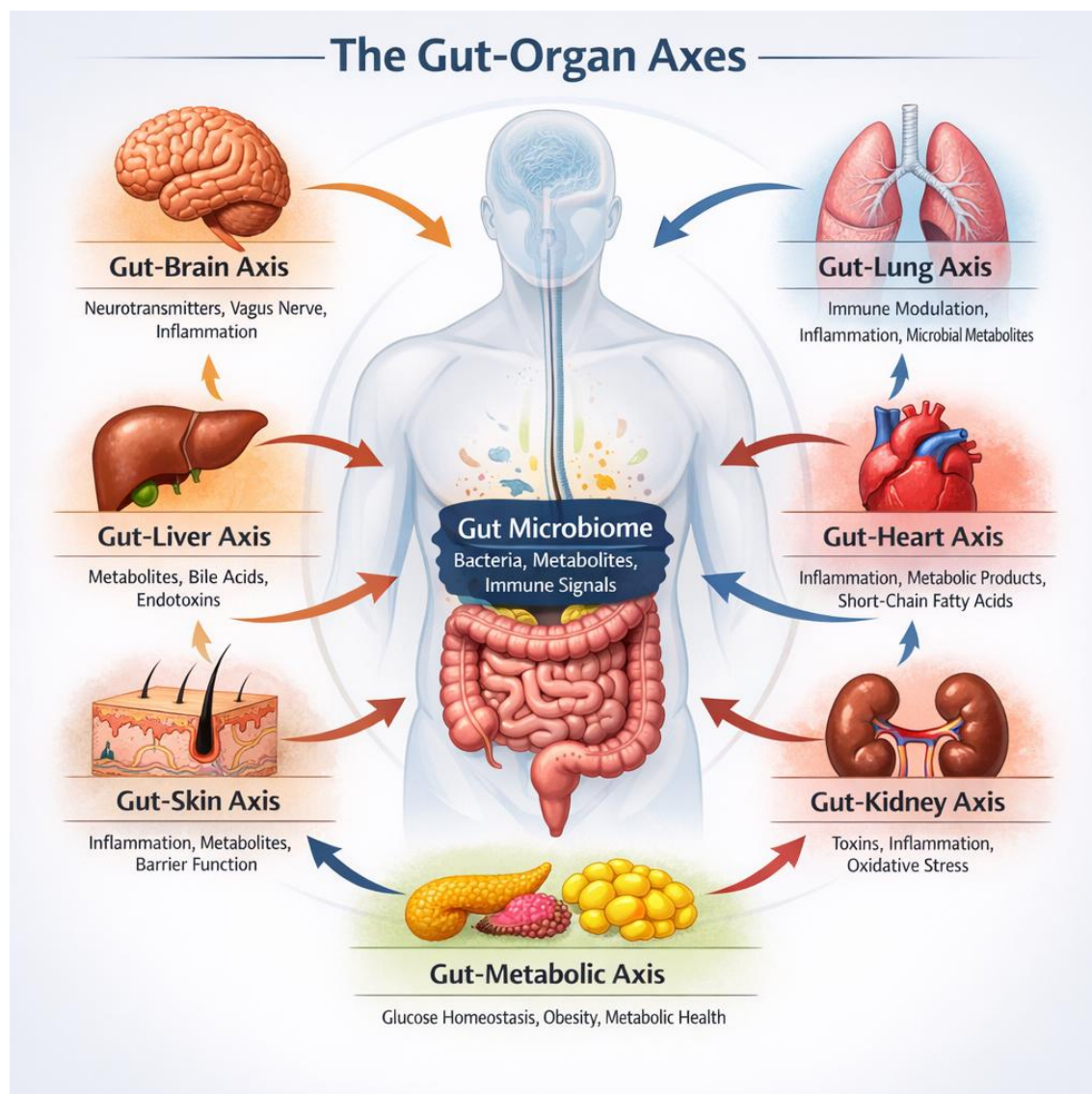


Figure 2. The gut–organ axes. Diagram illustrating major bidirectional communication pathways between the gut microbiota and the brain, lungs, liver, heart, kidneys, skin, and metabolic tissues, including representative mechanisms (neural, immune, endocrine, and microbial metabolites) and physiological outcomes. The figure was created using Microsoft Copilot.

Across the human lifespan, gut microbial composition is highly dynamic. In early life, type of birth, maternal transfer and breastfeeding establish a bifidobacteria rich microbiota that supports metabolic and immune development and microbial profiles correlate with breastfeeding dose and duration (Milani et al., 2017; Pannaraj et al., 2017). As children grow, microbial diversity increases, approaching an adult-like configuration dominated by Firmicutes and Bacteroidetes, influenced strongly by diet, environment, and household exposure (Stewart et al., 2018; Thursby & Juge, 2017).

In adulthood, the microbiota is comparatively stable yet individualized, shaped by habitual diet, medications, lifestyle, and other environmental factors (Fan & Pedersen, 2021).

With advancing age, compositional shifts often include reductions in beneficial taxa (e.g., bifidobacteria) and increases in proinflammatory organisms (e.g., Proteobacteria), contributing to frailty, metabolic alterations, and heightened vulnerability to chronic diseases (Badal et al., 2020; Odumaki et al., 2016; O'Toole & Jeffery, 2015). These changes underlie “inflammaging”, the chronic low-grade inflammation characteristic of older adults (Fransen et al., 2017; Ottman et al., 2012; Ticinesi et al., 2017).

Notably, recent multiomics analysis of the world longest lived individual revealed an exception to this age-associated trajectory, showing a gut microbiota composition enriched in *Bifidobacteriaceae*, particularly *Bifidobacterium*, at levels more typically associated with early life rather than advanced age. In this supercentenarian, *Bifidobacterium* abundance was markedly higher than in elderly control cohorts and accompanied by increased α -diversity and reduced proinflammatory taxa, suggesting a “rejuvenated” bacteriome reminiscent of infant-like microbial ecosystems. This finding challenges the conventional view of an inevitable decline of bifidobacteria with aging and supports the concept that preservation, or restoration, of early life associated microbial taxa may contribute to immune resilience, reduced inflammaging, and exceptional health span in extreme old age (Santos-Pujol et al., 2025).

Overall, the gut microbiome dynamic, life-stage-dependent nature (Figure 3) emphasizes its causal and modifiable role in health and disease, motivating strategies such as dietary modulation, prebiotics, probiotics, and postbiotics to promote resilience and prevent microbiome-associated pathology (Fan & Pedersen, 2021).

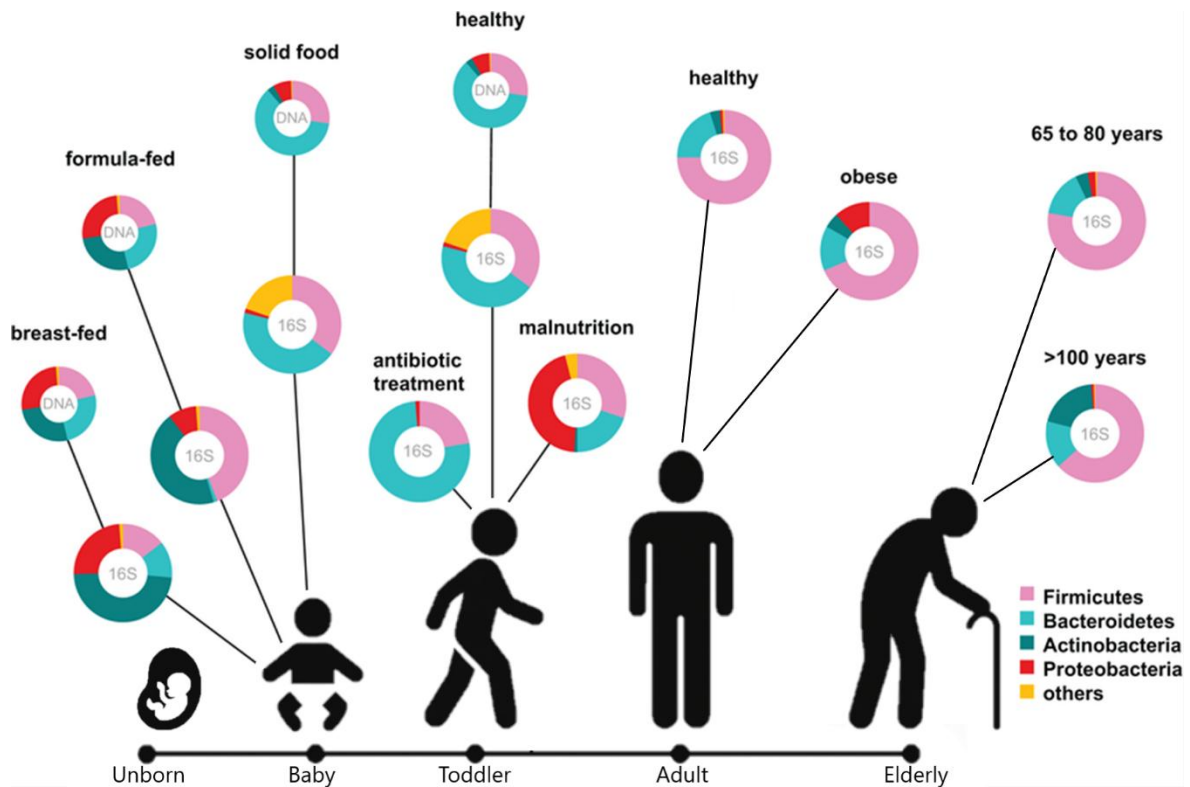


Figure 3. Developmental Dynamics of Human Gut Microbiota Across the Lifespan. Human gut microbiota undergoes profound changes from the prenatal stage through senescence. Neonates are generally considered to possess a sterile GI tract at birth. Microbial colonization begins during delivery, when the infant gut is exposed to microorganisms originating from the maternal reproductive tract and the surrounding environment. During the first two years of life, the gut microbiota exhibits considerable variability in composition and structure. Following the introduction of solid foods, particularly those rich in dietary fibres and complex carbohydrates, the microbial community becomes increasingly diverse and functionally stable. In later life stages, particularly during old age, the gut microbiota undergoes marked alterations characterized by reduced diversity and shifts in community composition, contrasting with the more robust and diverse profiles observed in younger (Ottman et al., 2012).

1.4 DYSBIOSIS

Dysbiosis refers to an imbalance or disruption in the composition of the gut microbiota, often linked to various health conditions and diseases. Factors like antibiotic use, poor diet, or environmental influences can lead to microbial dysbiosis, which may enhance inflammation, impair immune responses, and alter gut barrier integrity (Levy et al., 2017). Research indicates that dysbiosis can also contribute to various diseases such as metabolic disorders like obesity, type 2 diabetes, inflammatory bowel disease and neurodevelopmental disorders (Cani et al., 2018; De Filippis et al., 2020; Fan & Pedersen, 2021). Notably, specific bacterial groups, including Proteobacteria and pathobionts, may flourish in a dysbiotic gut, while beneficial species, such as certain bifidobacteria and Firmicutes strains, decrease. This shift disrupts the production of SCFAs, which play a crucial role in maintaining gut health and regulating immune responses (Zeng et al., 2017). Current studies suggest that therapeutic

interventions such as prebiotics and probiotics hold potential for re-establishing a balanced microbiome and restoring health in dysbiotic individuals (Suez et al., 2019).

1.5 ORIGIN OF THE CONCEPT "PROBIOTIC"

The term "probiotic" itself, derived from the Greek words pro (for) and bios (life), was first used in the early 20th century to describe substances that promote life.

The consumption of health-promoting bacteria, through fermented food products, and primarily from dairy origin, has been a part of human diets for centuries, although the health benefits were not well understood until more recently.

The origin of the “probiotic” concept is attributed to the Russian immunologist, zoologist and bacteriologist Elie Metchnikoff (1845-1916). In his book "The Prolongation of Life: Optimistic Studies", published in 1907, he hypothesized that the longevity of Bulgarian peasants could be associated with their consumption of a traditional fermented milk, rich in lactobacilli, a type of "friendly bacteria". He suggested that: “the dependence of intestinal microbes on food makes it possible to adopt measures to modify the microbial community in our bodies and replace dangerous microorganisms with useful ones” (Anukam, 2014). This idea was a pioneering concept for what would later become the modern understanding of probiotics and the impact of gut microbiome in health. Parallel to Metchnikoff's work, French paediatrician Henry Tissier observed that children suffering from diarrhea had a low presence of bacteria with a distinctive Y-shaped morphology in their stools. In contrast, these “bifid” bacteria were abundant in healthy children (Farré-Maduell & Casals-Pascual, 2019). He proposed that administering these bacteria to patients with diarrhea could aid in restoring healthy gut microbiota. These early findings of Metchnikoff and Tissier contributed to laying the foundation for the scientific exploration of using bacteria for probiotic purposes. The term “probiotic” is widely believed to have been introduced by Ferdinand Vergin in 1954 in his article titled “Anti-und Probiotika”. In this work, Vergin compared the detrimental effects of antibiotics and other antibacterial substances on the intestinal microbiota with the beneficial effects of certain bacteria, which he referred to as “probiotika” (Vergin, 1954). Later, in 1965, Lilly and Stillwell provided a more focused description of probiotics, defining them as microorganisms that stimulate the growth of other microorganisms (Lilly & Stillwell, 1965).

The definition of probiotics has undergone refinement over the years. The Food and Agriculture Organization/World Health Organization (FAO/WHO) expert consultation of probiotics in food published in 2001 (FAO/WHO, 2001) issued a definition for probiotics that was later retained in the Consensus Statement for probiotics elaborated by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2014 with minor modifications (Hill et al., 2014). According to this last definition, probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Probiotic microorganisms are organized within a structured taxonomic framework that includes genus, species, and strain levels. Among these, the most frequently represented genera are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Saccharomyces*, *Bacillus*, *Weizmannia*, and *Enterococcus*. In recent years, advancements in taxonomy have provided a more detailed classification of these microorganisms. For instance, the genus *Lactobacillus* was reclassified into 25 new genera in 2020 based on genomic data (Zheng et al., 2020b). To aid researchers in navigating this updated taxonomy, a collaborative effort among several academic institutions resulted in the creation of an online tool available at <http://lactobacillus.ualberta.ca/>. Such advancements underscore the evolving nature of probiotic research and emphasize the importance of precise strain identification in linking specific microorganisms to health benefits.

Today, probiotics are widely acknowledged for their beneficial effects on gut health, immune function, and the management of various chronic conditions, representing a significant advancement from their early conceptualization. However, it is crucial to emphasize that not all bacterial strains possess health-promoting properties.

To qualify as an effective probiotic, a bacterial strain must exhibit several key characteristics. In 2002 FAO/WHO formalized a list of criteria in the FAO/WHO Guidelines for the Evaluation of Probiotics in Food (FAO/WHO, 2002), which remain a cornerstone for later probiotic assessment. First, the microorganism must demonstrate the ability to survive the harsh conditions of the GI tract, including resistance to gastric acid, bile salts, and digestive enzymes (Begley et al., 2006; Hill et al., 2014; Jones et al., 2008). Additionally, the strain should adhere to the intestinal epithelium to ensure colonization and interaction with the host microbiota. The safety of the strain is also paramount, ensuring it is non-pathogenic and does not harbour transferable antibiotic resistance genes nor toxins (Imperial & Ibana, 2016). Also, the strain must allow large scale production and exhibit stability and viability during processing, storage and delivery to ensure sufficient viable counts reach the host at the time

of consumption. These factors together with functional efficacy, established through well-designed, randomized clinical trials (Binda et al., 2020; Torres-Maravilla et al., 2022), determine the strain probiotic potential and its utility in food or dietary supplements. Table 1 summarizes the main characteristics that a bacterial strain must exhibit to be considered a probiotic.

Table 1. Characteristics that a strain must possess to be defined as a probiotic.

Safety criteria	- Non-pathogenic - Absence of virulence factors - Absence of antibiotic resistance - Genetically stable - Origin (e.g., human)
Technological criteria	- Large scale production - High viability during processing and storage - Good organoleptic properties - Phage resistance
Physiological criteria	- Tolerance to gastric fluids - Bile tolerance - Adhesion to epithelial cells and mucosal surface
Clinical validation	- Demonstrated health benefits in humans, supported by evidence from randomized controlled clinical trials

1.6 PROBIOTICS FUNCTIONAL FEATURES

Probiotic microorganisms exert a variety of health-promoting functions that contribute to host general well-being. Probiotic functionality is mainly based on direct interaction with the host and interaction with host gut microbiota through a number of actions (Aponte et al., 2020), resumed in Table 2.

Table 2. Summary of functional features of probiotics and their mechanisms of action.

Functional Feature	Mechanism of Action	Health Impact
Strengthening the Intestinal Barrier	Stimulates mucin production, IgA secretion, and antimicrobial peptides (e.g., defensins)	Enhances gut integrity, prevents pathogen translocation, maintains homeostasis
Immunomodulatory, Anti-inflammatory, and Antioxidant Properties	Regulates immune signalling pathways; upregulates anti-inflammatory cytokines; activates antioxidant systems; reduces ROS	Improves mucosal and systemic immunity; reduces inflammation; mitigates chronic disease risk
Pathogen Inhibition	Competes for adhesion sites and nutrients; produces bacteriocins and organic acids; lowers intestinal pH	Suppresses pathogens (e.g., <i>Listeria monocytogenes</i> , <i>Salmonella</i> , <i>Helicobacter pylori</i>)
Nutritional and Metabolic Contributions	Produces SCFAs (acetate, propionate, butyrate); synthesizes vitamins (B-complex, K); enhances nutrient bioavailability.	Supports colonic health; improves nutritional status
Modulation of Gut Microbiota	Promotes beneficial taxa; inhibits harmful microorganisms	Maintains microbiota balance; supports metabolic and gut health
Systemic Health Benefits	Influences gut-brain axis; modulates lipid profiles; improves insulin sensitivity	Contributes to mental health, cardiovascular health, and metabolic regulation

As previously mentioned, one of the primary functions of probiotics is the modulation of the gut microbiota, which includes competitive exclusion of pathogens and support of beneficial microbial populations. For instance, research published in 2021 indicates that *Lactacaseibacillus rhamnosus* GG (LGG) can produce bacteriocins, organic acids, and hydrogen peroxide, which inhibit the proliferation of pathogens such as *Escherichia coli* (*E. coli*), *Salmonella*, and *Clostridium perfringens* (Guo et al., 2021a). Utilizing another mechanism, the acetate produced by *Bifidobacterium longum* NCC2705 serves as a vital co-substrate for butyrate synthesis and growth of *Eubacterium rectale*, a key commensal bacterium. This interaction contributes to maintaining a balanced and healthy microbiota composition (O’Callaghan & van Sinderen, 2016).

Probiotics also contribute to the maintenance and restoration of gut barrier integrity, protecting against increased intestinal permeability, commonly referred to as "leaky gut." For example, some *Lactiplantibacillus plantarum* strains have been reported to enhance the expression of tight junction proteins, thereby reinforcing the epithelial barrier (Alcántara et

al., 2023a; Barbaro et al., 2025; Bron et al., 2017). Furthermore, some *Bifidobacterium breve* can alleviate gut barrier dysfunction in inflammatory bowel disease (IBD) models by modulating host inflammatory responses and enhancing epithelial integrity (C. Huang et al., 2023).

Another important aspect of probiotic function is the immunomodulatory effect. For instance, strain *Lactocaseibacillus casei* Shirota (LcS) exhibits immunomodulatory effects by modulating cytokine production, thereby influencing inflammatory responses. Foey et al. (2022) found that LcS modulates macrophage-epithelial cell cytokine responses, affecting both pro-inflammatory and anti-inflammatory cytokine profiles in co-culture models.

Metabolic benefits, including the production of SCFAs and the regulation of lipid metabolism, are additional functions of probiotics. *Faecalibacterium prausnitzii* is known for its butyrate-producing capacity, which has profound effects on gut health by serving as an energy source for colonocytes and exhibiting anti-inflammatory properties (Duncan et al., 2004; Lenoir et al., 2020). Additionally, *Limosilactobacillus reuteri* has been studied for its role in improving cholesterol profiles by deconjugating bile salts, a mechanism that influences cholesterol metabolism. In a clinical trial, consumption of yogurt containing microencapsulated *L. reuteri* NCIMB 30242 led to significant reductions in LDL-cholesterol and total cholesterol among hypercholesterolemic adults, suggesting that bile salt deconjugation by this strain contributes to its cholesterol-lowering effects (M. L. Jones et al., 2012).

Probiotics can mitigate symptoms of lactose intolerance (Cano-Contreras et al., 2022), a condition caused by the inability to properly digest lactose due to insufficient lactase activity in the small intestine. Certain dairy-associated species, such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, produce β -galactosidase, an enzyme that breaks down lactose into its absorbable monosaccharides, glucose and galactose (Mater et al., 2006; Nguyen et al., 2012). This enzymatic activity can improve lactose digestion and reduce lactose malabsorption mitigating symptoms like bloating, diarrhea, and abdominal pain in individuals with lactose intolerance (Ibrahim et al., 2021). Specific strains of *Bifidobacterium longum* and *Lactobacillus acidophilus* have also been shown to play a role in improving lactose tolerance through similar mechanisms (Masoumi et al., 2021).

Probiotics do not necessarily need to alter the composition of the gut microbiota to exert beneficial effects. Numerous clinical studies have demonstrated that probiotics can significantly benefit human health, even in the absence of measurable changes in the

microbiota composition (Kaczmarczyk et al., 2022). Instead, probiotics may interact directly with gut epithelial cells, immune cells, dietary substrates, or resident commensal bacteria through their metabolic byproducts or specific signalling mechanisms, contributing to positive outcomes for the host. These interactions underline the complexity and multifunctionality of probiotic actions, which extend beyond simple microbial population shifts.

1.7 SIGNIFICANCE OF STRAIN-LEVEL CHARACTERIZATION FOR PROBIOTIC FUNCTIONALITY

The specificity of probiotic effects is strain-dependent, meaning that the health benefits observed in trials can only be attributed to the specific strain tested, and cannot be extrapolated from one strain to another within the same species (McFarland et al., 2018). For example, a strain effective in alleviating diarrhea cannot be assumed to promote immune stimulation or address other clinical conditions unless demonstrated. Even closely related strains within the same species may exhibit distinct functionalities, emphasizing the importance of strain-level characterization.

Each probiotic strain comprises descendants of a single microorganism, classified through genotypic identification and specific phenotypic traits.

Accurate strain identification is pivotal not only for validating the health benefits attributed to specific probiotics but also for ensuring rigorous quality control, facilitating product surveillance, and enabling robust epidemiological tracking. It serves as the cornerstone for linking a strain to its documented effects, ensuring that the health claims associated with probiotic products are scientifically substantiated and consistent with regulatory standards. The most reliable method for identifying microbial strains is whole genome sequencing (WGS), which encompasses both chromosomal and extrachromosomal elements. This approach enables precise identification at both species and strain levels. Reference databases, such as those provided by the National Center for Biotechnology Information (NCBI), support this process (<https://www.ncbi.nlm.nih.gov/>). A complete genome sequence offers significant benefits, including precise strain-specific identification and the ability to assess the presence or absence of potential risk factors (Binda et al., 2020).

Furthermore, advancing mechanistic research is essential to strengthen the evidence base for strain-specific benefits, supporting compliance with labeling and marketing regulations while building consumer trust and scientific credibility.

1.8 ORIGIN, CLINICAL EVIDENCE, AND GUIDELINE RECOGNITION OF AB-BIOTICS PROBIOTIC STRAINS

The probiotic strains constituting the AB-Biotics bacterial collection are predominantly of human origin. All probiotic strains included in the products listed in Table 3 derive from human sources, with a single exception: *Lacticaseibacillus paracasei* AB0918. This strain was isolated from a traditional fermented food from Angola (Africa) known as *omavele*, which consists of milk fermented inside a pumpkin (Embaló, 2014). The collection of this strain was carried out between 2009 and 2010 in collaboration with Universitat Autònoma de Barcelona.

The human-origin strains were initially isolated between 2005 and 2006 from healthy human donors, mainly from fecal or saliva samples collected from healthy children living in rural Amazonian communities. The only exception within this group is *Bifidobacterium longum* CECT 7894, which originates from a mother–infant collection of bifidobacteria (Astó et al., 2022). These strains were isolated in collaboration with the Spanish National Research Council (CSIC) from human milk and/or infant fecal samples obtained from healthy Spanish donors between 2005 and 2010.

Below is a summary of the clinical evidence supporting selected products utilized in this study.

Table 3. Overview of clinical trials with AB-BIOTICS listed products.

Indication / Population	Product / Strain (dose, duration)	Study design	Main outcomes	Reference
AB-KOLICARE (<i>B. longum</i> CECT 7894; <i>P. pentosaceus</i> CECT 8330)				
Infant colic n = 20 babies (age 21–120 days)	AB-KOLICARE (1×10 ⁹ CFU/day; 14 days)	Randomized, double-blind, placebo- controlled pilot trial	Pilot efficacy in infant colic-related symptoms; safety/tolerability noted	(Santas et al., 2015; Tintore & Cune, 2017)
Infant colic (Rome IV) n = 90 babies (age <2 months)	AB-KOLICARE (1×10 ⁹ CFU/day; 21 days)	Randomized double-blind, placebo-control- led trial	↓ crying duration & episodes vs. placebo from day 7/14/21; ↑ responder rate (≥50% crying reduction); no adverse effects	(Chen et al., 2021)

FGIDs (colic ± constipation) n = 36 babies (age 1 week–10 months)	AB-KOLICARE (10 drops/day; 2×10 ⁹ CFU/day; 14 days)	Prospective, observational pilot study	↓ FGID severity (colic and constipation), ↓ parental anxiety; no adverse effects; subgroup analyses show efficacy independent of feeding/delivery modes	(Astó et al., 2022)
Infant colic (Rome IV) n = 112 babies (age 2–12 weeks)	AB-KOLICARE (1×10 ⁹ CFU/day; 21 days) vs. <i>L. reuteri</i> DSM17938	Randomized, multicenter, single-blind, active-controlled trial	Higher response rate vs. <i>L. reuteri</i> on day 7 and 14; trend day 21; robust across sensitivity analyses; safe	(Moreno-Villares et al., 2024)
i3.1 (<i>L. plantarum</i> CECT 7484, <i>L. plantarum</i> CECT 7485, <i>P. acidilactici</i> CECT 7483)				
IBS-D (Rome III) n = 84 adults (age 20–70 years)	i3.1 (Low dose: 3–6×10 ⁹ CFU/day, High dose: 1–3×10 ¹⁰ CFU/day; 6 weeks)	Randomized, multicenter, double-blind, placebo-controlled trial	IBS-QoL improved from week 3; 55% responders vs. 17% placebo; visceral hypersensitivity improved; NNT=2.6	(Lorenzo-Zúñiga et al., 2014)
IBS-D / IBS-M (Rome IV) n = 55 adults (age 25–67 years)	i3.1 (3×10 ⁹ CFU/day; 6 weeks; ± antispasmodic)	Randomized, single-blind, placebo-controlled pilot trial	Higher responder rates for pain and diarrhea vs. placebo; higher QoL response (best in combo arm)	(Barraza-Ortiz et al., 2021)
Stress-related diarrhea n = 60 adults (age 20–<65 years)	i3.1 (3×10 ⁹ CFU/day; 4 weeks)	Randomized, double-blind, placebo-controlled trial	↓ stress-related diarrhea and fecal urgency; improved mental health & productivity; correlation with ↑ <i>Faecalibacterium</i>	(Sato et al., 2022)
Lactose intolerance n = 48 adults (age 20–72 years)	i3.1 (3×10 ⁹ CFU/day; 8 weeks)	Randomized, single-blind, placebo-controlled, prospective trial	↓ symptoms (pain/flatulence; diarrhea trend); 46% responders vs. 0 placebo; no LHBT change (suggests visceral sensitivity)	(Cano-Contreras et al., 2022)
Fructose intolerance n = 27 adults	i3.1 (3×10 ⁹ CFU/day; 30 days)	Prospective, two-cohort trial	↓ bloating and abdominal pain vs. baseline in symptomatic patients; no change in healthy controls	(Portincasa et al., 2022)

IBS (IBS-D/IBS-M) n = 89 adults (35-67 years)	(3×10 ⁹ CFU/day + vitamin D, 6 weeks)	Prospective, multicenter, observational trial	62.9% responders at day 42; significant improvements in IBS-SSS, IBS-QoL, VS.I and HADS	(Jouët et al., 2024)
AB-DENTALAC (<i>L. plantarum</i> CECT 7481 + <i>L. brevis</i> CECT 7480 ± <i>Pediococcus acidilactici</i> CECT 8633)				
Oral colonization and plaque prevention in healthy subjects with healthy n = 40 adults (age 18–55 years)	AB-DENTALAC (1×10 ⁹ CFU/day, 6 weeks)	Randomized, double-blind, placebo-controlled pilot trial	Significant oral colonization of probiotic vs. placebo; ~1-log increase in salivary levels of <i>L. brevis</i> and <i>L. plantarum</i> ; reduction in number of subjects with dental plaque (p = 0.016); plaque reduction correlated with <i>L. brevis</i> abundance; excellent tolerability	(Nart et al., 2021)
Adjunctive treatment of gingivitis n = 59 adults (age 18-55 years)	AB-DENTALAC (1×10 ⁹ CFU/day, 6 weeks)	Randomized, double-blind, placebo-controlled trial	No inter-group difference in mean Gingival Index (GI); significant reduction of sites with severe inflammation (GI = 3) in probiotic group; selective reduction of <i>Tannerella forsythia</i> only in probiotic arm (p = 0.008)	(Montero et al., 2017)
Post-operative pain and functional recovery after mandibular third-molar extraction n = 32 young people (age 14–25 years)	AB-DENTALAC (1×10 ⁹ CFU/day, 7 days)	Randomized, double-blind, placebo-controlled pilot trial	Significantly greater reduction in pain and eating difficulty on post-operative days 5–7 vs. placebo; no effect on swelling or infection rate; good safety profile; mechanistic link to GABA production by <i>L. brevis</i>	(Ferrés-Amat et al., 2020)

Oral malodor (halitosis) in adults with persistent malodor n = 35 adults (age 18–55 years)	AB-DENTALAC (1×10 ⁹ CFU/day, 30 days +30-day follow-up)	Randomized, single-blind, placebo-controlled pilot trial	~50% reduction in malodor scores and ~45% reduction in tongue coating during intervention; increased probiotic DNA detection; modulation of salivary cytokines (↑IL-10; ↓IL-8, TNF-α, IL-6); effects maintained during follow-up	(Enioutina et al., 2024)
AB-DR7 (<i>L. plantarum</i> CECT 7481)				
Upper respiratory tract infections (URTI) n = 109 adults (age 18–60)	<i>L. plantarum</i> DR7 (1 × 10 ⁹ CFU/day, 12 weeks)	Randomized, double-blind, placebo-controlled trial	Reduced nasal-symptom duration and URTI frequency; age-dependent immunomodulation (↑IL-4/IL-10 in <30 y; ↓IFN-γ/TNF-α in 30–60 y); enhanced NK-cell maturation	(Chong et al., 2019b)
URTI (re-analysis) (same cohort as Chong et al. 2019b)	<i>L. plantarum</i> DR7 (1 × 10 ⁹ CFU/day, 12 weeks)	Re-analysis of randomized, double-blind, placebo-controlled trial	Significant reduction in patient-days of URTI and fever (adjusted p < 0.001); 3.4-fold reduction in URTI-medication use; efficacy modulated by recent URTI history	(Altadill et al., 2021)
Stress-related bowel function and gut microbiota n = 111 adults (18–60)	<i>L. plantarum</i> DR7 (1 × 10 ⁹ CFU/day, 12 weeks)	Randomized, double-blind, placebo-controlled trial	Prevented stress-induced increase in bowel movements; preserved microbial diversity; gut taxa correlated with TPH2, DBH, TDO expression (gut–brain axis)	(Liu et al., 2020)

Stress & anxiety (moderately stressed adults) n =111 adults (age 18–60 years)	<i>L. plantarum</i> DR7 (1×10 ⁹ CFU/day, 12 weeks)	Randomized, double-blind, placebo-controlled trial	Reduced stress, anxiety, and total DASS-42 score (from week 8); Reduced plasma cortisol, reduced IFN-γ and TNF-α, increased IL-10; Improved selected CogState domains, stronger effects in adults > 30 years	(Chong et al., 2019a)
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Abbreviations:

FGIDs: Functional gastrointestinal disorders
IBS-D: Irritable Bowel Syndrome, Diarrhea-predominant
IBS-M: Irritable Bowel Syndrome Mixed/Alternating
GABA: gamma-aminobutyric acid
LHBT: Lactose Hydrogen Breath Test
QoL: Quality of Life
IBS-QoL: Irritable Bowel Syndrome Quality of Life Questionnaire
IBS-SSS: Irritable Bowel Syndrome Severity Scoring System
VSI: Visceral Sensitivity Index
HADS: Hospital Anxiety and Depression Scale
NK-cell: Natural-killer cell
DASS-42: Depression, Anxiety, and Stress Scale
TPH2: Tryptophan Hydroxylase 2
DBH: Dopamine Beta-Hydroxylase
TDO: Tryptophan 2,3-Dioxygenase

Definitions:

Rome III (2006): Symptom-based diagnostic criteria for functional gastrointestinal disorders, requiring chronic symptoms (≥3 months, onset ≥6 months before diagnosis) in the absence of structural disease; widely used in earlier clinical trials but allowing relatively heterogeneous patient populations.

Rome IV (2016): Updated criteria redefining these conditions as disorders of gut–brain interaction, with stricter symptom thresholds (e.g. abdominal pain as a core feature in IBS) and a positive diagnostic approach, improving pathophysiological relevance and population homogeneity in clinical research.

Bacillus coagulans Unique IS-2 is a strain licensed by AB-Biotics and originally isolated by the Indian company Unique Biotech Limited (Hyderabad, India) from a non-human source, specifically soil contaminated with healthy human fecal matter. The strain has been evaluated in 16 clinical trials, the results of which are summarized in Table 4.

Table 4. Overview of clinical trials with *Bacillus coagulans* Unique IS-2–based products.

Indication / Population	Product / Strain (dose, duration)	Study design	Main outcomes	Reference
Acute diarrhea n = 28 adults (age 18–55 years)	<i>Bacillus coagulans</i> Unique IS-2 (4×10^9 CFU/day, 10 days)	Open label, phase II clinical trial	↓ diarrhea duration, ↓ stool frequency, ↓ abdominal pain; safe	(Sudha & Bhonagiri, 2012)
Irritable bowel syndrome n = 141 children (age 4–12 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day, 8 weeks)	Randomized, double-blind, placebo-controlled trial	↓ pain, ↓ bloating, ↑ stool consistency, ↑ global relief	(Sudha et al., 2018)
Irritable bowel syndrome (Rome III criteria) n = 136 adults (age 18–60 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day, 8 weeks)	Randomized, double blind, placebo-controlled trial	↓ abdominal pain, ↑ complete spontaneous BMs, ↓ IBS severity	(Madempudi et al., 2019)
Functional abdominal pain n = 115 children (age 6–18 years)	Synbiotic (<i>B. coagulans</i> Unique IS-2 300 million CFU/day + FOS, 4 weeks)	Randomized, double blind, placebo-controlled trial	Higher response rate vs. placebo after treatment	(Saneian et al., 2015)
Functional constipation n = 100 adults (age 18–64 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day, 4 weeks)	Randomized, multicentric double blind, placebo-controlled trial	↑ bowel frequency, ↑ stool consistency, ↓ abdominal pain	(Madempudi et al., 2020)
Functional constipation (Rome III criteria) n = 150 adults (age 18–65 years)	<i>B. coagulans</i> Unique IS-2 + lactulose (2×10^9 CFU/day, 4 weeks)	Randomized, double blind, placebo-controlled, 3-arm trial	Faster symptom relief vs. lactulose alone	(Venkatarama n et al., 2023)
Functional constipation n = 60 children (age 2–14 years)	Synbiotic (<i>B. coagulans</i> Unique IS-2 (15×10^7 CFU/20kg/day, + FOS, 60 days)	Controlled trial	↑ stool frequency, ↑ global symptom improvement	(Saneian et al., 2013))

Small intestinal bacterial overgrowth (SIBO) n = 30 adults (age 21-80 years)	Synbiotic (<i>B. coagulans</i> Unique IS-2 + FOS, 6-month maintenance)	Randomized, double-blind pilot trial	↑ negative breath test, ↓ GI symptoms	(Khalighi et al., 2014)
Bacterial vaginosis (BV) n = 40 adult women (age 30-36 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day + antibiotics, 90 days)	Controlled, open-label (not blinded), non-randomized clinical study	↓ recurrence, ↓ BV symptoms vs. antibiotics alone	(Sudha et al., 2012)
Dental caries risk n = 48 children (age 5–15 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day, 14 days)	Randomized, double blind, placebo-controlled trial	↓ <i>S. mutans</i> and lactobacilli in saliva & plaque	(Sudha et al., 2020)
Plaque-induced gingivitis n = 30 Adults (age 18-50 years)	<i>B. coagulans</i> Unique IS-2 (300 million CFU/day, 3 weeks)	Randomized, double-blind, placebo-controlled pilot trial	↓ gingival inflammation and bleeding	(Jagadeesh et al., 2017)
Inflammatory bowel disease (ulcerative colitis) n = 100 adults (age 18-60 years)	<i>B. coagulans</i> Unique IS-2 (4×10^9 CFU/day + SMT, 4 weeks)	Randomized, double-blind, placebo-controlled trial	↓ disease severity, ↑ IL-10, improved QoL	(Bamola et al., 2021))
Hypercholesterolemia n = 30 adults (<30 years)	<i>B. coagulans</i> Unique IS-2 ($20-40 \times 10^9$ CFU/day, 60 days)	Open-label, single centre, noncomparative phase II clinical study	↓ total cholesterol, ↑ HDL	(Sudha et al., 2011)
Protein absorption & muscle strength n = 70 males (age 18-25 years)	<i>B. coagulans</i> Unique IS-2 + whey protein (2×10^9 CFU/day, 60 days)	Randomized, double-blind, placebo-controlled trial	↑ plasma amino acids, ↑ muscle strength	(Tarik et al., 2022)

Stool quality n= 144 adults (age 18-65 years)	<i>B. coagulans</i> Unique IS-2 (2×10^9 CFU/day, 4 weeks)	Randomized, double-blind, placebo- controlled trial	↑ BM frequency, ↑ stool consistency	(LeMoire et al., 2025)
COVID-19 adjunct therapy n = 56 adults with moderate disease (age 18-80 years)	<i>B. coagulans</i> Unique IS-2 (4×10^9 spores/day, 14 days)	Randomized, double-blind, placebo- controlled, three-arm trial	↓ ferritin, ↓ D-dimer	(Mirza et al., 2024)
BM: Bowel movement HDL: High-density lipoprotein (cholesterol)				

Among the AB-Biotics-products listed, several are supported by international probiotic guidelines, reflecting the robustness of their clinical evidence and the strain specific evaluation adopted by scientific societies. The following list summarizes which products or strains are included in international guidelines:

i3.1®

- **World Gastroenterology Organization (WGO)**, *Global Guidelines on Probiotics and Prebiotics*
 - Indication: Irritable Bowel Syndrome (IBS)
 - Years: 2017, 2023
- **British Society of Gastroenterology (BSG)**
 - Indication: IBS
 - Year: 2021
- **German Society for Gastroenterology, Digestive and Metabolic Diseases (DGVS./DGNM)**
 - Indication: IBS
 - Year: 2021
- **Russian Gastroenterological Association (RGA)**
 - Indication: IBS (adults)
 - Year: 2020

AB-DIGEST® (via strain-specific inclusion)

- **World Gastroenterology Organization (WGO)**
 - Strain: *Lactocaseibacillus rhamnosus* GG
 - Indications: Acute gastroenteritis (AGE), Antibiotic associated diarrhea (AAD), Nosocomial diarrhea, Functional abdominal pain
 - Year: 2023
- **ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition)**
 - Strain: *L. rhamnosus* GG
 - Indications: AGE and AAD in children
 - Year: 2023

Note: Guideline inclusion refers to **LGG**, a key strain contained in AB-DIGEST®, not to the branded product name itself.

AB-KOLICARE®

- **AEProbio (USA)**
 - Indication: Infant colic
 - Year: 2023

1.9 OTHER MICROBIOTA MODULATORS: PREBIOTICS AND SYNBIOTICS

Beyond probiotics, prebiotics and synbiotics play essential roles in microbiome modulation and overall health enhancement. Prebiotics, according to the current definition of the ISAPP, are “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). This updated concept extends beyond traditional bifidobacteria and lactobacilli stimulation, acknowledging that beneficial effects may also stem from taxa such as *Roseburia*, *Eubacterium*, and *Faecalibacterium* spp.

Prebiotics primarily consist of non-starch oligosaccharides and polysaccharides. These compounds occur naturally in foods such as cereals, legumes, vegetables and fruits, making them an integral part of a balanced diet. Furthermore, they can be synthetically produced through enzymatic processes to expand their applications (Davani-Davari et al., 2019). This class of compounds is characterized by three fundamental criteria that define their functionality and health benefits. Firstly, they must exhibit resistance to the digestive

processes of the GI tract, such as gastric acidity and enzymatic hydrolysis, to ensure they reach the colon intact. Secondly, they must be selectively fermented by specific gut microbiota, particularly beneficial bacterial groups, thereby promoting a favourable microbial balance. Finally, their metabolism results in the production of bioactive compounds, notably SCFAs, such as acetate, propionate, and butyrate, which contribute to gut health by enhancing intestinal barrier integrity, modulating immune responses, and reducing inflammation (Scott et al., 2008).

Common prebiotics include inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS). Recent studies highlight prebiotics potential in addressing metabolic syndrome, type 2 diabetes, and immune system disorders through enhanced gut microbial balance and immunomodulation (Bevilacqua et al., 2024; Holscher, 2017; O'Connor et al., 2017).

Human milk oligosaccharides (HMOs) are a unique class of prebiotics naturally found in human breast milk. They include a group of structurally diverse and bioactive carbohydrates, ranking as the third most abundant solid component after lactose and lipids in breast milk (Brockway et al., 2023). Over 200 distinct HMO structures have been identified, comprising combinations of five key monomers: glucose, galactose, N-acetylglucosamine (GlcNAc), fucose, and sialic acid (N-acetylneuraminic acid) (Bode, 2012; Zhang et al., 2021b). When these compounds reach the intestines, they selectively promote the growth of beneficial bacterial taxa such as *Bifidobacterium* spp. Beyond serving as a substrate for gut microbes and support infant gut health, HMOs modulate immune function, enhance gut barrier integrity, and protect against pathogens by acting as decoys for bacterial adhesion (Boll et al., 2024; Singh et al., 2022). Recent research has further demonstrated that maternal factors such as genetics, diet, and environmental conditions influence HMO composition and concentration, emphasizing their dynamic nature (Konieczna et al., 2024). For example, supplementation with probiotics during late pregnancy has been associated with increased levels of specific HMOs like 3-fucosyllactose and 3'-sialyllactose in colostrum (Seppo et al., 2019).

These findings highlight HMOs as key modulators of infant gut microbiota and immune development, with potential applications in designing infant formulas and functional foods. Synbiotics, on the other hand, are formulations that intentionally combine probiotics with prebiotics to achieve a synergistic effect, whereby the administered live microorganisms are supported by substrates that selectively enhance their growth and metabolic activity (Parhi

et al., 2024). According to the current ISAPP consensus definition, synbiotics are defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson et al., 2020). Emerging evidence suggests that synbiotics can effectively reduce gut dysbiosis, improve intestinal barrier function, and alleviate chronic diseases such as inflammatory bowel disease and colorectal cancer (Oh et al., 2020; K. Shimizu et al., 2018). For example, combining GOS with *Bifidobacterium longum* has been shown to enhance SCFAs production, suppress pathogens, and exhibit anti-inflammatory and anticancer properties. In a study of Marzorati et al. (2023), GOS supplementation increases beneficial bacteria, SCFAs, and lactic acid, while decreasing branched SCFAs, pH, and ammonium.

These microbiome modulators represent promising strategies for improving gut health and managing systemic diseases. However, further research is essential to optimize their formulations, dose-response relationships, and mechanisms of action, given their highly strain- and context-dependent effects.

1.10 POSTBIOTICS

As research advances, another group of functional products is gaining attention. Postbiotics are defined as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Aguilar-Toalá et al., 2018; Salminen et al., 2021; Żółkiewicz et al., 2020). They represent a promising alternative to probiotics by overcoming several limitations inherent to live microorganisms, notably improved stability, a prolonged shelf life, and enhanced resistance to environmental and processing stresses.

Unlike probiotics, postbiotics are not affected by antibiotic treatment and do not carry the risk of transmitting antibiotic-resistance genes. Moreover, they raise no concerns regarding pathogenicity in immunocompromised individuals or unintended microbial interactions (Liang & Xing, 2023; Salminen et al., 2021; Taverniti & Guglielmetti, 2011). Consequently, postbiotics are considered a safer alternative, particularly suitable for vulnerable populations such as newborns and individuals with heightened sensitivity.

On the downside, the absence of live microorganisms in postbiotics means they may not exert the same range of microbiota-modulating effects as probiotics, like colonize the gut or interact dynamically with the existing microbiota (Liang & Xing, 2023). This distinction

between probiotics and postbiotics emphasizes the need to evaluate the context in which each might be most beneficial.

1.11 FUNCTIONAL FOODS

The concept of functional foods emerged prominently in Japan during the 1980s and gained formal recognition with the establishment of the “Foods for Specified Health Use” (FOSHU) regulatory framework in 1991. This framework, introduced by Japan's Ministry of Health, Labor, and Welfare, aimed to ensure the safety and efficacy of foods with proven health benefits beyond basic nutrition. Functional foods are generally defined as any natural or processed food that not only satisfies nutritional needs but also provides additional physiological benefits, such as reducing disease risks or enhancing specific bodily functions (Damián et al., 2022; T. Shimizu, 2003). Functional foods may be fortified with a range of bioactive components, including vitamins, minerals, probiotics, prebiotics, postbiotics, and dietary fibres. Unlike dietary supplements, functional foods are consumed as part of a typical and regular diet, allowing for the delivery of health-promoting benefits through regular meals. Growing consumer interest in health and wellness has fueled rapid expansion of the functional foods market. This trend has driven innovations in food and beverage products targeting immunity, metabolic health, sports performance, gut health, and more, with gut health emerging as one of the most prominent areas.

Functional foods designed for gut health incorporate a variety of bioactive ingredients, each contributing specific benefits to maintain or restore GI homeostasis and associated systems. Between other key ingredients, probiotics have become a cornerstone of these applications. They represent one of the fastest-growing segments, due in part to increasing scientific evidence linking gut microbiota modulation with overall health (El-Sohaimy & Hussain, 2023; Grand view research, 2023).

Originally, functional foods were mainly the results of fermentation, such as yogurt, kimchi, kombucha, etc. Fermented foods have played a key role in human diets for millennia, historically serving to inhibit spoilage and pathogenic microorganisms (Taveira et al., 2021). Fermentation is a natural metabolic process that occurs in some bacteria and yeast that break down organic compounds to use the deriving energy for their sustainment, while releasing by-products such as alcohol, organic acids, gases, etc. These by-products hold a value in the food and beverage market as they can enhance not only the sensory, the safety and the

stability of the products but also improve their nutritional value (de la Fuente et al., 2021; Mukisa et al., 2017; Rizzi et al., 2024b; Ruiz Rodríguez et al., 2021). Numerous bioactive metabolites have been identified as result of bacterial protein, lipid, and carbohydrate metabolism. The synthesis of vitamins and antioxidants, as well as improved mineral bioavailability, has been widely reported for various lactic acid bacteria (LAB) species (Gänzle, 2015). A wide assortment of biologically active peptides from bacterial origin have also been described in fermented products displaying antioxidant, anti-microbial, anti-fungal, anti-inflammatory, anti-diabetic and anti-atherosclerotic activity, among others (Leeuwendaal et al., 2022; Şanlier et al., 2019).

In 2021, ISAPP released a consensus statement defining fermented foods as “foods made through desired microbial growth and enzymatic conversions of food components” and clarifying their distinction from probiotics. The statement also reviews the safety, health benefits, microbial ecology, systems biology, and regulatory aspects of fermented foods (Marco et al., 2021).

Thus, probiotic-fermented products may deliver a dual function to the host: they introduce well-characterized, health-supporting bacteria while enhancing nutritional value via active metabolites produced during fermentation (Y. Dong et al., 2024).

The rise of microbiota-oriented functional foods reflect a broader shift toward personalized nutrition and preventive health in the food industry. Regulatory frameworks such as FOSHU, and analogous systems in other parts of the world, are instrumental in fostering product credibility, consumer trust, and scientific substantiation of health claims.

1.12 PROBIOTICS IN FOOD SYSTEMS

Maintaining a healthy balance in the GI tract can be supported by consuming beneficial bacteria, either through dietary supplements (such as capsules containing viable cells) or through foods enriched with these microorganisms.

Probiotics can be applied across a wide range of food categories, including dairy products, beverages, snacks, confectionery, and bakery goods (Latif et al., 2023).

Food and beverage matrices consist of complex combinations of physicochemical parameters and conditions that can affect probiotic viability, including pH, water activity, temperature, osmotic stress, oxygen exposure, sensitivity to metabolites produced by other competing bacteria, presence of prebiotic components, etc. (Palanivelu et al., 2022; Sendra

et al., 2016; Terpou et al., 2019). Each matrix creates a unique environment that interacts with probiotics, influencing their survival during processing, storage, and GI transit. Additionally, probiotic strains exhibit distinct traits, growth behaviours, and survival requirements, making their responses to food matrices highly strain specific (Sendra et al., 2016). Consequently, it is essential to investigate tailored approaches and select probiotic strains individually, ensuring compatibility with the specific characteristics of the food matrix. Preserving the viability and the effectiveness of probiotic bacteria presents significant challenges that must be addressed during the development of functional food products.

Dairy products are among the most established and effective matrices for the delivery of functional ingredients, particularly probiotics. Fermented dairy systems offer a physiologically and technologically favourable environment for lactic acid bacteria and bifidobacteria, promoting their survival, metabolic activity, and stability throughout processing and refrigerated storage. In addition to supporting probiotic viability, fermentation contributes to desirable product attributes, including acidification, texture development, flavour formation, and enhanced microbiological safety. From a health perspective, probiotic-enriched dairy products have been demonstrated to modulate gut microbiota composition, improve nutrient bioavailability, and enhance product functionality, while also exerting antagonistic activity against gastrointestinal pathogens, including *Helicobacter pylori*, as evidenced by both in vitro and human intervention studies (De Souza Da Motta et al., 2022; Gao et al., 2021; Nami et al., 2019). Nevertheless, dairy-based delivery systems present inherent limitations, such as the requirement for refrigerated storage to maintain microbial viability and reduced suitability for individuals with lactose intolerance, milk protein allergies, or specific dietary preferences. Despite these constraints, dairy products remain one of the most established and consumer-accepted vehicles for probiotic delivery, owing to their favourable sensory characteristics, technological versatility, and long-standing role in fermented food consumption.

Table 5. Main probiotic species used in the production of fermented milks (Denkova et al., 2012).

GENUS	SPECIES
<i>Lactobacillus</i>	<i>L. acidophilus</i> ; <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>L. crispatus</i> ; <i>L. johnsonii</i>
<i>Lacticaseibacillus</i>	<i>L. casei</i> ; <i>L. paracasei</i> ; <i>L. rhamnosus</i>
<i>Lactiplantibacillus</i>	<i>L. plantarum</i>
<i>Limosilactobacillus</i>	<i>L. fermentum</i> ; <i>L. reuteri</i>
<i>Ligilactobacillus</i>	<i>L. salivarius</i>
<i>Lactococcus lactis</i>	<i>L. lactis</i>
<i>Bifidobacterium</i>	<i>B. adolescentis</i> ; <i>B. bifidum</i> ; <i>B. breve</i> ; <i>B. infantis</i> ; <i>B. animalis</i> subsp. <i>lactis</i> ; <i>B. longum</i>
<i>Enterococcus</i>	<i>E. faecalis</i> ; <i>E. faecium</i>
<i>Pediococcus</i>	<i>P. acidilactici</i>
<i>Propionibacterium</i>	<i>P. freudenreichii</i>
<i>Saccharomyces</i>	<i>S. cerevisiae</i> var. <i>boulardii</i>
<i>Streptococcus</i>	<i>S. thermophilus</i>

Non-dairy probiotic carriers, including fruit and vegetable juices, cereal and nuts-based beverages, and herbal drinks, present a promising alternative to dairy-based products, addressing consumer new dietary preferences and ethical considerations.

For instance, the presence of prebiotic fibres in plant-based milks can synergistically enhance probiotic viability. However, these matrices face significant challenges, particularly in maintaining probiotic viability during storage due to their lower buffering capacity and potential for acidity or oxidative stress. Moreover, ensuring the survival of probiotics along the manufacturing processes and storage conditions without altering the product sensory qualities is one of the main limitations (Min et al., 2019).

Different strategies are being explored to enhance stability and efficacy of probiotic in non-dairy matrices such as strain selection, fortification with prebiotics like inulin, fermentation and innovative approaches, such as encapsulation technologies and secondary packaging (Aspri et al., 2020; Fenster et al., 2019).

Bakery products, such as bread and biscuits, represent an unconventional yet promising matrix for probiotics. Bakery items are shelf-stable and do not require refrigeration. Their dry, low-moisture environment can protect probiotics during storage.

The high-temperature baking process, however, poses a substantial challenge, as most probiotics are sensitive to heat. Advances in encapsulation technologies, post-baking applications and use of sporulated probiotic are addressing these limitations, enabling bakery

products to deliver probiotics effectively and ensuring functional and nutritional benefits (Almada-Érix et al., 2022; Bahmani et al., 2016; Mani-López et al., 2023).

Spore-forming bacteria such as *Heyndrickxia coagulans*, and *Bacillus subtilis*, from the *Bacillaceae* family, are emerging as robust alternatives to conventional probiotics. Species within these genera exhibit exceptional stability during harsh processing and storage conditions due to their ability to form resilient endospores, a property absent in common LAB, that makes them particularly suitable for incorporation into dry food systems, beverages, and heat-treated products.

Heyndrickxia coagulans was first described as *Bacillus coagulans* by Hammer in 1915 (Hammer, 1915), based on strains isolated from coagulated evaporated milk. Subsequent isolates showing phenotypic similarities to *Lactobacillus* led to its temporary classification as *Lactobacillus sporogenes* in early taxonomic literature, though later DNA-based analyses returned it to *Bacillus* (Drago & De Vecchi, 2009). In 2020, phylogenomic studies demonstrated that *Bacillus coagulans* formed a distinct lineage separate from core *Bacillus* species, leading to its reassignment as *Weizmannia coagulans* (Gupta et al., 2020). However, 2023 genome-based reclassification revealed that *Weizmannia* was not sufficiently distinct from *Heyndrickxia*. As a result, *W. coagulans* was formally transferred to *Heyndrickxia coagulans*, which is now the validly published name (Narsing Rao et al., 2023).

In this thesis the commercial name “*Bacillus coagulans* Unique IS-2” has been used to identify the strain *Heyndrickxia coagulans* Unique IS-2.

Microencapsulation has emerged as a promising technique to protect probiotics during processing. For instance, *Lactobacillus acidophilus* LA-5 encapsulated using alginate-fish gelatin microencapsulation maintained higher cell viability during bread baking and maintained its levels throughout a seven-day storage period, highlighting its potential for functional bakery products (Hadidi et al., 2021).

Confectionery products and snacks, including chocolates, gummies, cereal bars, etc., offer another innovative matrix for probiotics. These products are particularly attractive for their ability to target specific demographics, such as children, through appealing taste and convenient formats. The low water activity of these products provides a stable environment for probiotics during storage. However, challenges include maintaining probiotic viability during production processes that may involve elevated temperatures or extended handling

times. Furthermore, the typically high sugar content of these products may detract from their health benefits.

The selection and formulation of an appropriate food matrix is a critical determinant of both the functional efficacy and commercial success of probiotic-containing products. Dairy probiotic products continue to dominate the market due to their historical proven efficacy and established processing technologies to support microbial viability. However, in response to increasing consumer demand for diversified and plant-based alternatives, the development of non-dairy matrices, including bakery products, snacks, confectionery, and other categories is gaining significant momentum.

Each matrix type presents a distinct set of advantages and limitations, influencing factors such as sensory acceptance, microbial survival, regulatory status, and industrial scalability. Addressing these challenges requires continued research and innovation.

As consumer interest in functional foods and personalized nutrition continues to grow, the advancement of adaptable and effective probiotic delivery systems will remain a key focus in the field of food biotechnology and health-oriented product development.

1.13 EUROPEAN REGULATORY FRAMEWORK FOR PROBIOTIC FOODS

In September 2025, the European Food Safety Authority (EFSA) released a new consolidated guidance that reshapes how microorganisms used in the food chain should be characterised for safety evaluation (EFSA Scientific Committee, 2025). The “2025 EFSA Guidance on the Characterisation of Microorganisms Used in the Food Chain” harmonises scientific criteria previously distributed across multiple regulatory documents covering novel foods, food enzymes, feed additives and genetically modified microorganisms, and integrates them into a single, comprehensive standard that reflects current scientific methods. A major update is the requirement to use WGS as the core tool for taxonomic identification of bacteria, yeasts, filamentous fungi and bacteriophages, and the extension of WGS-based classification to filamentous fungi for the first time. For microalgae and protists, EFSA maintains a combined morphological and marker-based sequencing approach, noting that WGS is not yet universally reliable for these groups. The guidance also introduces a “WGS first” strategy for antimicrobial resistance (AMR): AMR screening must start from genome data to distinguish intrinsic from acquired resistance genes, followed, when necessary, by minimum inhibitory concentration (MIC) testing using updated, species-specific interpretive

cutoffs. This is complemented by the introduction of MIC requirements for yeasts and filamentous fungi whenever viable cells might remain in the final product. Figure 4 shows the decision tree on the assessment of AMR in bacteria and bacteriophages. For microalgae and protists, EFSA maintains a combined morphological and marker-based sequencing approach, noting that WGS is not yet universally reliable for these groups. The guidance also introduces a “WGS-first” strategy for antimicrobial resistance: AMR screening must start from genome data to distinguish intrinsic from acquired resistance genes, followed, when necessary, by minimum inhibitory concentration (MIC) testing using updated, species-specific interpretive cut-offs. This is complemented by the introduction of MIC requirements for yeasts and filamentous fungi whenever viable cells might remain in the final product.

EFSA also clarifies expectations for assessing pathogenicity and toxigenic potential across all microbial groups (e.g., for *Bacillus* spp., cell cytotoxicity test). Applicants must begin with literature reviews and genomic analyses and perform phenotypic or quantitative testing only when a potential safety concern is indicated. Requirements for demonstrating the absence of viable cells or residual DNA from production strains have been updated as well: applicants now need to analyse at least 1 g/mL of final product (rather than working from a 10 g/mL sample) and include positive controls with low-level microbial spikes, while the requirement to target strain specific DNA sequences has been relaxed. On genetic modifications, the new framework modernises and partially replaces the 2011 guidance, introducing a clearer structure for environmental risk assessment depending on whether the microorganism is genetically modified and whether it is used as an active agent or as biomass. EFSA also clarifies how Qualified Presumption of Safety (QPS) qualifications should be verified and explicitly opens the door for certain genetically modified strains to qualify for QPS status when the modification does not raise safety issues, or when it removes an element of concern.

Although the guidance does not apply to all sectors, such as microbial plant protection products, biocides, cosmetics or cleaning agents, it provides a unified set of requirements for microorganisms intended for food and feed applications. This consolidation simplifies dossier preparation by allowing applicants to develop a single core characterisation package that can be adapted to different regulatory uses and improves consistency across EFSA scientific panels. Overall, the updated guidance aims to facilitate more predictable and transparent safety assessments while supporting the development of innovative microbial products that meet modern scientific standards.

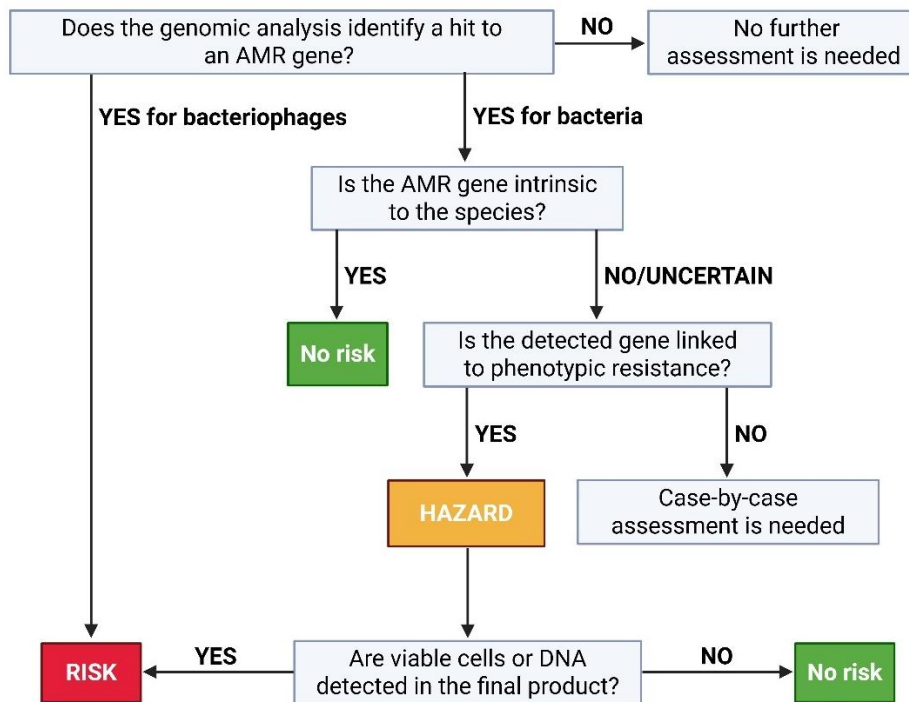


Figure 4. Decision tree on the risk assessment of antimicrobial resistance in bacteria and bacteriophages (EFSA Scientific Committee, 2025). The figure was created in <https://BioRender.com>.

2 PROJECT PLAN AND OBJECTIVES

2.1 GENERAL OBJECTIVE

To evaluate the applicability, performance, and functional impact of selected AB-Biotics probiotic strains across a wide range of fermented and non-fermented food and beverage matrices by assessing their viability, stability, metabolic activity, and sensory acceptance, with the aim of establishing rational strain–matrix selection criteria that maximize functional performance, product stability, and consumer acceptance, and of generating a robust scientific basis to support industrial innovation and the expansion of AB-Biotics’ functional food portfolio.

2.2 SPECIFIC OBJECTIVES

- 1 To evaluate the viability and survival kinetics of probiotic strains in fermented and non-fermented food matrices, under realistic storage conditions.
2. To assess the compatibility of probiotic formulations with common industrial processes, including pasteurization, baking, freezing, mixing, and prolonged storage.
3. To determine the impact of probiotic incorporation or fermentation on physicochemical, microbiological, and sensory parameters of food products.
4. To investigate the potential of probiotic fermentation as a strategy to enhance functionality through the production of bioactive metabolites.

2.3 WORK PLAN SUMMARY

Owing to the heterogeneity of matrices and probiotic strains investigated, the experimental design is described separately and in detail at the beginning of the Results and Analysis section for each matrix. Table 6 provides an overall summary of the work plan.

Table 6. Work plan summary.

Matrix	Probiotic Product	Storage	Shelf life	Analysis
NON-FERMENTED FOOD AND BEVERAGE MATRICES				
Dairy Products				
Cheese cream	Blend I3.1	4 ± 2 °C	4 months	CFU, pH
Frozen Dessert				
Ice creams	Blend I3.1	-18 ± 2 °C	18 months	CFU, pH, titratable acidity, overrun, melting, sensory analysis
Confectionary Products				
Peanut butter	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	12 months	CFU, pH, water activity, sensory analysis
Chocolate creams	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	12 months	CFU, pH, water activity, sensory analysis
Chocolate	Blend AB-KOLICARE Blend I3.1	25 ± 5 °C	15 / 24 months	CFU, pH, water activity, colour
Popping candy	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	12 months	CFU, pH, water activity, sensory analysis
Gummies	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	12 months	CFU, pH, water activity
Snack Products				
Energetic bars	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	12 months	CFU, pH, water activity, texture, sensory analysis
Bakery products				
Butter cookies	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C	4 / 12 months	CFU, pH, water activity, colour, sensory analysis
Beverage Products				
Orange juice	<i>P. acidilactici</i> CECT 7483 <i>L. plantarum</i> CECT7484 <i>L. plantarum</i> CECT7485 <i>B. longum</i> CECT7894 <i>B. coagulans</i> Unique IS-2	4 ± 2 °C	2 / 4 / 12 months	CFU, pH, total soluble solids, physical stability, colour
Isotonic Drink	<i>B. coagulans</i> Unique IS-2	25 ± 5 °C / 30 ± 2 °C	15/18 months	CFU, pH, total soluble solids, turbidity, colour, sensory analysis
Isotonic Drink Powder	<i>B. coagulans</i> Unique IS-2 Blend AB-DIGEST	30 ± 2 °C, 65% RH / 40 ± 2 °C, 75% RH	1.5/6 months	CFU, water activity, colour, sensory analysis
FERMENTED FOOD AND BEVERAGE MATRICES				
Dairy Products				
Drinkable fermented milk	Blend I3.1	4 ± 2 °C	2 months	CFU, pH
Spoonable fermented milk	Blend I3.1	4 ± 2 °C	2 months	CFU, pH
Set fermented milk	<i>L. plantarum</i> CECT 7485 <i>L. brevis</i> CECT 7480 <i>L. plantarum</i> CECT 7481 (DR7®) <i>L. paracasei</i> AB0918 Blend PROBIOMIND	4 ± 2 °C	35 days	CFU, pH, titratable acidity, colour, secondary oxidation, acidification kinetics, coagulation kinetics, texture, sensory analysis

Bakery products				
Sandwich Bread	<i>Bacillus coagulans</i> Unique IS-2	25 ± 5 °C	28 days	CFU, pH, aw, colour, texture, weight loss during baking, sensory analysis
Beverage Products				
Alcohol-free beer	<i>Bacillus coagulans</i> Unique IS-2	25 ± 5 °C	6 months	CFU, pH
Plant-based Beverage Product				
Tiger nut beverage	<i>P. acidilactici</i> CECT 7483 <i>L. plantarum</i> CECT7481 (DR7®) <i>L. plantarum</i> CECT 7484 <i>L. plantarum</i> CECT 7485	4 ± 2 °C	2 months	CFU, pH, physical stability
Oat and Soy beverage	<i>L. paracasei</i> AB0918 + starter (<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> and <i>S. thermophilus</i>)	4 ± 2 °C	35 days	CFU, acidification kinetics, pH, titratable acidity, colour, viscosity, secondary oxidation, sensory analysis
Fruit juices	<i>L. brevis</i> CECT 7480 <i>L. plantarum</i> CECT 30292 <i>L. plantarum</i> CECT7481 (DR7®) <i>L. plantarum</i> CECT 7484 <i>L. plantarum</i> CECT7485	4 ± 2 °C	2 months	CFU, pH, total soluble solids, targeted and untargeted metabolomic analysis, total phenolic content, antioxidant capacity, sensory analysis

*CFU= colony forming units. Microbiological analyses have been performed to assess the viability of probiotics and the microbiological quality.

3 MATERIAL AND METHODS

3.1 PROBIOTIC STRAINS

The strains utilized in this study (Table 7) were provided by AB-Biotics S.A. (KANEKA Corp., Sant Cugat del Vallès, Spain) as lyophilized preparations of vegetative cells and were stored at $-20\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in sealed aluminium bags until use, with the exception of *B. coagulans*, which was supplied in its spore form and stored at room temperature ($25 \pm 5\text{ }^{\circ}\text{C}$) in sealed aluminium bags.

Table 7. Overview of probiotic single strains and blends included in the study.

Species	Strain	Origin	Commercial product
<i>Bifidobacterium longum</i> subsp. <i>Longum</i>	KABP042 (CECT7894)	Human feces (breast-feed infant)	AB-DIGEST, AB-KOLICARE
<i>Pediococcus pentosaceus</i>	KABP041 (CECT8330)	Human feces (healthy child)	AB-DIGEST, AB-KOLICARE
<i>Lacticaseibacillus rhamnosus</i>	GG (ATCC53103)	Human feces (healthy adult)	AB-DIGEST
<i>Pediococcus acidilactici</i>	KABP021 (CECT7483)	Human feces (healthy child)	I3.1, AB21
<i>Lactiplantibacillus plantarum</i>	KABP022 (CECT7484)	Human feces (healthy child)	I3.1, AB21
<i>Lactiplantibacillus plantarum</i>	KABP023 (CECT7485)	Human feces (healthy child)	I3.1, AB21, PROBIOMIND
<i>Levilactobacillus brevis</i>	KABP052 (CECT7480)	Human saliva (healthy child)	AB-DENTALAC, PROBIOMIND
<i>Lactiplantibacillus plantarum</i>	DR7, KABP051 (CECT7481)	Human feces (healthy child)	AB-DENTALAC, PROBIOMIND, AB-DR7, MINDBIOME
<i>Lactiplantibacillus plantarum</i>	KABP033 (CECT30292)	Fermented silege	AB21
<i>Lacticaseibacillus paracasei</i>	AB0918 (CECT30827)	Fermented milk (Omavele)	-
<i>Heyndrickxia coagulans</i>	Unique IS-2	Soil contaminated with human feces	<i>Bacillus coagulans</i> Unique IS-2

3.2 MATRICES

In this study, a diverse range of food and beverage matrices was employed. Some products were purchased from local supermarkets in Barcelona, Spain, and inoculated with probiotics or starters without additional formulation (i.e., peanut butter, chocolate spreads, tiger nut beverage, soy beverage, oat beverage, orange juice, peach juice, and apple juice). Other

matrices were developed through formulation and production processes conducted either in the UAB pilot plant or the UAB laboratory (i.e., cereal bars, butter cookies, fermented milk, sandwich bread).

A third group of matrices was provided by AB-Biotics' potential customers and contract manufacturing partners as part of collaborative projects (i.e., cream cheese, ice cream, chocolate bars, chocolate bites, chocolate chunks, popping candy, gummies, isotonic drinks in powder and liquid formats, fermented milk, and non-alcoholic beer). Some specific details regarding the ingredients, quantitative formulation, and production processes of these matrices cannot be disclosed, as they constitute proprietary information protected under a non-disclosure agreement (NDA). Data generated from these formulations were used exclusively for scientific evaluation (e.g., physicochemical characterization, sensory analysis, etc.) and do not compromise the scientific integrity of the study. General information regarding ingredient composition and processing steps is provided where possible without breaching confidentiality obligations.

3.2.1 Peanut Butter

A commercial organic peanut butter was used (Table 8). Specifically, Veritas Crema de cacahuete 100% ECO (Veritas, Barcelona, Spain).

Ingredients: 100% organic roasted peanuts

Table 8. Commercial peanut butter nutritional information.

Nutritional value per 100 g	
Energy	613 kcal / 2537 kJ
Fats	50.00 g
<i>Saturated Fats</i>	7.70 g
Carbohydrates	9.30 g
<i>Sugars</i>	4.90 g
Proteins	28.00 g
Salt	0.02 g

The probiotic-enriched peanut butter was prepared by incorporating the appropriate dose of lyophilized *Bacillus coagulans* Unique IS-2 spores into the commercial product (inoculum of 8.52 log CFU/g). Prior to addition, the probiotic powder was premixed with sunflower oil to facilitate its homogeneous distribution in the peanut butter matrix. The samples were stored at 25 ± 5 °C in a sealed plastic container.

3.2.2 Chocolate Creams

Three commercial chocolate creams were selected (Table 9):

1. Milk chocolate cream (MCC) = Nutella (Ferrero S.p.A., Alba, Italy)

Ingredients: Sugar, palm oil, hazelnuts (13%), defatted cocoa (7.4%), skimmed milk powder (6.6%), whey powder, emulsifiers (soy lecithin), vanillin.

2. Vegan dark chocolate cream (VDCC) = Dark Chocolate Spread La Vida Vegan (Brinkers Food, Enschede, Netherlands).

Ingredients: Sunflower oil, cane sugar, defatted cocoa powder, sunflower lecithin, bourbon vanilla powder.

3. Vegan hazelnut chocolate cream (VHCC) - Hazelnut Chocolate Spread La Vida Vegan (Brinkers Food).

Ingredients: Sunflower oil, cane sugar, hazelnuts, cocoa butter, defatted cocoa powder, sunflower lecithin, rice syrup powder, bourbon vanilla powder.

Table 9. Commercial chocolate creams nutritional information.

	MCC	VDCC	VHCC
	Nutritional value per 100 g		
Energy	539 kcal / 2252 kJ	547 kcal / 2292 kJ	581 kcal / 2421 kJ
Fats	30.9 g	38 g	38 g
<i>Saturated Fats</i>	10.6 g	5.9 g	5.4 g
Carbohydrates	57.5 g	39 g	54 g
<i>Sugars</i>	56.3 g	35 g	48 g
Fibre	-	9.3 g	3.2 g
Protein	6.3 g	7.6 g	4.2 g
Salt	0.107 g	0.03 g	0.01 g

The probiotic-enriched chocolate creams were prepared by incorporating the appropriate dose of lyophilized *B. coagulans* Unique IS-2 spores into the commercial products (inoculum of 8.60 log CFU/g). Prior to addition, the probiotic powder was premixed with sunflower oil to facilitate its homogeneous distribution in the chocolate creams. The samples were stored at 25 ± 5 °C in a sealed plastic container.

3.2.3 Orange Juice

A commercially available ultra-high temperature (UHT) treated orange juice was used (Table 10). Specifically, the product 'Zumo 100% natural y 100% exprimido Hacendado' (Mercadona, Albalat dels Sorells, Spain).

Ingredients: 100% orange juice.

Table 10. Commercial orange juice nutritional information.

	Nutritional value per 100 ml
Energy	44 kcal / 184.10 kJ
Fats	0.10 g
<i>Saturated Fats</i>	0 g
Carbohydrates	9.90 g
<i>Sugars</i>	9.30 g
Proteins	0.70 g
Fibre	0.60 g
Salt	0 g

Three tests were conducted, using different probiotic strains and product preparation.

Test 1. Orange juice supplemented with four different probiotic strains

To prepare the experimental formulations, the appropriate dosage of four lyophilized probiotic strains (inoculum of at 7.96 log CFU/ml) was directly mixed into commercial orange juice and stored at 4 ± 2 °C. This procedure resulted in four distinct versions of the juice, each supplemented with a specific probiotic strain:

- *Bifidobacterium longum* CECT 7894
- *Pediococcus acidilactici* CECT 7483
- *Lactiplantibacillus plantarum* CECT 7484
- *Lactiplantibacillus plantarum* CECT 7485

Test 2. Orange juice supplemented with *B. coagulans* Unique IS-2

For this experiment, the probiotic-enriched orange juice was prepared by directly mixing the commercial product with the appropriate dose of lyophilized *B. coagulans* Unique IS-2 spores, using two inoculation levels (6.78 and 7.78 log CFU/mL). The fortified juices were subsequently stored at 4 °C.

Test 3. Pasteurized orange juice supplemented with *B. coagulans* Unique IS-2

- For this experiment, pasteurized probiotic-enriched orange juice was obtained by mixing the commercial product with the appropriate dosage of lyophilized *B.*

coagulans Unique IS-2 spores (inoculum of 7.78 log CFU/ml). The mixture was subsequently pasteurized at 82 °C for 45 s using a milk pilot pasteurization unit (Garvía, S.A. Barcelona), after which it was aseptically bottled in sterile containers and stored at 4 °C, protected from light.

3.2.4 Fermented Tiger Nut Beverage

For the present study, a commercially available UHT treated tiger nut beverage was selected as the base matrix (Table 11). Specifically, the product used was Bebida Chufa sin azúcar EcoMil (Nutriops S.L., Murcia, Spain).

Ingredients: water, tiger nuts (12%), sunflower lecithin (emulsifier), and carob seed flour (thickener).

Table 11. Commercial tiger nut beverage nutritional information.

	Nutritional value per 100 ml
Energy	39 kcal / 161 kJ
Fats	2.1 g
<i>Saturated Fats</i>	0.5 g
Carbohydrates	4.2 g
<i>Sugars</i>	2.7 g
Proteins	0.5 g
Sodium	0.04 g

Four experimental tests were conducted using distinct probiotic strains. For each formulation, the appropriate dosage of lyophilized probiotic powder was directly incorporated into the commercial tiger nut beverage (inoculum 7.96 log CFU/ml). After the inoculation, the mixtures were either directly stored at 4 ± 2 °C (probiotic-supplemented versions) or subjected to fermentation at 37 °C for 24 hours prior to refrigeration at 4 ± 2 °C (probiotic-fermented versions). This procedure yielded a total of eight beverage variants, four supplemented and four fermented, each containing a specific probiotic strain:

- *Lactiplantibacillus plantarum* CECT 7481
- *Lactiplantibacillus plantarum* CECT 7485
- *Lactiplantibacillus plantarum* CECT 7484
- *Pediococcus acidilactici* CECT 7483

3.2.5 Fermented Soy and Oat Beverages

Two commercially available UHT treated beverages, one soy-based and one oat-based, were selected as the base matrices (Table 12). Specifically:

- Soy beverage = Yosoy Soja 0% Azúcar (Liquats Vegetals S.A., Girona, Spain)

Ingredients: water, 10% soy, sea salt

- Oat beverage = Yosoy Avena (Liquats Vegetals S.A.)

Ingredients: water, 15% oat

Table 12. Commercial soy and oat beverages nutritional information.

	Soy beverage	Oat beverage
	Nutritional value per 100 ml	
Energy	30 kcal / 125 kJ	47 kcal / 198 kJ
Fats	1.6 g	0.7 g
<i>Saturated Fats</i>	0.2 g	0.1 g
Carbohydrates	0.7 g	8.2 g
<i>Sugars</i>	0 g	5.4 g
Proteins	3.0 g	1.6 g
Dietary fibre	0.4 g	0.7
Salt	0.03 g	0.03 g

Commercial products were inoculated with the yogurt starter culture Lyofast SY42 (Sacco System, Cadorago, Italy), composed of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (inoculum: 0.01 UC/L according to manufacturer dosage instructions), combined with a defined amount of lyophilized probiotic *L. paracasei* AB0918 powder (inoculum 7 log CFU/mL). Prior to inoculation, both the probiotic and the starter culture were separately premixed with Milli-Q water to ensure homogeneous distribution. The inocula were then incorporated into the pre-heated soy or oat beverage at 43 °C. Fermentation was conducted at 43 °C until the product reached pH of 4.6, yielding the probiotic-fermented product (PF).

For each beverage matrix, a control sample (C) was prepared under identical conditions, using only the starter culture without the probiotic strain. Both formulations were subsequently stored at 4 °C.

3.2.6 Fermented Fruit Juices

Within the scope of applying probiotics in fermented fruit juices, two trials were conducted:

TEST 1: Screening and Functional Evaluation of Probiotic Strains for Flavonoid Biotransformation in Orange Juice

For this study, the same commercial product described before (see section 3.2.3) was selected.

To prepare the experimental formulations, the appropriate dosage of five lyophilized probiotic strains was directly mixed into commercial orange juice (inoculum 7.96 log CFU/ml), fermented 24 h at 37°C and then stored at 4 ± 2 °C. This procedure resulted in five distinct versions of fermented juice, each with a specific probiotic strain:

- *Levilactobacillus brevis* CECT 7480
- *Lactiplantibacillus plantarum* CECT 30292
- *Lactiplantibacillus plantarum* CECT 7484
- *Lactiplantibacillus plantarum* CECT 7485
- *Lactiplantibacillus plantarum* CECT 7481

TEST 2: Functional Evaluation of Probiotic Strain *L. plantarum* CECT 7481 in Orange, Apple and Peach Juices

The description of the material used in this trial is provided in the publication “*Lactiplantibacillus plantarum* KABP051: Stability in Fruit Juices and Production of Bioactive Compounds During Their Fermentation”, specifically in Section 4.2.4.3.”

3.2.7 Sandwich Bread

Sandwich bread was manufactured in the UAB pilot plant using the ingredients and formulation listed in Table 13.

Table 13. Sandwich bread ingredient list and quantitative formulation.

Ingredient	Commercial product	%	g
White flour	Farina Gallo Força (Grupo Gallo, El Carpio, España)	59.91%	2,995.27
Water (40% ice)	Aigua Font Vella (Aguas Danone–Font Vella, Barcelona)	32.28%	1,614.00
Pressed yeast	Levadura fresca Levital (Levital S.A., Valladolid, Spain)	1.76%	88.00
Sugar	Sucrebó blanc (José Llopart S.A., Sant Boi de Llobregat, Spain)	2.35%	117.55
Salt	Sal marina fina Bonpreu (Bon Preu SAU, Les Masies de Voltregà, Spain)	0.94%	47.00
Sunflower oil	Oli de gira-sol Bonpreu (Bon Preu SAU)	0.88%	44.00
Vinegar	Vinagre de vi blanc Bonpreu (Bon Preu SAU)	0.35%	17.55
Sodium propionate	(Quimidroga S.A., Barcelona)	0.27%	13.55
Sorbic acid	MIRCAP® SB 85-H (VEDEQSA-Lamirsa Group, Terrassa, Spain)	0.09%	4.45
Bread improver	Gamma Panoleci (Royal Zeelandia Group, Zierikzee, Netherlands)	0.71%	35.55
Monocalcium phosphate	C8017 (Sigma-Aldrich, Co., St. Louis, MO, United States)	0.18%	9.00
<i>B. coagulans</i> Unique IS-2	(AB-Biotics S.A.)	0.29%	14.27
Total		100.00%	5,000.19

The bread production process was carried out following a standardized protocol comprising five sequential stages: mixing, resting, fermentation, baking, and cooling. Initially, 5 Kg of dough was prepared using a two-speed spiral mixer. All dry and liquid ingredients were added together into the spiral mixer IM12D (Mecnosud Srl; (Flumeri, Italia), including the lyophilized *B. coagulans* Unique IS-2 spores, pre-blended with sunflower oil to facilitate the incorporation (inoculum 8.46 log CFU/g). The ingredients were mixed for 1 minute at low speed followed by 10 minutes at high speed to ensure proper gluten development. After mixing, the dough was divided, pre-shaped into round units of 550 g, and allowed to rest for 10 minutes at room temperature to relax the gluten and ease further handling. The dough was then placed into molds and allowed to ferment in a climatic chamber KBF 720 (BINDER GmbH, Tuttlingen, Germany) set at 45°C and 85% relative humidity for 70 minutes, allowing for optimal yeast activity and volume expansion. Subsequently, the fermented dough was baked in a convection oven fan-assisted S200 (Sveba Dahlen AB; Fristad, Sweden) at 210 °C for 22 minutes to develop the final structure and crust, as well as to ensure microbiological safety. Finally, the baked loaves were cooled at room temperature for 120 minutes to stabilize their internal structure and prevent moisture accumulation before being packaged in sterile plastic bags and stored at 25 ± 5 °C.

3.2.8 Cereal Bar

Cereal bars were manufactured in the UAB laboratory using the ingredients and formulation listed in Table 14.

Table 14. Cereal bar ingredient list and quantitative formulation.

Ingredient	Control		Probiotic	
	%	g	%	g
Buckwheat flakes	27.80%	69.50	27.64%	69.10
Fructose	21.80%	54.50	21.80%	54.50
Dried cranberry	15.00%	37.50	15.00%	37.50
Puffed millet	3.00%	7.50	3.00%	7.50
Puffed amaranth	3.00%	7.50	3.00%	7.50
Puffed quinoa	3.00%	7.50	3.00%	7.50
Sunflower seeds	6.50%	16.25	6.50%	16.25
Pumpkin seeds	6.50%	16.25	6.50%	16.25
Carob syrup	6.00%	15.00	6.00%	15.00
Inulin	5.00%	12.50	5.00%	12.50
Glycerol	1.00%	2.50	1.00%	2.50
Coconut oil	1.00%	2.50	1.00%	2.50
Ascorbic acid	0.40%	1.00	0.40%	1.00
<i>B. coagulans</i> Unique IS-2	—	—	0.16%	0.40
Total	100.00%	250.00	100.00%	250.00

Ingredient	Commercial product
Buckwheat fflakes	Copos de trigo sarraceno sin gluten NaturGreen (Laboratorios Almond, S.L., Librilla, Spain)
Fructose	Fructosa El Granero Integral (Biogran, S.L.)
Dried cranberry	Arándano Rojo Entero Deshidratado INTRACMA (Bibe Diet, S.L., Viladecans, Spain)
Puffed millet	Mijo hinchado El Granero Integral (Biogran, S.L., Alcobendas, Spain)
Puffed amaranth	Amaranto hinchado ecológico Eco-Salim (Grupo Salim, Masquefa, Spain)
Puffed quinoa	Quinoa hinchada Bio Naturitas Essentials (Naturitas, Barcelona)
Sunflower seeds	Semilla de girasol tostadas Bio Naturitas Essentials (Naturitas)
Pumpkin seeds	Pipas de calabaza peladas Bio Naturitas Essentials (Naturitas)
Carob syrup	Sirope de algarroba Bio Sol Natural (Sol Natural, Vilassar de Mar, Spain)
Inulin	Inulina de ágave Bio Naturitas Essentials (Naturitas)
Glycerol	(Barcelonesa, Barcelona)
Coconut oil	Coco Bio Naturitas Essentials (Naturitas)
Ascorbic acid	(Barcelonesa, Barcelona)
<i>B. coagulans</i> Unique IS-2	(AB-Biotics S.A.)

The probiotic cereal bars were prepared following a standardized thermal and mechanical process optimized to ensure both product consistency and probiotic viability. Initially, the cranberries were weighed and reduced to half their original size to ensure an even distribution and optimal texture. Separately, melted coconut oil, glycerol, and *B. coagulans* Unique IS-2 spores were weighed, mixed thoroughly to form a uniform distribution, and stored until further use (inoculum: 8.20 log CFU/g). Fructose and carob syrup were also weighed and combined in preparation for thermal processing. The remaining dry ingredients were weighed, mixed thoroughly to ensure homogeneity, and stored separately.

The fructose and carob syrup mixture were heated under constant stirring until it reached 110 °C, approaching the boiling point, in order to achieve the necessary viscosity for binding the dry components. Prior to combining with the hot syrup, the probiotic-lipid mixture was blended with the dry ingredients to enable uniform distribution of the spores. Immediately after the syrup reached the target temperature, it was rapidly incorporated into the dry-probiotic mixture and mixed thoroughly to form a cohesive mass. The hot mixture was quickly spread into rectangular molds and pressed firmly to ensure compaction and uniform shape. The molded bars were then placed in a freezer at -20 °C for 15 minutes to allow structural stabilization. Once cooled, the bars were vacuum-packed in individual plastic pouches and stored at 25 ± 5 °C.

3.2.9 Butter Cookies

Butter cookies were manufactured in the UAB pilot plant using the ingredients and formulation listed in Table 15.

Table 15. Butter cookies ingredient list and quantitative formulation.

Ingredient	Control		Test 1	
	%	g	%	g
White flour (Type 00)	46.06%	251.00	46.00%	251.00
Butter	22.02%	120.00	22.00%	120.00
Sugar	18.35%	100.00	18.33%	100.00
Egg	11.93%	65.00	11.91%	65.00
Leavener	1.28%	7.00	1.28%	7.00
Salt	0.37%	2.00	0.37%	2.00
<i>B. coagulans</i> Unique IS-2	-	-	0.11%	0.62
Total	100.00%	545.00	100.00%	545.62

Ingredient	Test 2		Test 3	
	%	g	%	g
White flour (Type 00)	45.97%	251.00	45.91%	251.00
Butter	21.98%	120.00	21.95%	120.00
Sugar	18.31%	100.00	18.29%	100.00
Egg	11.90%	65.00	11.89%	65.00
Leavener	1.28%	7.00	1.28%	7.00
Salt	0.37%	2.00	0.37%	2.00
<i>B. coagulans</i> Unique IS-2	0.18%	1.00	0.31%	1.71
Total	100.00%	546.00	100.00%	546.71

Ingredient	Commercial product
White flour (Type 00)	Harina de trigo Hacendado (Mercadona S.A.)
Butter	Mantequilla Hacendado (Mercadona S.A.)
Sugar	Azúcar blanco Hacendado (Mercadona S.A.)
Egg	Huevos frescos Hacendado (Mercadona S.A.)
Leavener (Baking powder)	Levadura química Hacendado (Mercadona S.A.)
Salt	Sal marina Hacendado (Mercadona S.A.)
<i>B. coagulans</i> Unique IS-2	(AB-Biotics S.A.)

The cookie production process was carried out following a standardized protocol comprising four sequential stages: mixing, shaping, baking, and cooling. Initially, melted butter and egg were creamed until a homogeneous mixture was obtained. The lyophilized *B. coagulans* Unique IS-2 spores were then incorporated by premixing it with the liquid ingredients to

ensure its even distribution (inocula: 8.06 CFU/g in Test 1, 8.26 CFU/g in Test 2, 8.50 CFU/g in Test 3). Subsequently, all dry ingredients were gradually added under continuous blending to form a uniform dough. Upon reaching the desired consistency, the dough was portioned and shaped into individual cookies. These were then baked in a convection oven fan-assisted S200 (Sveba Dahlen AB) at 175°C for 13 minutes. Finally, to stabilize their internal structure and prevent moisture accumulation, the baked cookies were cooled at room temperature for 60 minutes prior to storage in airtight plastic containers at 25 ± 5 °C.

3.2.10 Cream Cheese

A prototype probiotic cream cheese was supplied by an industrial collaborator for research purposes. Although individual ingredient concentrations were not disclosed, the full ingredient list is provided: quark cheese, parmesan cheese, olive oil, white pepper, potassium sorbate, fibre, protein, xanthan gum, trisodium citrate, probiotic blend I3.1.

The manufacturing of the probiotic-fortified cream cheese was carried out under controlled conditions to ensure product quality and maintain probiotic viability. All ingredients, except the probiotic, were accurately weighed and homogenized in a stainless-steel mixing vessel equipped with a system allowing both heating and refrigeration. The mixture was then subjected to thermal treatment for pasteurization and texture development. Upon reaching 80 °C, it was rapidly cooled to 40 °C using the integrated cooling system. At this point, the lyophilized probiotic blend I3.1, pre-mixed with olive oil to facilitate incorporation, was added through an aseptic port to minimize contamination risk (inoculum 7.95 log CFU/g). Gentle yet thorough mixing was performed to achieve uniform distribution and the target probiotic concentration. Finally, the fortified cream cheese was portioned into individual plastic containers and hermetically sealed with aluminium foil lids prior to storage at 4 °C.

3.2.11 Ice Cream

Four probiotic ice cream prototypes (Table 16) were supplied by an industrial collaborator for research purposes. Although individual ingredient concentrations were not disclosed, the full ingredient list is provided below.

1. Chocolate ice cream

Ingredients: Whole fresh milk, water, inverted sugar, whey proteins, soluble corn fibre, cocoa powder, Belgian chocolate chips 4% (cocoa mass, sugar, cocoa butter, anhydrous butter [milk], emulsifier [soy lecithin (E 322)], natural vanilla flavour), sugar, 70% chocolate coating (cocoa mass, sugar, defatted cocoa powder, cocoa butter, emulsifier [E-322], and flavour), stabilizers (alginates, carrageenans, locust bean gum, guar gum, mono- and diglycerides of fatty acids), salt, probiotic blend I3.1

2. Vanilla ice cream

Ingredients: Whole fresh milk, water, inverted sugar, whey proteins, soluble corn fibre, caramel (glucose and fructose syrup, water, sugar, skimmed condensed milk, caramel powder [whey, butter, maltodextrin, sugar, skimmed milk, natural flavour], thickeners [E-440, E-415], salt, preservative [E-202], flavours, acidulant [E-334], coloring [E-160c]), sugar, 82% butter, skimmed milk powder, Madagascar Bourbon vanilla paste (Bourbon vanilla 1.65%, coloring [beta-carotene], thickener [modified starch], preservatives [potassium sorbate and sodium benzoate]), stabilizers (alginates, carrageenans, locust bean gum, guar gum, mono- and diglycerides of fatty acids), probiotic blend I3.1.

3. Yogurt (with red berry coulis) ice cream

Ingredients: Water, whole milk, natural yogurt 10% (whole milk, skimmed milk powder, and lactic cultures), inverted sugar syrup, milk proteins, forest fruit-flavoured syrup (glucose and fructose syrup, inverted sugar syrup, sugar, flavours, acidulant: citric acid, and beetroot powder [beet juice and maltodextrin]), fermented milk (fermented skimmed milk powder, whey, acidity regulator: calcium phosphate), sugar, acidulant: citric acid and flavour), soluble corn fibre, sugar, butter, dextrose, milk powder, stabilizer (sodium alginate, carrageenans, locust bean gum, guar gum, mono- and diglycerides of fatty acids), probiotic blend I3.1.

4. Mango ice cream

Ingredients: Water, mango pulp, whey proteins, soluble malt fibre, dextrose, inverted sugar, sugar, stabilizers (alginates, carrageenans, locust bean gum, guar gum, mono- and diglycerides of fatty acids), probiotic blend I3.1.

Table 16. Ice cream prototypes nutritional information.

	Chocolate	Vanilla	Yogurt	Mango
	Nutritional value per 100 g			
Energy	153 kcal / 640 kJ	147 kcal / 618 kJ	152 kcal / 642 kJ	105 kcal / 446 kJ
Fats	4.3 g	3.5 g	3.2 g	0.2 g
<i>Saturated Fats</i>	2.8 g	2.6 g	2.3 g	0.2 g
Carbohydrates	17.6 g	18.7 g	20 g	18.2 g
<i>Sugars</i>	15.2 g	16.2 g	17 g	16.4 g
Protein	8.0 g	8.1 g	8.4 g	5.8 g
Fibre	5.8 g	3.9 g	4.2 g	3.6 g
Salt	0.16 g	0.14 g	0.10 g	0.04 g

The production of the functional probiotic ice cream involved a controlled, multi-stage process. Initially, all liquid and dry ingredients were blended to form a homogeneous mix. This mix then underwent homogenization, followed immediately by pasteurization to ensure microbial safety and optimal texture. The mix was then rapidly cooled to 40°C, and the probiotic blend I3.1, pre-mixed with sterilized water to facilitate incorporation, was aseptically introduced into the ice cream base and thoroughly mixed to ensure uniform distribution (inoculum: 7.51 log CFU/g in Test 1 and 7.38 log CFU/g in Test 2). Following probiotic incorporation, the mix was further cooled to 4-5 °C and underwent an ageing period. Subsequently, the aged mix was frozen in an ice cream freezer, incorporating air to achieve the desired overrun. Finally, the soft ice cream was accurately portioned into paper boxes hermetically sealed with a plastic foil and stored at $-18 \pm 2^{\circ}\text{C}$.

3.2.12 Chocolate Bars, Bites and Chunks

Prototype probiotic chocolate products (Table 17) were supplied by two industrial collaborators for research purposes.

For the evaluation of probiotics in chocolate, three tests were conducted, each using different probiotic strains and distinct chocolate formulations.

Test 1. Chocolate bars: Milk chocolate bars enriched with probiotic blend I3.1 (MC1 + I3.1).

Test 2. Chocolate bites: Milk chocolate bites enriched with probiotic blend I3.1 (MC2 + I3.1) or with probiotic blend AB-KOLICARE (MC2 + AB-KOLICARE), and dark chocolate enriched with probiotic blend I3.1 (DC2 + I3.1) or with probiotic blend AB-KOLICARE (DC2 + AB-KOLICARE).

Test 3. Chocolate chunks (74% cocoa): Cocoa dark chocolate chunks enriched with probiotic blend I3.1 (DC3 + I3.1).

The formulations of Test 1 and Test 2 were not disclosed while, for Test 3, although individual ingredient concentrations were not disclosed, the full ingredient list is provided: cocoa, sugar, cocoa butter, milk protein (8%), emulsifier (sunflower lecithin), vanilla extract, probiotic lactic ferments: *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, *L. plantarum* CECT 7485.

Table 17. Chocolate chunks prototype (Test 3) nutritional information.

	Nutritional value per 100 g
Energy	531 kcal / 2222 kJ
Fats	39 g
<i>Saturated Fats</i>	24 g
Carbohydrates	24 g
<i>Sugars</i>	18 g
Dietary fibre	12 g
Proteins	16 g
Salt	0.06 g

Following refining and conching, the chocolate mass was maintained at its melting temperature (dark: 45–50 °C; milk: 40–45 °C) to ensure complete dissolution of pre-existing cocoa butter crystals. Tempering was then performed to promote the formation of stable crystals, following standard temperature curves: dark chocolate melt at 45–50 °C, cool to 27–28 °C, and reheat to 31–32 °C; milk chocolate melt at 40–45 °C, cool to 26–27 °C, and reheat to 29–30 °C. At this stage, a lyophilized probiotic blend (I3.1 or AB-KOLICARE), pre-mixed in tempered cocoa butter at approximately 35 °C, was incorporated through an aseptic addition port and mixed under gentle agitation at ≤ 32 °C (dark) or ≤ 30 °C (milk) to ensure uniform distribution.

Inocula:

- Test 1: I3.1 at 8.38 log CFU/g
- Test 2: I3.1 at 9.00 and 9.48 log CFU/g; AB-KOLICARE at 9.30 and 9.78 log CFU/g
- Test 3: I3.1 at 8.76 log CFU/g.

The tempered chocolate was then portioned into molds, vibrated to eliminate entrapped air, passed through a controlled cooling tunnel to facilitate proper setting, and subsequently subjected to static crystallization at 18–20 °C prior to finishing and packaging. The finished products were stored at 25 ± 5 °C.

3.2.13 Popping Candy

A prototype probiotic popping candy in sticks was supplied by an industrial collaborator for research purposes (Table 18 and 19).

Table 18. Popping candy ingredient list and quantitative formulation.

Ingredient	Formula %	g (stick)
Popping candy granules	50.50 %	1.01 g
Pineapple juice powder	22.00 %	0.44 g
Fructose	10.00 %	0.20 g
Inulin	10.00 %	0.20 g
Vitamin C	2.50 %	0.05 g
Flavour	1.00 %	0.02 g
Silicon dioxide	1.00 %	0.02 g
<i>Bacillus coagulans</i> Unique IS-2	3.00 %	0.06 g
Total	100 %	2.00 g

Table 19. Popping candy prototype nutritional information.

Nutritional value per 100 g	
Energy	351.3 kcal / 1470 kJ
Fats	0 g
<i>Saturated Fats</i>	0 g
Carbohydrates	92.2 g
<i>Sugars</i>	76.6 g
Proteins	0.1 g
Dietary fibre	9 g
Sodium	4 mg

The production of the functional probiotic popping candy involved blending ready-made popping candy granules with lyophilized *B. coagulans* Unique IS-2 spores (inoculum: 9.48 log CFU/) and the other dry ingredients in a suitable mixing apparatus. The mixing and subsequent packaging were conducted within a strictly controlled temperature and humidity (< 25 °C, < 40%) environment to preserve both the probiotic viability and the integrity of the popping effect. Finally, the blend was filled into individual multilayer flexible laminate stick packs and stored at 25 ± 5 °C.

3.2.14 Gummies

A prototype probiotic gummy was supplied by an industrial collaborator for research purposes. Although individual ingredient concentrations were not disclosed, the full ingredient list is provided: maltitol syrup, erythritol, sorbitol, pectin, citric acid, sodium citrate, natural flavouring (strawberry flavouring), glazing agent (carnauba wax and vegetable oil), black carrot juice concentrate, and the *B. coagulans* Unique IS-2 spores.

The production process of the probiotic gummies began with the preparation of a syrup base by mixing maltitol syrup, erythritol, and water, while pectin was separately dissolved in water through melting and stirring to create a pectin solution. These mixtures were then combined and underwent blending, cooking, and concentration to form a uniform syrup and gelling solution. At this stage, the probiotic spores of *B. coagulans* Unique IS-2 (inocula: 8.82 log CFU/g and 9.12 log CFU/g), were added along with acid, flavour, colour, and additional water. The resulting mixture was blended, deaerated under vacuum to remove entrapped air, filtered, and poured into molds. After drying and demolding, the gummies were coated to enhance stability and appearance, then packaged and stored at 25 ± 5 °C.

3.2.15 Isotonic Drinks

Four prototypes of bottled isotonic drink (without probiotic) were supplied by an industrial collaborator for research purposes. Their qualitative composition is shown in Table 20.

Table 20. Isotonic drinks ingredient list and product features.

	Ingredients	Characteristics
FORMULATION 1 (F1)	Water, Sodium lactate, Natural Blueberry flavour, Potassium chloride, Malic acid, Natural Flavour, Molasses (*), Magnesium chloride, Calcium chloride, Steviol glycosides, Salt, FD&C Red No. 40, FD&C Blue No. 1 *Adds a negligible amount of sugar	NO Sugar - Natural Flavour, pH 4.0
FORMULATION 2 (F2)	Water, Artificial blueberry flavour, Malic acid, Potassium chloride, Sodium chloride, Natural flavours, Magnesium chloride, Calcium chloride, Sucralose (7.8 mg/100 mL), Acesulfame K (3.9 mg/100 mL), FD&C Red No. 40 dye, FD&C Blue No. 1 dye	NO Sugar - Artificial Flavour, pH 2.8
FORMULATION 3 (F3)	Water, Dextrose monohydrate, Sodium lactate, Natural blueberry flavour, Potassium chloride, Malic acid, Molasses, Magnesium chloride, Calcium chloride, Steviol glycosides, Salt, FD&C Red No. 40, FD&C Blue No. 1	Sugar - Natural Flavour, pH 4.0
FORMULATION 4 (F4)	Water, Dextrose monohydrate, Sodium lactate, Artificial blueberry flavour, Potassium chloride, Magnesium chloride hexahydrate, Calcium chloride dihydrate, Sodium chloride, FD&C Red No. 40 dye, FD&C Blue No. 1 dye.	Sugar - Artificial Flavour, pH 4.0

To assess the performance of the probiotic strain *B. coagulans* Unique IS-2 in isotonic drink formulations, three experimental tests were conducted.

Test 1: Four experimental formulations were prepared by directly incorporating the appropriate dose of lyophilized *B. coagulans* Unique IS-2 spores into the four versions of preformulated isotonic beverages (inoculum: 6.78 log CFU/ml). All formulations shared the same base but differed in specific features, including the presence or absence of sugar, the use of natural versus artificial flavours, and, in one case, pH.

Prior to addition, the probiotic inoculum was dissolved in Milli-Q water to ensure homogeneous distribution. The probiotic-enriched products were then stored at 30 °C ± 1 °C in the absence of light.

Test 2: The same four formulations used in Test 1 were replicated; however, the storage conditions were modified to 25 °C ± 5 °C under light exposure and transparent packaging. This allowed for the assessment of probiotic viability and product integrity under more variable ambient conditions.

Test 3: Formulation 3 (F3) was selected for further investigations. In this test, a high-temperature short-time (HTST) pasteurization process was applied using Tetra Therm® Aseptic Pilot (Tetra Pak, Pully, Switzerland). The isotonic beverage was first supplemented with lyophilized *B. coagulans* Unique IS-2 spores, previously dissolved in Milli-Q water to ensure homogeneous distribution, followed by thermal treatment at 90 °C for 20 seconds to ensure microbial safety. After pasteurization, the product was aseptically filled into sterile containers and stored at 25 °C ± 5 °C under dark conditions.

3.2.16 Reconstitutable Isotonic Drink Powder

A prototype isotonic drink in powder format (without probiotic) was supplied by an industrial collaborator for research purposes (Table 21). Although individual ingredient concentrations were not disclosed, the full ingredient list is provided: dextrose, sodium citrate, citric acid, potassium chloride, natural flavour, natural strawberry and kiwi flavour, calcium citrate, dipotassium phosphate, silicon dioxide, magnesium citrate, malic acid, steviol glycosides, sodium chloride, FD&C ed No. 40.

Table 21. Reconstitutable isotonic drink powder prototype nutritional information.

	Nutritional value per 100 g
Energy	267 kcal / 184.10 kJ
Fats	0 g
<i>Saturated Fats</i>	0 g
Sodium	2200 mg
Total Carbohydrate	67 g
<i>Total Sugars</i>	67 g
Protein	0 g
Calcium	52 mg
Potassium	376 mg
Magnesium	25.2 mg
Chloride	345 mg

Two different probiotic reconstitutable isotonic drink powders were prepared using the same base formulation. One was enriched with the probiotic blend AB-DIGEST, and the other with *B. coagulans* Unique IS-2 spores.

The required dosage of lyophilized probiotic strains was directly incorporated into the bulk base, thoroughly mixed, and then packed in aluminium foil sticks (inocula: 9.48 log CFU/g for AB-DIGEST and 8.60 log CFU/g for *B. coagulans* Unique IS-2).

This process yielded two distinct versions of reconstitutable isotonic drinks, each supplemented with a specific probiotic strain, and stored under two different conditions:

- Zone IVa (hot/humid): 30 °C $\pm$ 2 °C / 65 % $\pm$ 5 % RH
- Accelerated conditions: 40 °C $\pm$ 2 °C / 75 % $\pm$ 5 % RH

3.2.17 Fermented Milk

For the evaluation of probiotics in fermented milk, two prototype products, one drinkable and one spoonable, were supplied by an industrial collaborator for research purposes and used in Test 1 and 2.

Due to confidentiality constraints, the list of ingredients used in the formulation cannot be disclosed in this thesis.

A third trial (Test 3) was performed, and different probiotic versions of set fermented milk were manufactured in the UAB laboratory using the commercial milk (Table 22).

Ingredients: whole milk, starter culture, probiotic

Table 22. Nutritional information of whole UHT milk used in Test 3 Llet sencera Bonpreu UHT (Bonpreu).

Nutritional value er 100 ml	
Energy	63 kcal / 262 kJ
Fats	3.6 g
<i>Saturated Fats</i>	2.5 g
Carbohydrates	4.6 g
<i>Sugars</i>	4.6 g
Proteins	3.0 g
Salt	0.13 g
Calcium	110 mg

TEST 1: Drinkable yogurt was produced through controlled fermentation of pasteurized milk using the starter culture Y239A (Sacco System), composed of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, in co-fermentation with the probiotic blend I3.1. The process began with milk pasteurization. After cooling, the starter culture and probiotic were added, each having been separately premixed with sterile water to ensure uniform distribution. Fermentation was then carried out in bulk tanks until the target pH was achieved. Subsequently, the product was cooled and mixed with additional ingredients such as sweeteners and functional additives. Finally, it was mechanically stirred to obtain a

smooth, low-viscosity texture characteristic of drinkable yogurt, before packaging under strict hygienic conditions, and stored at 4 °C.

TEST 2: Spoonable yogurt was produced through controlled fermentation of pasteurized milk using the starter culture Y239A (Sacco System), in co-fermentation with the probiotic blend I3.1. The process began with milk pasteurization. After cooling, the starter culture and probiotic were added, each having been separately premixed with sterile water to ensure uniform distribution. Fermentation was then carried out in bulk tanks until the target pH was achieved. The coagulated mass was then gently stirred to achieve a creamy, spoonable texture without excessive whey separation and additional ingredients such as sweeteners and functional additives were incorporated before packaging under strict hygienic conditions, and stored at 4 °C.

TEST 3: Whole milk was inoculated with the yogurt starter culture Lyofast SY42 (Sacco System) at a dosage of 0.01 UC/L, in accordance with the manufacturer's instructions. The milk was subsequently supplemented with either a single probiotic strain: *Lactobacillus paracasei* AB0918, *Lactobacillus plantarum* CECT 7481, *L. plantarum* CECT 7485, or *Lactobacillus brevis* CECT 7480; or with a multi-strain probiotic formulation (PROBIOMIND), composed of a 1:1:1 ratio of *L. plantarum* CECT 7481, *L. plantarum* CECT 7485, and *L. brevis* CECT 7480. Prior to inoculation, the starter culture and probiotic preparations were separately pre-mixed with Milli-Q water to ensure homogeneous dispersion within the milk matrix.

The inocula were then incorporated into the pre-heated milk at 43 °C. A control batch fermented exclusively with the starter culture (Lyofast SY42) was included for comparative purposes. All probiotic treatments were standardized to an initial inoculation level of 10⁷ CFU/mL to ensure adequate viability. The lyophilized probiotic and starter cultures were rehydrated in water prior to inoculation to ensure uniform distribution throughout the milk. Fermentation was conducted at 43 °C until the target pH of 4.6 was achieved, after which samples were immediately cooled and stored at 4 °C.

3.2.18 Alcohol-free Beer

A prototype probiotic alcohol-free beer was supplied by an industrial collaborator for research purposes (Table 23). Although individual ingredient concentrations were not

disclosed, the full ingredient list is provided: water, barley malt, hops, yeast, *B. coagulans* Unique IS-2 spores.

Table 23. Alcohol-free beer prototype nutritional information.

Nutritional value per 100 ml	
Energy	4.3 kcal / 18 kJ
Fats	0.0 g
<i>Saturated Fats</i>	0.0 g
Carbohydrates	1.0 g
<i>Sugars</i>	<0.05 g
Proteins	0.07 g
Salt	< 0.007 g

The manufacturing process began with standard brewing operations, including mashing, lautering, boiling, and wort clarification, followed by fermentation under controlled conditions. Alcohol removal was achieved through vacuum evaporation to obtain an alcohol-free product while preserving sensory attributes. The beer was then subjected to pasteurization to ensure microbiological stability. After thermal treatment, the product was cooled to the designated filling temperature under hygienic conditions. At this stage, carbonation was adjusted by injecting food-grade carbon dioxide (CO₂) to restore the desired level of dissolved gas, ensuring proper mouthfeel and sensory quality. Immediately after carbonation, the lyophilized *B. coagulans* Unique IS-2 spores, previously mixed with sterile water, were aseptically incorporated through a dedicated addition port to maintain viability and prevent contamination (inocula: 7.36 log CFU/g and 7.66 CFU/g). Gentle mixing ensured uniform distribution of the probiotic cultures without compromising their integrity. The fortified beer was subsequently dosed into sealed cans and stored at 25 ± 5 °C.

3.3 ASSESSMENT OF PRODUCT STABILITY

In order to determine the impact of the addition of probiotics and their metabolism on the studied matrices, product stability and quality were investigated by microbiological, physicochemical and sensorial analysis.

For each specific matrix, the experimental design was adjusted to answer specific questions, whether dictated by commercial or scientific needs.

3.3.1 Microbiological Analyses

3.3.1.1 Probiotic Viable Cell Count

Probiotic viable cell count (Colony Forming Units; CFUs) was determined by the standard plate count method in triplicate. Serial decimal dilutions of probiotic products were prepared using buffered peptone water (CM1049, Oxoid Ltd., Hampshire, England) or, in the case of Bifidobacteria enumeration, buffered peptone water supplemented with 0,1% L-Cysteine (168149, Sigma-Aldrich).

For lactic acid bacteria (LAB) enumeration, 1 mL of the appropriate dilutions were inoculated onto de Man, Rogosa, and Sharpe agar (MRS) (CM0361B, Oxoid Ltd.) by pour plate technique and incubated anaerobically, using Anaerogen™ 2.5 L bags (Thermo Fisher Scientific Inc., Waltham, MA, USA) and anaerobiosis hermetic jars, at 37 °C for 48 h. In the case of Bifidobacteria, 1 mL of the appropriate dilutions were inoculated onto MRS agar plates supplemented with 0,1% L-Cysteine and incubated anaerobically at 37 °C for 48 h.

For *B. coagulans* spores enumeration, before the plating, the serial dilutions were subjected to a 30-minute heat shock at 75 °C, to activate dormant spores (Jafari et al., 2016), and then, 1 mL of the appropriate dilutions were inoculated onto Glucose Yeast extract BC agar (GYEA BC) (M2102, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated aerobically at 37 °C for 48 h.

In products containing both a starter culture and an added probiotic strain, the enumeration of probiotic viable cells was determined by differential counting. Specifically, the colony-forming units (CFU) attributed to the probiotic were calculated as the difference between the total CFU count (MRS plates) obtained from samples containing both the probiotic and the starter culture, and the CFU count obtained from the corresponding control samples containing only the starter culture. This approach assumes that the starter culture contributes consistently to the total microbial population under the same formulation and incubation conditions, and that no significant interaction affects its growth in the presence of the probiotic. Therefore, subtracting the starter control CFU from the combined culture CFU allows for an estimation of the viable probiotic population within the product.

3.3.1.2 Microbiological Quality Assessment

To discard external contamination, each product was analysed at the beginning and at the end of the storage period to determine the presence of total coliforms and specifically *E. coli*, mesophilic aerobic bacteria, aerobic spore forming bacteria, psychrotrophic bacteria, yeasts

and moulds, utilizing the media and conditions specified in the Table 24. For all media, 0,5 mL of sample in the case of liquids, or 0,5 mL of the first dilution in the case of solids, were inoculated in the surface of the prepared plates. Detection limit was 2 CFU/mL for liquid samples and 20 CFU/g for solid samples. For liquid products subjected to HTST processing, microbiological enumerations were performed both before and after the heat treatment to assess the effectiveness of the thermal step in reducing or eliminating background microbial contamination.

Table 24. Media and grow condition for microbial contamination count.

Microorganism	Media	Grow Conditions
Coliforms and <i>E. coli</i>	Chromogenic Coliform Agar (CCA) (CM1205, Oxoid Ltd.)	Aerobic incubation 24 h at 37 °C
Aerobic Mesophilic Bacteria	Plate Count Agar (PCA) (CM0325, Oxoid Ltd.)	Aerobic incubation 72 h 30 °C
Aerobic spore forming bacteria	Plate Count Agar (PCA) (CM0325, Oxoid Ltd.)	Aerobic incubation 48 h at 37 °C (after heat treatment at 80°C/10 min)
Psychrotrophic Bacteria	Plate Count Agar (PCA) (CM0325, Oxoid Ltd.)	Aerobic incubation 7 days at 7°C
Yeasts and moulds	Rose-Bengal Chloramphenicol Agar (DMT101, Laboratorios MICROKIT, Madrid, Spain)	Aerobic incubation 7 days at 30 °C

3.3.2 Physicochemical Analyses

3.3.2.1 Basic Physicochemical Parameters

3.3.2.1.1 pH

The pH of the samples was measured using a pH-Meter (pH-Meter BASIC 20, Crison Instruments, S.A., Barcelona) at a temperature of 25°C. Liquid samples were analysed directly after tempering to the measurement temperature. For solid matrices, the samples were first minced to achieve homogeneity, then mixed with Milli-Q water to achieve a 50% (w/v) suspension. The resulting mixture was stirred thoroughly to ensure uniform distribution of the sample in the liquid phase before pH measurement. The pH meter was calibrated prior to each measurement session using standard buffer solutions (pH 4.0, pH 7.0 and pH 9.21) to ensure accuracy and reproducibility.

3.3.2.1.2 Titratable Acidity

Titrateable acidity (TA) was determined in homogenized samples following a standardized titration method. Ten grams of each homogenized sample were weighed and mixed with 10 g of Milli-Q water in a clean beaker. The mixture was tempered to 25 °C, ensuring consistent temperature conditions for all measurements. The pH electrode of a calibrated pH meter was immersed in the suspension, ensuring full contact with the sample for accurate readings. While stirring the mixture gently to maintain homogeneity, titration was carried out using a 0.1 N sodium hydroxide (NaOH) solution ± 0.0002 N and free of carbonate. The NaOH solution was added dropwise, and the pH of the mixture was continuously monitored. The endpoint of the titration was set at a pH of 8.30 ± 0.01 , which corresponds to the neutralization of the acidic components in the sample. The volume of NaOH required to reach the endpoint was recorded to the nearest 0.05 mL and used to calculate the TA, expressed as a percentage of lactic acid equivalents per 100 g of product. Lactic acid was chosen as the standard for calculation due to its relevance in food and beverage acidity profiling. All measurements were performed in triplicate to ensure reproducibility.

3.3.2.1.3 Water Activity

Water activity (aw) was determined using a dew point water activity meter (AQUALAB Pre, Addium Inc., Pullman, WA, USA) to evaluate the potential of food matrices for microbial growth. Samples were finely minced to ensure homogeneity and tempered to 25 °C, as aw measurements are sensitive to temperature fluctuations. Each sample was placed in the instrument chamber, where equilibrium relative humidity (ERH) of the headspace was measured and converted to aw, reflecting the availability of free water for microbial proliferation and chemical reactions. Measurements were performed in triplicate, expressed as mean $\pm$ standard deviation.

3.3.2.1.4 Total Soluble Solids

The total soluble solids (TSS) content of the samples was determined using an optical refractometer (Analogue Hand-Held Refractometer PREF-B32-001, Labbox Labware S.L., Barcelona). Prior to measurement, the refractometer was calibrated using distilled water at 20 °C to ensure accuracy. Samples were tempered to 20 °C to minimize temperature-related variations, as TSS measurements are temperature-dependent and standard readings are referenced at this temperature. A small aliquot of each sample was placed on the prism surface of the refractometer, ensuring full coverage without air bubbles. The prism cover

was closed gently to distribute the liquid evenly, and the instrument scale was observed through the eyepiece to determine the TSS value, which was expressed as degrees Brix (°Brix). The °Brix scale represents the percentage of TSS in the sample, primarily sugars, but also includes other soluble components such as acids and minerals. After each measurement, the prism was thoroughly cleaned with distilled water and dried with lint-free tissue to prevent cross-contamination between samples. All readings were performed in triplicate, and the average value was reported to ensure reproducibility and accuracy.

3.3.2.1.5 Colour

Colour measurements were conducted using a portable colorimeter (CR-400, Konica Minolta Inc., Japan). This instrument operates by illuminating the sample with a light source and measuring the reflected light at specified angles. The results were recorded in the CIE 1976 (L*, a*, b*) colour space. CIE L*, a* and b* values were measured with an illuminant of D65 and a standard observer angle of 10°. L* represents the lightness, with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser. Chromatic components are represented by a* and b* axes. Positive values of a* are red and negative values are green, while positive values of b* are yellow and negative values are blue. ΔE^* (total colour difference) was calculated using the formula $\Delta E^* = \sqrt{((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)}$ (ISO/CIE 11664-4:2019). The colorimeter was calibrated according to the manufacturer's guidelines.

3.3.2.2 *Physical Stability and Structural Properties*

3.3.2.2.1 Viscosity

The apparent viscosity of the samples was assessed using a viscometer (FUNGILAB VISCO BASIC Plus L, Fungilab S.A., Barcelona). The spindle and RPM (Revolutions Per Minute) settings were selected based on the characteristics of the fluid being analysed. Specifically, spindle L1 was used at 50 RPM for oat beverages and spindle L3 was used at 1 RPM for soy beverages. The samples, stored at 4 ± 2 °C in 1L Tetra Brik® slim package, prior to measurement, were agitated five times in a 180° motion and transferred to a graduated cylinder to ensure consistent volume. After setting up and starting the measurement, the first two readings were discarded allowing the apparatus stabilization. Five subsequent measurements were recorded in centipoise (1 cP = 0.001 Pa*s) for accurate viscosity determination.

3.3.2.2.2 Texture

The texture of various matrices was analysed using a texture analyser device (Texture Analyzer Plus-Upgrade, Stable Micro Systems Ltd., Godalming, United Kingdom) using specific probes and test conditions for each matrix (Table 25). Prior to testing, the equipment was calibrated for weight and height. Measurements were performed with the matrix maintained at 4 ± 2 °C for yogurt and at 25 ± 5 °C for the cereal bar and bread. The parameters analysed were deformability (N/mm), hardness (N), and work (mJ) that were processed using the software “Exponent” accompanying the instrument.

Table 25. Test parameters for texture analysis in different food matrices.

Matrix	Test Type	Test Speed (mm/sec)	Target Mode	Trigger Force (N)	Selected Probe
Fermented Milk	Compression	2.00	Strain 25%	0.049	PIR - 1” DIA CYLINDER DELRIN RADIUSED
Cereal Bar	Compression	1.00	Distance 5,000 mm	0.049	P/20 - 20mm DIA CYLINDER ALUMINIUM
Bread	Compression	2.00	Strain 40%	0.049	P36R - 36mm DIA ALUMINIUM RADIUSED AACC

3.3.2.2.3 Physical Stability Assessment

The physical stability assessment was performed using a stability analyzer (Turbiscan™ LAB Stability Analyzer, Formulacion, Toulouse, France) to measure the stability of dispersions by detecting changes in particle concentration over time. Using Static Multiple Light Scattering (SMLS) technology, the Turbiscan instrument scans a sample along its height to monitor the Transmission (T) and Backscattering (BS) of light. Any sedimentation, creaming, or particle migration in the sample causes a variation in light intensity, allowing for the quantification of these destabilizing phenomena.

To provide a comprehensive assessment, the stability results were evaluated based on two key quantitative parameters to characterize the sample behaviour over time. Firstly, the BS profiles were utilized to provide a spatially resolved understanding of the destabilization phenomena. The difference in the BS signal between the initial and final scans ($\Delta BS = BS_{final} - BS_{initial}$) was calculated for each vertical segment of the sample. This analysis effectively mapped the specific zones where particle migration occurred: an increase in BS

at the top of the sample indicated creaming, while an increase at the bottom signified sedimentation.

Additionally, the overall kinetic stability of the emulsions was evaluated using the Turbiscan Stability Index (TSI), calculated according to the method described by Mengual et al. (1999), which quantifies the overall variation in light transmission and backscattering over time as a measure of sample destabilization according to the following formula:

$$TSI = \sqrt{\frac{\sum_{i=1}^n (X^i - X_{BS})^2}{n - 1}}$$

where X^i represents the backscatter mean every 1 min of measurement, X_{BS} is the mean of X^i , and n is the total number of scans.

A higher TSI value directly correlates with a greater magnitude and rate of change within the sample, thereby indicating a lower overall stability.

3.3.2.2.4 Turbidity

Turbidity of liquid samples was measured using a portable turbidity meter (Hach 2100Q, Hach Company, Loveland, CO, USA), following ISO 7027 and EPA Method 180.1 standards. The Hach 2100Q turbidity meter was calibrated before use according to the manufacturer's instructions using StablCal™ calibration standards of 20, 100, and 800 nephelometric turbidity units (NTU). Calibration was verified using a secondary turbidity standard to ensure the accuracy and reliability of measurements. Samples were gently homogenized and transferred to clean, dry turbidity vials, ensuring no bubbles or scratches on the vial surfaces. Turbidity readings were recorded in NTU.

3.3.2.3 *Aeration and Thermal Behaviour*

3.3.2.3.1 Overrun

Overrun is defined as the increase in volume of ice cream as a result of air incorporation during the churning and freezing process. For this study, overrun was calculated as the percentage increase in volume of the ice cream compared to the initial mix volume prior to churning and freezing.

The overrun was calculated using the following equation:

$$\text{Overrun (\%)} = [(W_{mix} - W_c)/W_{mix}] * 100$$

Where W_{mix} is the weight of a predefined volume of the mix before churning, and W_c is the weight of the same volume of churned ice cream.

A calibrated 100 mL laboratory measuring cylinder was tared, filled with ice cream and weighted. Care was taken to avoid excessive melting during volume measurement to ensure the accuracy of the results. A bigger sample of ice cream was set aside and let to complete melting under agitation to release the trapped air for 2 h, then the same cylinder used above was tared, filled with ice cream and weighted. The two weights were used to calculate the overrun %.

3.3.2.3.2 Melting Percentage and First Drop Fall Time

The melting properties of the ice cream were determined according to the method described by Muse & Hartel (2004) and Koxholt et al. (2001), with minor modifications.

Samples (70 g), previously stored at -18°C for 24 h (MI), were placed on a mesh with a pore density of 56 holes/cm². A beaker was positioned beneath the mesh to collect the melted portion (MD), and the time required for the first drop of melted ice cream to fall was recorded as the “first drop time”.

After recording the first drop time, the mass of the melted ice cream was collected and weighed at 5-minute intervals to monitor the melting progression over time. The melting percentage at each time point was calculated using the following equation:

$$\text{Melting Percentage (\%)} = (\text{MD} / \text{MI}) * 100$$

3.3.2.3.3 Weight Loss Analysis During Baking

To quantify moisture loss during baking, samples were weighed using an analytical balance (accuracy ± 0.001 g) immediately before and after baking. Weight loss was expressed as a percentage of the initial weight, calculated using the formula:

$$\text{Weight loss (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

3.4 FUNCTIONAL PROPERTIES

3.4.1 Fermentation and Coagulation Functionalities

3.4.1.1 Evaluation of Coagulation Parameters

The coagulation was monitored by Optigraph® System (Ysebaert, Frépillon, France), based on a near-infrared optical device. The Optigraph calculates three coagulation parameters from the coagulation curves: time for detecting the onset of coagulation (CT), which is indicated by the maximum of the first derivate curve; aggregation rate (AR), calculated from

the slope of the linear region of the curve; and gel density index (GH), calculated as the difference between D1 (the optical density, of the milk after coagulation has occurred) and D0 (initial optical density, or turbidity, of the milk before coagulation starts). Milk samples were pre-heated in a water bath until 43 °C and subsequently inoculated with 1% starter and the probiotic strain under study. The device cuvettes were filled with 10 mL of inoculated milk and monitored for 8 h at 43 ± 2 °C. Each milk sample was analysed in triplicate (3 cuvettes).

3.4.1.2 Evaluation of Fermentation Parameters

The progression of milk fermentation was characterized by continuously monitoring acidification kinetics using a calibrated pH meter (Portavo 904, Knick International, Berlin, Germany). Throughout incubation, the instrument recorded real-time pH changes within the matrix, enabling the construction of detailed acidification curves. Fermentation was allowed to proceed until the samples reached a terminal pH of 4.6, a value widely recognized as indicative of casein coagulation and the onset of gel formation in acidified dairy systems. From the resulting pH–time profiles, several kinetic descriptors were calculated to comprehensively characterize the fermentation dynamics. The coagulation time (T_e) was defined as the time required for the system to reach pH 4.6, the isoelectric point of casein (Kern et al., 2020). The maximum acidification rate (V_m) was determined from the inflection point of the acidification curve, corresponding to the steepest decline in pH and thus to the phase of most intense metabolic activity. The time to reach V_m (T_m) was derived from the temporal location of this inflection point. Additionally, the latency time (T_a) was characterized as the interval required for an initial pH decrease of 0.08 units, a sensitive indicator of the end of the lag phase and the onset of exponential acid production (Medeiros et al., 2015). All fermentations were conducted in triplicate.

3.4.2 Antioxidant and Bioactive Properties

3.4.2.1 Folin–Ciocalteu Assay

Total phenolic content was determined by using an adaptation of the Folin–Ciocalteu (F-C) assay. In brief, 1.97 mL of Milli-Q water and 175 µL of Folin-Ciocalteu's phenol reagent 1.9-2.1N (Sigma-Aldrich) were added to 100 µL of the appropriate filtered and diluted sample, standard or blank. Milli-Q water, instead of juice, was used for the blank. After 8 min of incubation at room temperature, 375 µL of 20% Na_2CO_3 solution were added, and

the mixture was incubated for 30 min in complete darkness at 40 °C. The final mixture absorbance was measured using a spectrophotometer INFINITE 200 PRO (Tecan Group Ltd., Männedorf, Switzerland) at 765 nm. Gallic acid (Sigma-Aldrich) standard calibration curve (0-500 mg/L) was used for the quantification of total phenolic compounds, and the results were expressed as gallic acid equivalent (GAE) using units of mg/L.

3.4.2.2 DPPH Assay

The antioxidant capacity was determined using an adaptation of the 2,2-difenil-1-picrilhidrazil (DPPH) radical scavenging method (Suárez-Jacobo et al., 2011; Sun et al., 2022). Fifty µL of each juice sample were filtered and diluted until obtaining absorbance values within the calibration curve. Juice samples, standards and blank were mixed with 1.95 mL of methanolic DPPH (Merck KGaA, Darmstadt, Germany) 0.1 mM solution and kept at complete darkness for one hour at room temperature. Milli-Q water instead of juice was used for the blank preparation. The final mixture absorbance was measured using a spectrophotometer INFINITE 200 PRO (Tecan Group Ltd.) at 515 nm.

A Trolox (Merck KGaA) five-point standard calibration curve (0,1-0,7 mM) was used for the quantification of antioxidant capacity, and the results were expressed as Trolox equivalent (TE) using units of µmols/L.

3.4.2.3 TBARS Determination (MDA-TBA Method)

Freshly fermented samples were subjected to accelerated oxidative conditions by incubation at 50 °C for 48 h prior to TBARS (thiobarbituric acid reactive substances) analysis, which was used as an index of lipid peroxidation.

Secondary oxidation products, particularly malondialdehyde (MDA), were quantified using a spectrophotometric TBARS assay. Briefly, samples were mixed with 0.15 M potassium chloride (KCl) in a 1:3 (v/v) ratio and centrifuged at $1,000 \times g$ for 5 minutes. An aliquot of 50 µL from the upper phase was then mixed with 50 µL of 8.1% sodium dodecyl sulfate (SDS), 375 µL of 20% acetic acid, and 375 µL of 0.7% thiobarbituric acid (TBA).

The TBA reagent was prepared by first adjusting the pH to 12 to ensure full solubilization and reactivity, followed by acidification to pH 3.0–3.5 to favour formation of the MDA–TBA chromogenic adduct.

The mixture was agitated and incubated at 95 °C for 1 hour. Absorbance was then measured at 532 nm using a spectrophotometer INFINITE 200 PRO (Tecan Group Ltd.).

The concentration of TBARS in the samples was determined by comparison with an external calibration curve generated using 1,1,3,3-tetramethoxypropane (TMP), a standard precursor of MDA. Each formulation was analysed in triplicate, and the results were expressed as MDA $\mu\text{mol/L}$.

All reagents used in this analysis were purchased from Merck KGaA.

3.4.3 Metabolomic Analyses

3.4.3.1 Computational Screening of Beta-Glucosidase Enzymes: *BglI1*, *BglI2*, *BglI3*

In silico analyses were conducted to screen AB-Biotics strain genomes for the presence of β -glucosidase genes potentially involved in aglycone generation. DNA sequences of the beta glucosidase genes *bglH1*, *bglH2* and *bglH3* (Vaccalluzzo et al., 2022) were retrieved from the NCBI with accession numbers OK127688, OK127687 and OK127686, respectively, and subjected to BLAST analysis (Altschul et al., 1990) against all available AB-Biotics genomes.

3.4.3.2 HPLC-QTOF-Based Targeted Metabolomics of Orange juice Flavonoids

Metabolomic analyses were performed by MS-Omics (Copenhagen, Denmark) as follows. Processing was performed using MassHunter software. Analyses were conducted using a high-performance liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer (HPLC-QTOF). The HPLC component was an Agilent 1200 series (Agilent Technologies Inc., Santa Clara, USA), including a quaternary pump G1311A, inline degasser G1322A, thermostated autosampler G1367B, and column oven G1316A; connected to an Agilent G6530A Accurate Mass Q-TOF mass spectrometer, featuring an electrospray ionization (ESI) source using JetStream technology. A diode-array detector (DAD, G1315B) was also integrated (Data acquisition B.05.00 and Qualitative Analysis B.07.00; Agilent Technologies Inc.). Chromatographic separations were achieved using USTA HPLC-21:ZORBAX Eclipse XDB-C18 column (150 mm $\times$ 4.6 mm, 5 μm particle size; Agilent Technologies Inc.) at a constant temperature of 40 $^{\circ}\text{C}$. The injection volume was 2 μL and the mobile phase consisted of water containing 0.1% formic acid (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B), delivered at a flow rate of 1 mL/min. Data were acquired in extended dynamic range mode (2 GHz) with a low mass cut-off set at m/z 1700.

Orange juice samples were analysed in parallel with the reference standards of hesperidin, naringin, hesperetin, and naringenin. The standards were prepared at a concentration of 100 µg/mL in appropriate solvent, 10% Dimethyl sulfoxide (DMSO) and combined in equal parts to generate a multipoint calibration standard at 25 µg/mL. Before injection, the samples were subjected only to centrifugation and filtration through 0.22 µm syringe filters (MilliporeSigma, Burlington, MA, USA), with no further treatments applied.

Quantification was based on calibration curves generated from reference standards, covering the peak area ranges observed in the samples to ensure accurate concentration estimates. Results are reported both as peak areas and corresponding concentrations (µg/mL, ppm). Since all injections were performed at the same volume and samples were analysed undiluted, no further adjustments were necessary.

3.4.3.3 Untargeted Metabolomics of Semi-Polar Metabolites in Orange, Peach, and Apple juices by LC-MS/MS

Metabolomic analyses were carried out in collaboration with MS-Omics (Copenhagen, Denmark) as follows. The analysis was performed using a UPLC system (Vanquish, Thermo Fisher Scientific Inc.) coupled high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific Inc.).

Samples were extracted as follows: 75 µL samples were mixed with 25 µL aqueous solution of zinc nitrate hexahydrate (5% w/v) and ammonium thiocyanate (20% w/v). After centrifugation at 15,000× g for 15 min at 4 °C, supernatant was filtered through a 0.22 µm filter. An electrospray ionization interface was used as ionization source. Analysis was performed in polarity switching ionization mode.

The UPLC (Ultra-performance liquid chromatography) was performed using a slightly modified version of the protocol described by Doneanu et al., (2011). Peak areas were extracted using Compound Discoverer 3.2 (Thermo Fisher Scientific Inc.).

Identification of compounds was performed at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; Level 2a: identification by retention times (compared against in-house authentic standards) and accurate mass (with an accepted deviation of 3 ppm); Level 2b: identification by accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; and Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm).

3.4.3.4 GC-MS-based Targeted Metabolomics of Short-chain Fatty Acids in Orange, Peach, and Apple juices

Metabolomic analyses were carried out in collaboration with MS-Omics as follows. Juice samples were centrifuged ($14,000 \times g$), filtered ($0.2 \mu\text{m}$), and stored at -20°C until needed. For the SCFA analyses, samples were acidified using hydrochloride acid, and deuterium labelled internal standards were added. Samples were analysed in a randomised order without extraction. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$; Phenomenex Inc., Torrance, CA, USA) installed in a GC (Agilent 7890B GC System; Agilent Technologies Inc.) coupled with a time-of-flight MS (PegasusR BT, LECO Instrumentos S.L., Madrid, Spain). The system was controlled by ChromaTOFR (LECO Instrumentos S.L). Raw data were converted to netCDF format using Chemstation (Agilent Technologies Inc.), before the data were imported and processed in Matlab R2014b (Mathworks Inc., Natick, MA, USA) using the PARADISE software version 6 described by Johnsen et al. (2017).

3.4.3.5 Targeted Metabolomics of Bioactive Compounds in Orange, Peach, and Apple juices by LC-MS/MS

Metabolomic analyses were carried out in collaboration with MS-Omics as follows. A targeted analysis following the method described previously in Section 3.4.3.3 utilizing specific standards, was conducted to verify the identity and the concentration of the following compounds: Nicotinic acid (CAS: 59-67-6), Phenyllactic acid (CAS: 828-01-3), Acetylcholine (CAS: 51-84-3) and N-Acetyl-L-glutamine (CAS: 2490-97-3).

3.5 SENSORY ANALYSIS

Sensory evaluation of various probiotic food matrices was performed by an untrained panel composed of individuals of mixed gender and age, aiming to assess general consumer acceptance as well as the possible impact of the probiotic on the organoleptic characteristics of the product. Detailed demographic data will be presented alongside the results of each test.

Different types of sensory analysis were implemented according to the specific requirements and nature of each food matrix.

3.5.1 Comparative Sensory Tests

Comparative sensory tests are primarily designed to detect sample differences and assess the sensory effects of product modifications, like probiotic enrichment. This study used two primary comparative methods:

3.5.1.1 *Blind Comparison Tests*

This methodology involved presenting panellists with multiple samples coded with random three-digit numbers to conceal their identity and formulation. This approach allowed an objective evaluation of several sensory attributes, including flavour, texture, aroma intensity and acceptability and general acceptability.

Acceptability attributes were evaluated using a 9-point hedonic scale, where 1 corresponded to “Dislike extremely” and 9 to “Like extremely”. Attributes assessed in terms of magnitude (e.g. aroma, sweetness, acidity) were rated using a 9-point intensity scale, anchored at 1 = “Extremely low” and 9 = “Extremely high”. For attributes not related to intensity, such as texture, panellists scored the parameter according to its perceived level within the same 9-point structured scale, from very low to very high expression of the attribute, rather than intensity per se.

In addition, a binary yes/no question was included to assess purchase intent, providing insight into prospective consumer acceptance.

This type of blind sensory evaluation has been widely applied to a variety of food matrices, including cereal bars, fermented milk, fermented vegetable drinks, fermented orange juice, isotonic beverage and reconstitutable isotonic drink powders.

3.5.1.2 *Guided Comparative Tests*

For guided comparative analyses, panellists were provided with a clearly identified control sample alongside one or more coded test samples. The primary objective was to facilitate a direct comparison of the test samples against the control concerning predefined organoleptic parameters.

Differences in sensory attributes were scored using a 7-point scale ranging from negative to positive values (−3 to +3), where 0 indicates no difference compared to the control. The algebraic sign (negative or positive) reflects lower or greater perception of the attribute, respectively: −3 = extremely lower, −2 = moderately lower, −1 = slightly lower, 0 = no difference, +1 = slightly greater, +2 = moderately greater, and +3 = extremely greater.

On this scale, a score of 0 signified no discernible deviation from the control, while ± 1 indicated a minimal deviation, ± 2 represented a notable deviation, and ± 3 denoted a considerable deviation. This scale design enabled a detailed assessment of both the direction (e.g., more or less intense, better or worse) and magnitude of sensory differences, particularly those attributable to probiotic enrichment or other modifications in formulation. In addition, general acceptability for these products was measured independently using a 9-point hedonic scale identical to that employed in the blind tests.

This methodology was applied to cookies, vegan dark chocolate cream, peanut butter cream, vegan hazelnut chocolate cream, and milk chocolate cream.

3.5.2 Acceptability Tests

Acceptability tests are designed to evaluate consumer preference of individual samples presented in isolation, without direct comparison to other products. This methodology is fundamental to understanding the overall acceptability of a product from the consumer's perspective. Panellists evaluate specific organoleptic attributes using a 9-point hedonic scale for each organoleptic attribute, following the same criteria and definitions as previously described for blind comparison tests.

In this study, acceptability tests were performed on ice cream, popping candy, and bread. These evaluations furnished valuable insights into the sensory quality and market potential of single probiotic-enriched products without the confounding effect of sample comparisons.

3.6 STATISTICAL ANALYSIS

All experiments were carried out in triplicate, ensuring robustness and reproducibility. The values reported are expressed as mean values $\pm$ SD. The selection of appropriate statistical analyses was determined based on the structure and objective of each experiment.

Prior to the selection of statistical tests, the Shapiro-Wilk normality test was used to determine whether the data was normal, which was then complemented by a visual inspection of the data distribution through Q-Q plots. As most datasets appeared to follow a normal distribution based on these plots, parametrical statistical tests were applied in subsequent analyses.

For physicochemical and microbiological data, comparisons between two groups (e.g., control vs. probiotic inoculated samples) were evaluated using Student's t-test. The choice

between paired and unpaired Student's t-test was based on the study design, depending on whether the sample was measured under different conditions (paired) or whether independent samples were compared (unpaired). For experiments involving comparisons between more than two groups (e.g., evaluating the impact of different probiotic strains on a food matrix), a one-way analysis of variance (ANOVA) was performed. In studies assessing the influence of two independent factors (e.g., probiotic strain and storage time), a two-way ANOVA was conducted. Following both one-way and two-way ANOVA analyses, Tukey's multiple comparison test was applied to determine statistically significant differences between sample means.

Sensory evaluation data were subjected to statistical analyses using similar criteria. For guided comparison tests involving one probiotic-enriched sample and a control, an Unpaired Student's t-test was applied, as the sensory panel assessed different product samples. In cases where more than two samples were compared (e.g., different probiotic treatments), a one-way ANOVA followed by Tukey's post-hoc multiple comparisons test was used.

All statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, Inc., 225 Franklin Street, Floor 26, Boston, MA 02110, USA).

Statistical significance was set as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4 RESULTS AND DISCUSSION

4.1 CHAPTER 1: ASSESSMENT OF AB-BIOTICS PROBIOTIC STRAINS STABILITY AND IMPACT IN DIFFERENT NON-FERMENTED FOOD AND BEVERAGE MATRICES

4.1.1 Dairy Products

4.1.1.1 Cream Cheese

The stability and properties of the cream cheese enriched with the probiotic blend I3.1 were assessed according to the experimental design outlined in Figure 5.

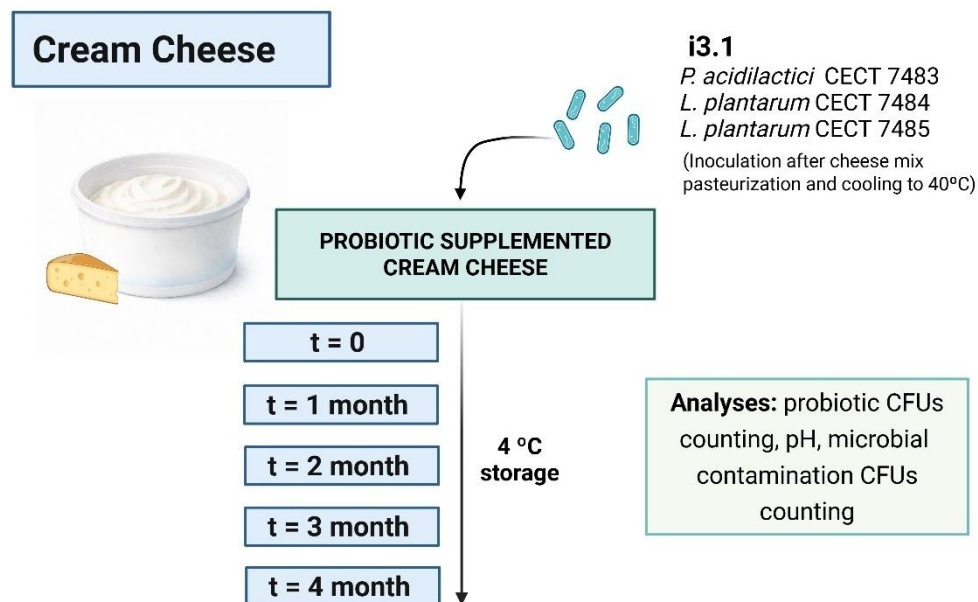


Figure 5. Schematic illustration of the experimental design. Cream cheese was formulated with the probiotic blend I3.1 and stored at 4 ± 2 °C for four months. Analyses were performed immediately after inoculation and subsequently at 1, 2, 3, and 4 months of refrigerated storage. Evaluated parameters included viable probiotic counts (CFU), pH, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

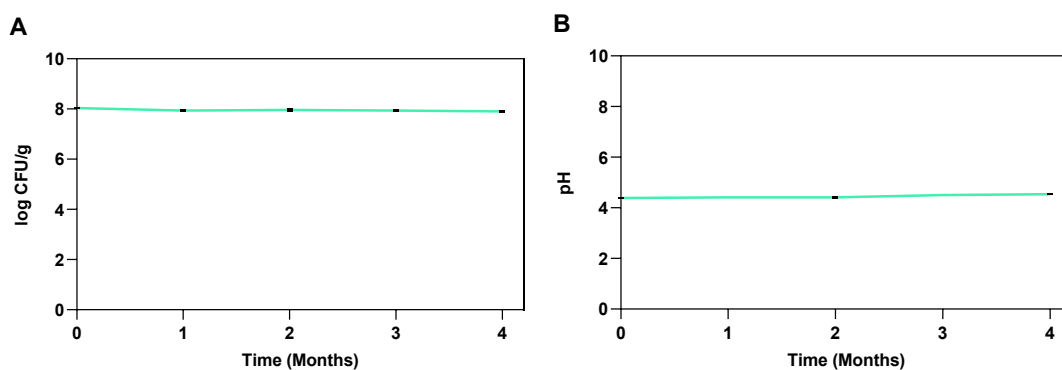


Figure 6. Probiotic viability expressed as log CFU/g (A) and pH (B) of cream cheese fortified with probiotic blend I3.1 (*P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, *L. plantarum* CECT 7485), over 4 months storage at 4 °C.

The incorporation strategy for the I3.1 probiotic blend into the pasteurized cream cheese matrix, as detailed in Section 3.2.10 (Materials and Methods), was designed to ensure a functional probiotic concentration in the final product. The target viable cell concentration per 50 g serving was set at 7.78 log CFU/g, corresponding to the clinically validated daily dosage of the blend. To compensate for potential viability losses during refrigerated storage, a 1.5-fold overage was applied, yielding an initial target concentration of 7.95 log CFU/g. As shown in Figure 6-A, the initial viable count of the I3.1 blend was 8.03 ± 0.02 log CFU/g, decreasing only marginally to 7.90 ± 0.01 log CFU/g at the end of storage ($\Delta = -0.13$ log CFU/g; $p < 0.05$). This minimal decline demonstrates that the over-formulation strategy was effective and that the finished product contained the intended therapeutic dose throughout storage.

The high stability observed aligns with previous research using cream cheese matrices as probiotic carriers. Santini et al. (2012) evaluated *Lactobacillus paracasei* Lpc-37 in a dried tomato-flavoured cream cheese, with the probiotic co-inoculated alongside mesophilic starter cultures and commercial rennet. Under these conditions, probiotic counts remained above 7.5–8 log CFU/g across 21 days, demonstrating a survival profile highly comparable to that observed for the I3.1 blend in the present study, despite differences in strain identity and production process.

Similarly, Hernández-Figueroa et al. (2025) incorporated *Lactobacillus acidophilus* LA-5, alongside different starter mixtures, into reduced-fat cream cheese manufactured through fermentation, meaning the probiotic acted both as a starter culture component and as a functional adjunct. Although the conditions differ from the current study, which involves a pasteurized, non-fermented cream cheese with post-process probiotic addition, the findings

remain relevant: all formulations maintained 8–9 log CFU/g over 30 days, confirming that cream cheese, irrespective of fat level or whether fermented or pasteurized, generally affords strong probiotic protection. Moreover, Speranza et al. (2018) demonstrated that functional rennet-coagulated cream cheese supplemented with *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and *Lactobacillus reuteri* DSM 20016, together with inulin or FOS, sustained probiotic counts above 8.3 log CFU/g over 28 days while preserving desirable texture and flavour attributes.

Together, these studies confirm that cream cheese, fermented or pasteurized, full-fat or reduced-fat, tends to provide a protective environment for probiotic microorganisms, likely due to its buffering protein matrix, and lipid-associated microhabitats that stabilize cellular membranes during cold storage (Wang et al., 2009).

The pH of the I3.1 cream cheese remained stable at 4.44 ± 0.07 throughout the storage period (Figure 6-B). The absence of post-acidification indicates that the metabolic activity of the I3.1 blend was minimal under refrigerated conditions. This pattern is consistent with cream cheese systems where metabolism is constrained by temperature and matrix buffering. For reduced-fat cream cheeses with *Lactobacillus rhamnosus*, Ningtyas et al. (2019) reported only modest pH fluctuations over 35 days, with batch means spanning approximately 4.59–4.72 depending on formulation (non-encapsulated vs. encapsulated cells; with or without β -glucan or phytosterols).

In other studies cited before, Speranza et al. (2018) observed pH values of ~4.99–5.00 during 28 days at 4 °C, showing minimal drift under cold storage again.

By contrast, Hernández-Figueroa et al. (2025) reported that cream cheese formulations with initial pH values ranging from 4.45 to 4.85 underwent post-acidification of ~0.5–0.8 units over 30 days, indicating ongoing metabolic activity in starter culture based systems. Although refrigeration reduced the rate of pH decline, it did not prevent post acidification. Finally, in Santini et al. (2012), pH showed small changes, remaining stable at around pH 6 during 21 days, and probiotic batches exhibited slightly higher pH and lower titratable acidity than controls, indicating limited post-acidification when the probiotic was present. Altogether, the present results demonstrate that pasteurized, post-inoculated cream cheese is an excellent and stable vehicle for the delivery of the I3.1 probiotic blend.

4.1.2 Frozen Desserts

4.1.2.1 Ice Cream

Two tests were conducted to evaluate the stability and properties of various ice cream formulations enriched with the probiotic blend I3.1 as shown in the experimental design outlined in Figure 7.

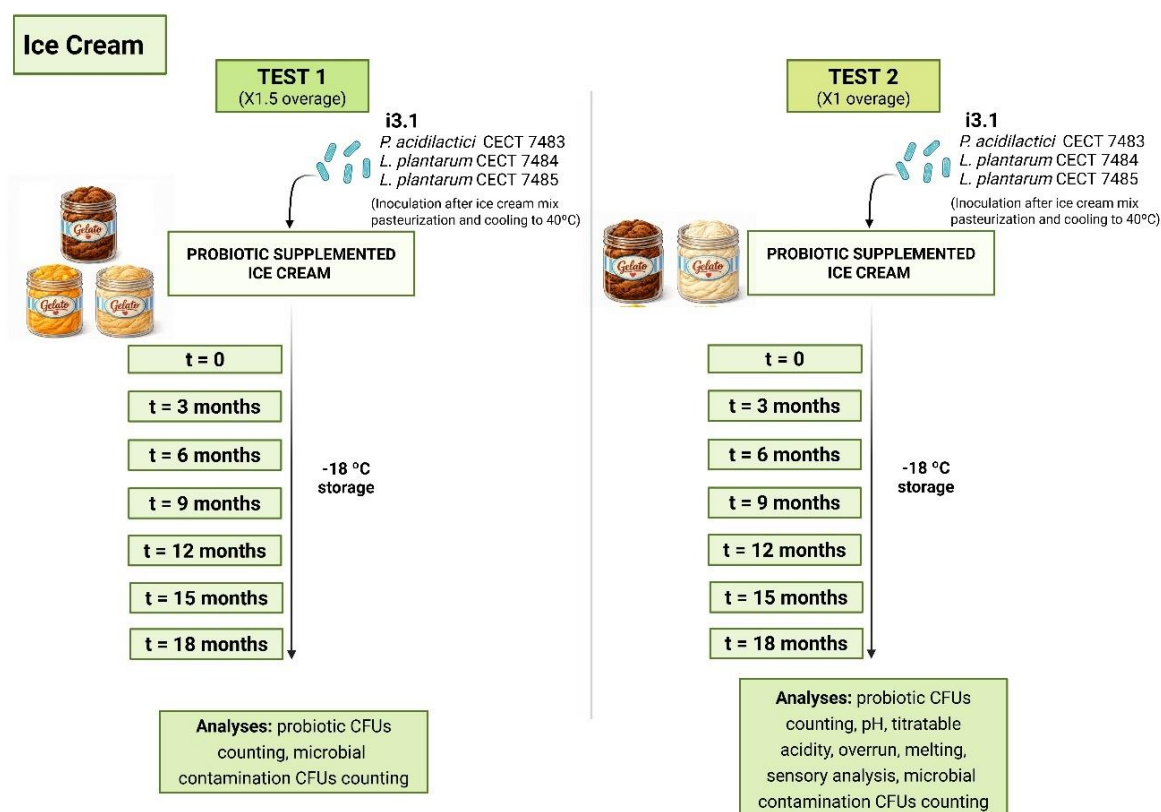


Figure 7. Schematic illustration of the experimental design. Multiple ice cream formulations were supplemented with the probiotic blend I3.1 and stored at -18 ± 2 °C for 18 months. Two inclusion levels were evaluated: a 1.5-fold overage relative to the clinical dose (Test 1) and a no-overage condition corresponding to the exact clinical dose of 3×10^9 CFU (Test 2). Samples were analysed immediately after production and at 3, 6, 9, 12, 15, and 18 months of storage. Assessed parameters included viable probiotic counts (CFU) and microbiological safety contamination (at the beginning and at the end of storage) in both Test 1 and Test 2, while Test 2 additionally included measurements of pH, TA, overrun, melting behaviour, and a sensory analysis using 14-days samples. The figure was created in <https://BioRender.com>.

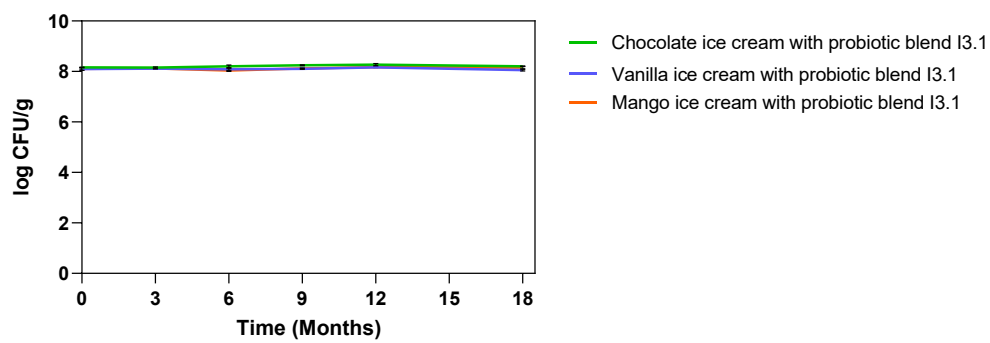


Figure 8. Test 1. Viability of probiotic blend I3.1, expressed as log CFU/g, in fortified ice cream formulations (Chocolate, Vanilla, Mango) during 18 months of storage at -18 ± 2 °C.

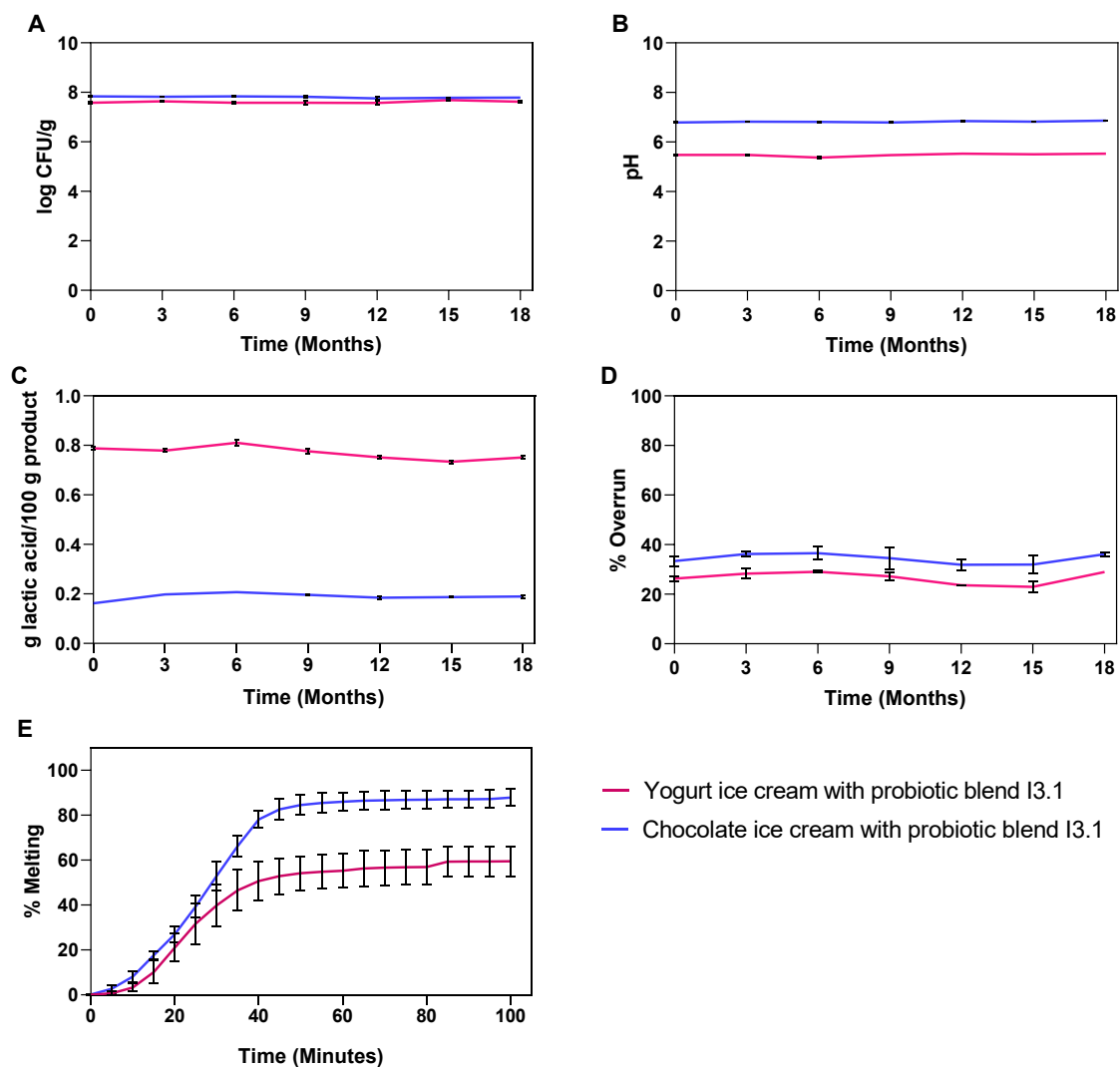


Figure 9. Test 2. Probiotic viability and physicochemical characteristics of ice cream formulations (Chocolate and Yogurt) fortified with probiotic blend I3.1 during 18 months of frozen storage. A) Viability of probiotic blend I3.1 expressed as log CFU/g. B) pH. C) TA expressed as g of lactic acid / 100 g of product. D) Overrun. E) Melting curve.

The I3.1 probiotic blend was integrated into ice cream formulations using a method tailored to achieve its clinically effective concentration in the finished product, as detailed in the Section 3.2.11 (Materials and Methods). In Test 1, the target probiotic concentration was set at 7.33 log CFU/g per 140 g serving, while in Test 2 it was set at 7.38 log CFU/g per 125 g serving, both corresponding to the clinically validated daily dosage of the blend. A 1.5-fold overage was applied in Test 1 to counteract possible loss during storage, resulting in an initial inoculum of 7.51 log CFU/g, whereas no overage was applied in Test 2.

In Test 1, chocolate, vanilla, and mango ice creams fortified with probiotic blend I3.1 demonstrated exceptional stability, with average concentrations of 8.21 ± 0.05 , 8.10 ± 0.04 , and 8.12 ± 0.05 log CFU/g, respectively, throughout the storage period (Figure 8). These concentrations successfully exceeded the target dose of 7.33 log CFU/g, indicating that the initial 1.5-fold over-formulation was more than sufficient to effectively deliver the desired probiotic load in all ice cream versions.

Similarly, in Test 2, both yogurt and chocolate ice cream varieties showed robust probiotic viability. The CFU counts for both flavours remained consistent around 7.61 ± 0.06 log CFU/g and 7.80 ± 0.03 log CFU/g, respectively, for the full 18-month storage duration (Figure 9-A). In this test, the probiotic concentration also exceeded the target of 7.38 log CFU/g per 125 g serving, confirming the results of the first test.

Probiotic viability in ice cream is inherently challenged by various stresses encountered during formulation, processing and storage, such as unfavourable acidity, redox potential, ice crystal formation, oxygen exposure or high sugar concentration (Mohammadi et al., 2011). However, the results observed in this study demonstrate that the I3.1 probiotic blend successfully withstands both the ice cream manufacturing process and the conditions of the finished product. These results are consistent with previous findings in the literature. For instance, Nousia et al. (2011) evaluated the viability of *Lactobacillus acidophilus* LMGP-21381 in ice cream and found that the freezing process did not result in significant viability loss after prolonged storage (~45 weeks) at -15°C and -25°C . Similarly, Haynes & Playne (2002) investigated the stability of probiotic cultures, including *L. acidophilus*, *B. lactis*, and *L. paracasei* subsp. *paracasei*, in low-fat ice cream stored at -25°C for up to 12 months. That study concluded that direct addition of frozen concentrated probiotic cultures is an effective strategy for producing both dairy- and water-based probiotic ice cream capable of maintaining viable cell counts above 1×10^6 CFU/g throughout extended storage.

The physicochemical properties of chocolate and yogurt flavours were monitored in Test 2 (Figure 9, panels B to E).). The pH of the yogurt ice cream remained stable around 5.48 ± 0.05 , while the chocolate ice cream maintained a consistent pH of approximately 6.86 ± 0.03 . Correspondingly, the TA, expressed as grams of lactic acid per 100 grams, remained stable throughout the storage period at approximately 0.77 ± 0.03 for the yogurt ice cream and 0.19 ± 0.01 for the chocolate ice cream. These values are consistent with typical ranges reported for similar ice creams (Goff et al., 2025). The stability of both pH and TA suggests that the incorporation of the probiotic blend does not negatively impact the fundamental chemical properties of the product over time, an essential factor for preserving its sensory characteristics, structural integrity, and microbiological safety (Pimentel et al., 2022).

The overrun percentage, representing the volume of air incorporated into the ice cream matrix, displayed stable and consistent values over storage, ranging between $26.61 \pm 2.58\%$ for yogurt and $34.38 \pm 2.76\%$ for chocolate ice cream. These levels lie well within the typical 15–50% range for premium ice creams (Almena et al., 2020) indicating that the formulations and freezing conditions efficiently supported uniform aeration.

Comparable aeration behaviour has been reported in other functional ice cream systems. Mehmetoğlu & Tarakçı, (2023) observed 20.83–23.67% overrun in propolis enriched ice creams, with values remaining stable across 60 days of storage. Similarly, Demir & Arslan, (2023) reported overruns between 26.83% and 46.29% across ice creams containing different stabilizers, underscoring how formulation and processing parameters can modulate aeration capacity. Overall, the differences among studies can be attributed to variations in recipe composition, stabilizer systems, production technique and freezer performance, while the present results demonstrate robust and reproducible overrun behaviour consistent with high quality frozen desserts.

Melting profiles assessed during storage showed consistent differences between formulations. The chocolate ice cream exhibited a significantly faster melting rate, reaching $85.38 \pm 4.40\%$ melt after 55 min, at which point the melting rate began to stabilize. In contrast, the yogurt ice cream melted more slowly, achieving $54.81 \pm 7.56\%$ melt after 55 min before stabilizing, highlighting compositional and structural differences influencing melting behaviour. A controlled melting rate is generally considered desirable for consumer satisfaction, as it contributes to perceived quality and texture during consumption.

These trends were further supported by the first-drop time, which was markedly longer for the yogurt ice cream (29 ± 5 min) compared to the chocolate ice cream (15 ± 2 min),

confirming a delayed melting onset in the yogurt matrix. Such behaviour is consistent with mechanisms described by Wu et al. (2025), who report that ice creams with stronger internal structure, arising from higher serum phase viscosity, protein interactions, or more effective fat–air network stabilization, exhibit longer induction times and slower drip-through rates during meltdown tests.

The sustained stability of all assessed parameters throughout the prolonged storage period underscores the structural and functional robustness of the probiotic ice cream formulations under freezing conditions.

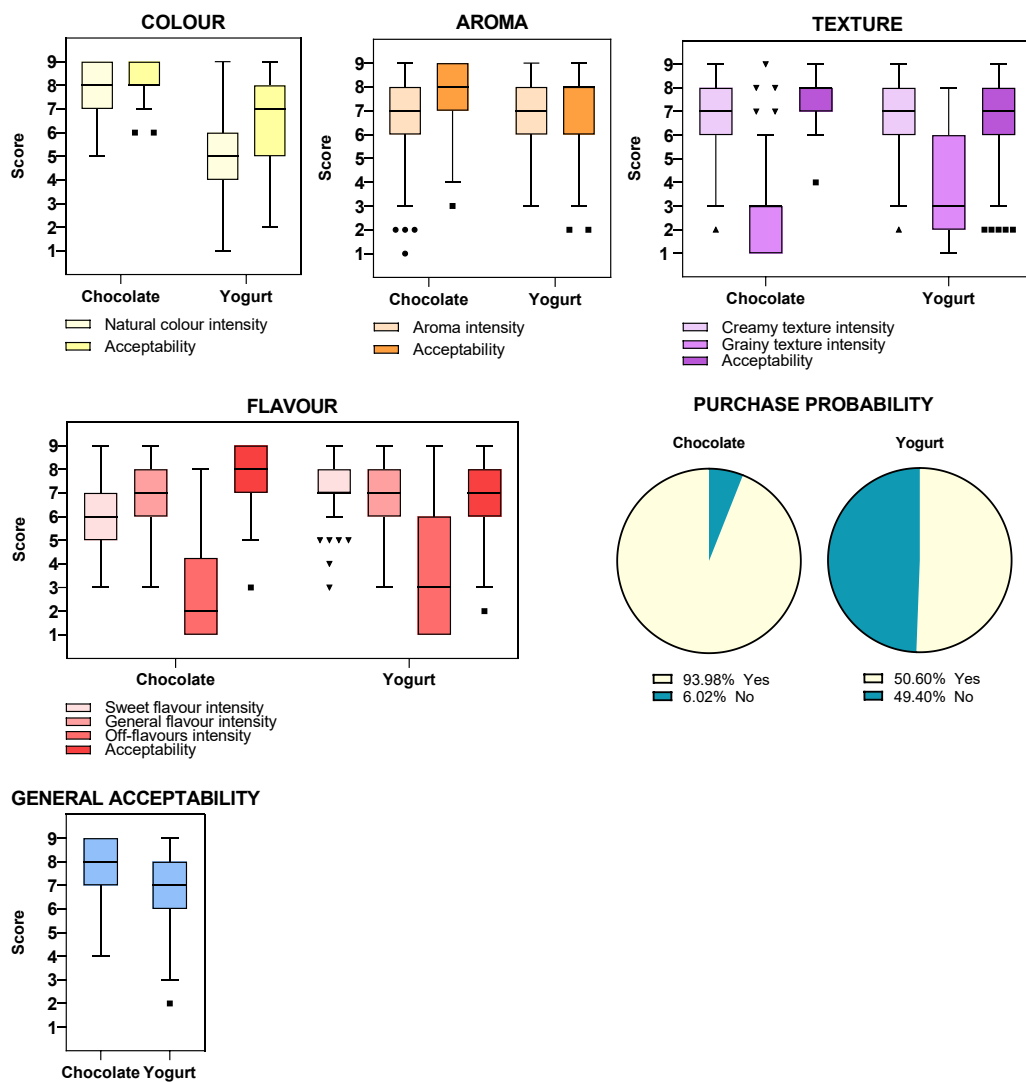


Figure 10. Sensory analysis results of chocolate ice cream (Chocolate) and yogurt ice cream (Yogurt), fortified with probiotic blend I3.1. The boxplots illustrate the scores for sensory attributes, categorized by intensity and acceptability, while the pie charts illustrate the scores of the purchase probability, as evaluated by 83 untrained panellists. Separated dots represent outliers, corresponding to individual panellist scores lying outside the whisker range ($> 1.5 \times \text{IQR}$).

The sensory assessment of Test 2 (chocolate and yogurt ice creams) was conducted two weeks after sample production and involved 83 untrained panellists (39% female and 61% male; 18–64 years), as summarized in Figure 10. As no control samples were included, panellists were informed that they were evaluating ice creams enriched with probiotics; consequently, their assessments focused on the intrinsic product characteristics rather than on comparisons with non-enriched counterparts.

Overall, the chocolate ice cream demonstrated superior consumer acceptability and purchase intent compared to yogurt variant. The chocolate ice cream achieved a general acceptability score of 7.94 ± 1.02 , leading to a high purchase probability of 93.98%. In contrast, the yogurt ice cream showed a lower general acceptability score of 6.76 ± 1.47 , with a considerably reduced purchase probability of 50.60%.

Detailed attribute analysis revealed specific drivers for these preferences. The chocolate ice cream was perceived with higher natural colour intensity (7.93 ± 1.01) and colour acceptability (8.18 ± 0.94). The yogurt version received lower scores in both attributes, 5.14 ± 1.89 and 6.43 ± 1.78 . Panellists pointed out that the colour of the coulis in the yogurt ice cream appeared too intense, making it seem artificial rather than natural. While aroma intensity was slightly higher for yogurt ice cream (7.01 ± 1.54 vs. 6.48 ± 1.74 for chocolate), the aroma acceptability was notably higher for chocolate (7.73 ± 1.35 vs. 7.18 ± 1.73 for yogurt). Texture analysis indicated that chocolate ice cream exhibited higher creamy texture intensity (7.10 ± 1.47) and better general texture acceptability (7.66 ± 1.02) with less perceived graininess (2.82 ± 1.87). Conversely, the yogurt ice cream had a lower creamy texture intensity (6.77 ± 1.84) and general texture acceptability (6.47 ± 1.82), coupled with higher graininess (3.54 ± 2.20), likely influenced by the presence of red berry coulis.

Regarding flavour, both ice creams were scored similarly in terms of general intensity (6.88 ± 1.38 for chocolate and 6.88 ± 1.24 for yogurt), but the chocolate ice cream scored higher in general acceptability (7.81 ± 1.14 vs. 6.73 ± 1.59 for yogurt), while sweet intensity was higher for the yogurt ice cream (7.31 ± 1.26 vs. 5.99 ± 1.31 for chocolate). Both formulations exhibited relatively low scores for off-flavours (2.87 ± 2.32 for chocolate and 3.69 ± 2.28 for yogurt), indicating no significant off-notes.

These findings suggest that while both probiotic ice cream formulations were generally accepted, the chocolate matrix provided a more appealing sensory profile overall, contributing to its higher consumer preference and purchase probability.

The higher perceived creaminess and overall flavour balance in the chocolate variant appear to be key factors. The lower performance of the yogurt ice cream, particularly in texture and overall acceptability, highlights potential challenges in balancing the tartness of yogurt/coulis with desired sweetness and achieving optimal texture in a probiotic-fortified dairy matrix.

Collectively, these findings underscore the substantial potential of ice creams as an ideal food matrix for the delivery of the I3.1 probiotic blend, primarily due to its exceptional stability and strong consumer acceptance observed in this study. Nevertheless, it is evident from the sensory results that, irrespective of probiotic inclusion, the yogurt with red berry coulis formulation would benefit from further optimization to achieve a more favourable consumer response.

4.1.3 Confectionery Products

4.1.3.1 Peanut Butter

The stability and properties of peanut butter enriched with the probiotic strain *B. coagulans* Unique IS-2 were assessed according to the experimental design outlined in Figure 11.

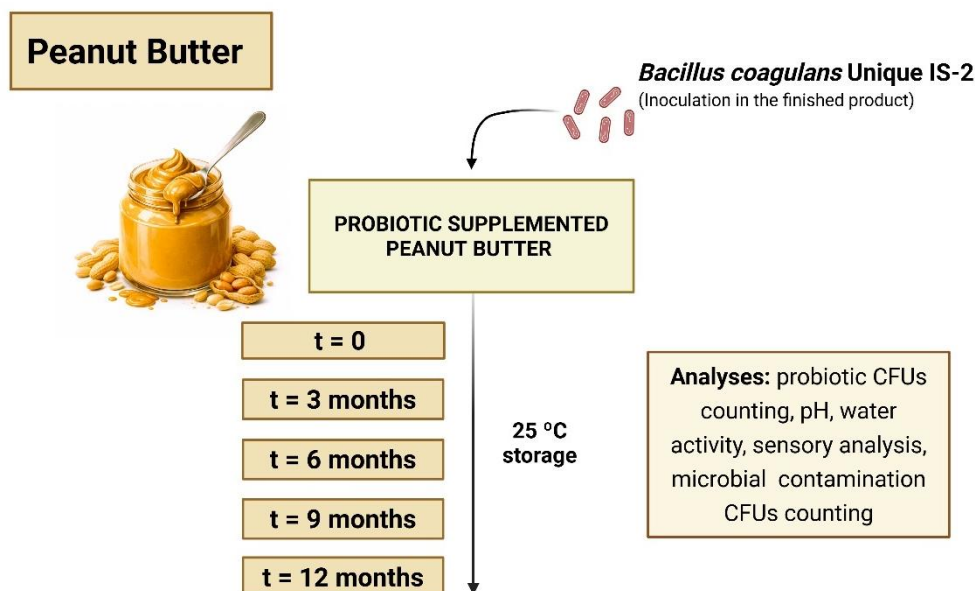


Figure 11. Schematic illustration of the experimental design. Peanut butter was fortified with the probiotic strain *B. coagulans* Unique IS-2 and stored at 25 ± 5 °C for 12 months. Analyses were performed immediately after inoculation and at 3, 6, 9, and 12 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), pH, aw, sensory attributes, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

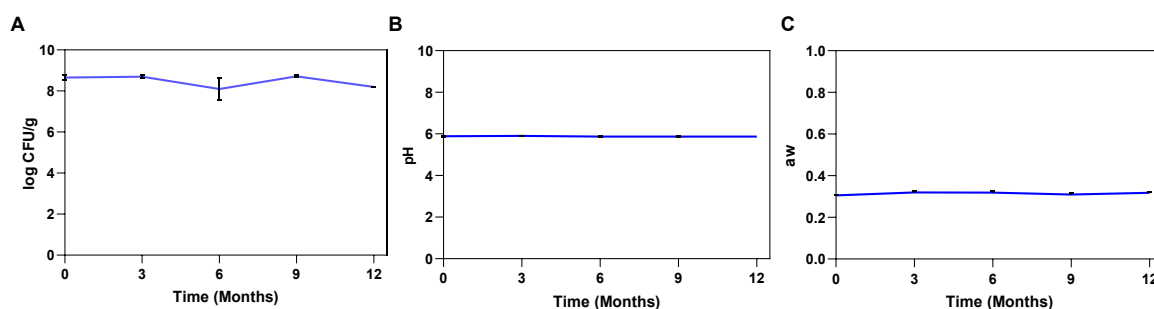


Figure 12. Probiotic viability and physicochemical characteristics of peanut butter fortified with *B. coagulans* Unique IS-2 during 12 months of storage at room temperature (25 ± 5 °C). A) Viability of *B. coagulans* spores expressed as log CFU/g. B) pH. C) aw.

The strategy for *B. coagulans* Unique IS-2 spores incorporation into the peanut butter, as detailed in Section 3.2.1 (Materials and Methods), was designed to ensure a functional probiotic concentration in the final product. The target probiotic concentration was set at 8.12 log CFU/g per 15 g serving, which corresponds to the clinically validated daily dosage of the strain. To compensate for potential viability loss during extended storage at room temperature, a 2.5-fold overage was applied, resulting in an initial inoculum of 8.52 log CFU/g.

As shown in (Figure 12-A), the probiotic spore count remained relatively stable across the storage period, with values around 8.47 ± 0.30 log CFU/g. After 12 months, the viable spore count showed no decline, demonstrating the matrix's excellent protective capacity for spore-forming probiotics. In a study by Klu et al. (2014) the authors evaluated the survival of four commercial probiotic blends composed by strains of lactobacilli, bifidobacteria, streptococci and lactococci in full-fat and reduced-fat peanut butter throughout 12 months at 4 °C, 25 °C, and 37 °C. In that study, even under optimal refrigerated conditions, most probiotic mixtures exhibited a reduction of approximately 1 log CFU/g after 12 months, and greater losses were observed at higher temperatures. The high viability observed in our study underscores the exceptional protective effect of the matrix for *B. coagulans* Unique IS-2 spores, exceeding the stability typically reported for non-spore-forming strains in similar food systems. In this line, Majeed et al. (2016) demonstrated that *B. coagulans* MTCC 5856 retained high viability (>99.06%) in various functional peanut butters, up to 12 months of storage, indicating excellent stability and suitability for consumer products.

The pH (Figure 12-B) remained stable throughout the storage period at approximately 5.88 ± 0.02 . This suggests that the incorporation of *B. coagulans* spores did not trigger fermentation or other biochemical changes that could alter the product's acidity.

In parallel, aw (Figure 12-C) values remained low and consistent at 0.314 ± 0.006 . Maintaining low aw levels (<0.6) is critical, as it significantly limits microbial proliferation and enzymatic activity, thereby contributing to extended shelf life and supporting the long-term viability of the probiotic strain (Tapia et al., 2020).

The combination of low aw and stable pH reinforces the conclusion that the probiotic peanut butter is both microbiologically and chemically stable over extended storage. Interestingly, a study by Klu & Chen (2015) demonstrated that peanut butter matrices significantly enhanced the survival of probiotic strains during simulated GI passage compared to controls lacking the matrix. Taken together, these findings indicate that peanut butter is an excellent matrix for maintaining probiotic viability, even throughout gastrointestinal transit.

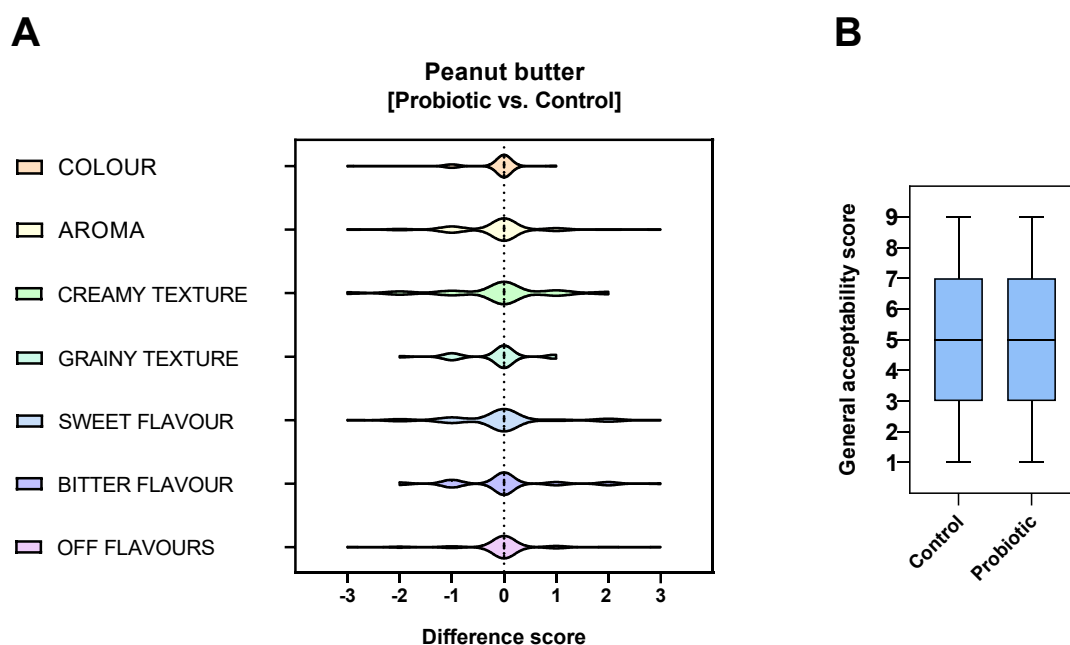


Figure 13. Sensory analysis results of peanut butter formulations without (Control) and with the probiotic strain *B. coagulans* Unique IS-2 (Probiotic). A) Violin plot represents the distribution of scores reflecting the perceived differences for various sensory attributes. B) Boxplot summarizes general acceptability scores, as rated by 51 untrained panellists.

To assess consumer acceptance, a sensory analysis was performed one week after sample production, comparing the probiotic peanut butter with a control sample without probiotics. The evaluation involved 51 untrained panellists (68.6% female, 27.5% male, 3.9% other; 18–70 years) and is summarized in Figure 13. The violin plot illustrates the difference score analysis across various sensory attributes, including colour, aroma, creamy texture, grainy texture, sweet taste, bitter taste, and off-flavours, comparing the control and the probiotic-

enriched peanut butter. The analysis revealed no statistically significant differences between the two samples for any of the analysed parameters ($p > 0.05$).

General acceptability score, presented in the accompanying box plot, shows comparable means and dispersion between control and probiotic samples. This suggests that the inclusion of *B. coagulans* Unique IS-2 spores did not negatively impact overall consumer preference. Granato et al., (2010) emphasized that sensory acceptance is a critical factor in the success of functional foods, and the data presented here support the market viability of the probiotic peanut butter.

The results collectively demonstrate that peanut butter constitutes an effective and protective delivery matrix for the incorporation and long-term stability of *Bacillus coagulans* Unique IS-2 spores. The matrix supported the preservation of spore viability throughout extended storage at ambient temperature while maintaining stable physicochemical properties and sensory quality, confirming its technological suitability for developing shelf-stable probiotic foods.

4.1.3.2 Chocolate Creams

The stability and properties of different chocolate creams: milk chocolate cream (MCC), vegan dark chocolate cream (VDCC), and vegan hazelnut chocolate cream (VHCC); fortified with the probiotic strain *B. coagulans* Unique IS-2 spores, were assessed according to the experimental design outlined in Figure 14.

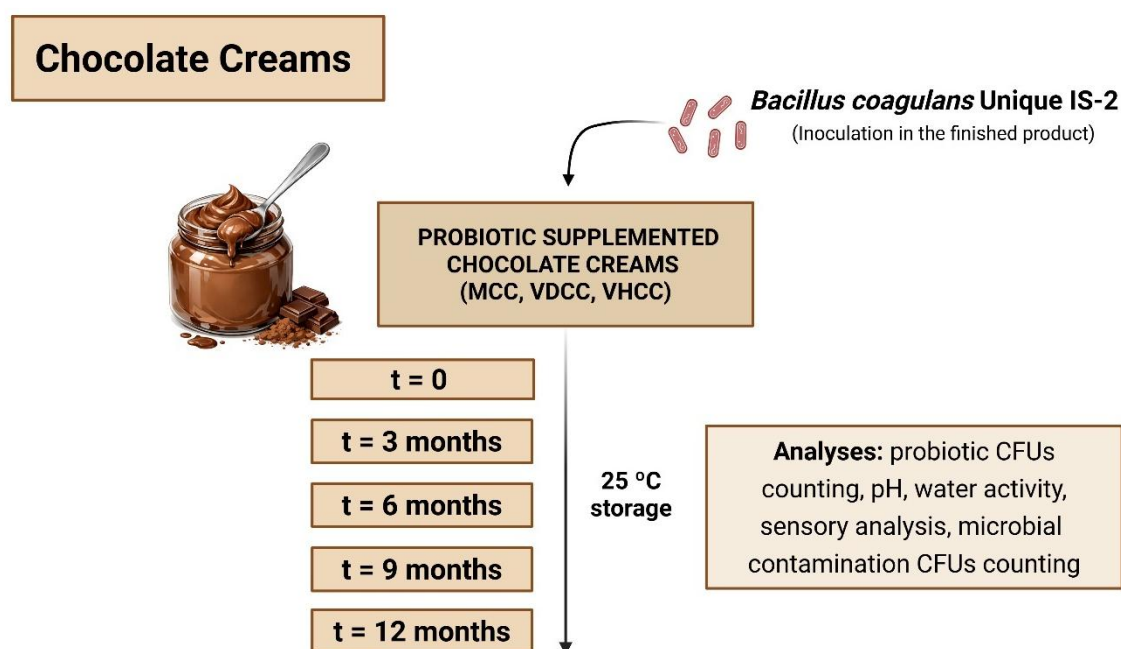


Figure 14. Schematic illustration of the experimental design. Chocolate creams were fortified with the probiotic strain *B. coagulans* Unique IS-2 and stored at 25 ± 5 °C for 12 months. Analyses were performed immediately after inoculation and at 3, 6, 9, and 12 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), pH, aw, sensory attributes, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

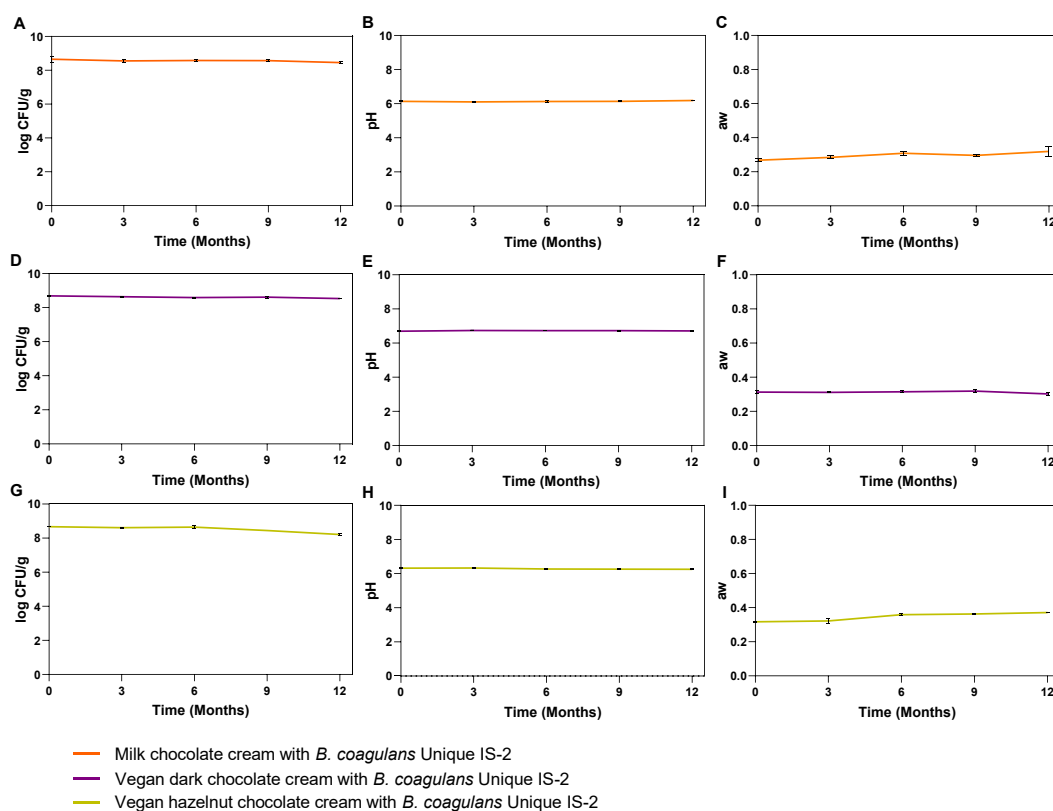


Figure 15. Probiotic viability and physicochemical characteristics of MCC / VDCC / VHCC fortified with *B. coagulans* Unique IS-2 during 12 months of storage at room temperature (25 ± 5 °C). A-D-G) Viability of *B. coagulans* spores expressed as log CFU/g. B-E-H) pH. C-F-I) aw.

The inoculation of *B. coagulans* Unique IS-2 spores into different commercial chocolate creams, as detailed in Section 3.2.2 (Materials and Methods), was designed to ensure a functional probiotic concentration in the final product. The target probiotic concentration was set at 8.12 log CFU/g per 15 g serving, which corresponds to the clinically validated daily dosage of the strain. To compensate for potential viability loss during extended storage at room temperature, a 3-fold overage was applied, resulting in an initial inoculum of 8.60 log CFU/g.

As shown in Figure 15, all three formulations effectively maintained probiotic viability throughout 12 months of storage at 25 °C, although VHCC exhibited a moderately lower retention. Mean viability values across the storage period were 8.54 ± 0.06 log CFU/g for MCC, 8.61 ± 0.06 log CFU/g for VDCC, and 8.52 ± 0.19 log CFU/g for VHCC. The overall viability loss between initial inoculation and month 12 was limited, with reductions of -0.20 log CFU/g for MCC ($p > 0.05$), -0.16 log CFU/g for VDCC ($p < 0.05$), and -0.46 log CFU/g for VHCC ($p < 0.05$), confirming that all matrices supported long-term spore stability, albeit to different extents. These findings align with previous research demonstrating the robustness of *B. coagulans* in related matrices. Majeed et al., (2016) reported that *B. coagulans* MTCC 5856 retained over 96.81% viability in chocolate fudge frosting and hot fudge toppings during processing and 12 months of storage at room temperature, confirming its excellent stability in chocolate-based systems. *Enterococcus faecium* CRL 183, although not being an sporulated microorganism, similarly maintained counts above 8 log CFU/g in chocolate spreads over 180 days at 20 °C, indicating strong probiotic resilience under ambient storage conditions (Cozentino et al., 2022). Interestingly, beyond storage stability, Yonejima et al. (2015) demonstrated that chocolate coating significantly enhanced the acid tolerance and enzymatic activity of *Lactobacillus brevis* subsp. *coagulans*, maintaining viability up to 100-fold higher than powders or beverages under simulated gastric conditions, highlighting the protective role of chocolate matrices on probiotic functionality.

The pH values of the chocolate spreads remained relatively stable over time, with only minor declines observed. MCC maintained a pH around 6.13 ± 0.03 , while the vegan variants hovered near 6.72 ± 0.02 for the VDCC and 6.29 ± 0.03 for the VHCC, respectively.

Water activity values were consistently low across all formulations, remaining below 0.6 throughout the study period and specifically: 0.295 ± 0.020 for MCC, 0.312 ± 0.006 for the VDCC and 0.346 ± 0.024 for the VHCC, respectively. This is ideal for shelf-stable products

and supports the long-term survival of *B. coagulans* spores. Moreover, low aw inhibits the growth of spoilage organisms and contributes to the extended shelf life observed in these products (Tapia et al., 2020).

Taken together, these results confirm that chocolate spreads are a suitable carrier for *B. coagulans* Unique IS-2 spores, offering both microbial stability and physicochemical integrity over extended storage periods.

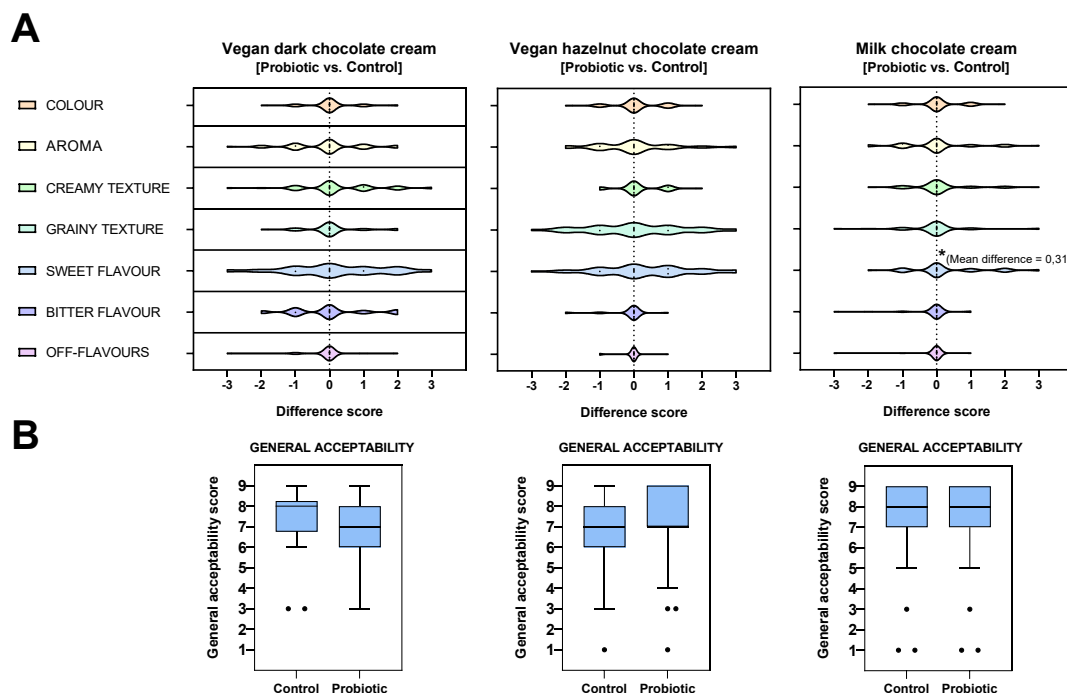


Figure 16. Sensory analysis results of three different chocolate cream formulations without (Control) and with the probiotic strain *B. coagulans* Unique IS-2 (Probiotic). A) Violin plots represent the distribution of scores reflecting the perceived differences for various sensory attributes. B) Boxplots summarize general acceptability scores, as rated by 51 untrained panellists. (Significance: * $p < 0.05$).

To assess consumer acceptance, a sensory analysis was performed one week after sample production, comparing the probiotic peanut butter with a control sample without probiotics. The evaluation involved 51 untrained panellists (68.6% female, 27.5% male, 3.9% other; 18–70 years) and is summarized in Figure 16.

The panel evaluated three types of chocolate cream: milk chocolate, vegan dark chocolate, and vegan hazelnut chocolate, each with and without the probiotic. Violin plots illustrating the difference scores between control and probiotic samples across seven sensory attributes (colour, aroma, creamy texture, grainy texture, sweet flavour, bitter flavour, and off-flavours) revealed minimal perceived differences.

Most distributions were centred around zero, indicating that the addition of *B. coagulans* spores did not significantly alter the sensory profile of the products. A slight but statistically significant increase in sweet flavour ($\Delta = +0.31$) was detected in the probiotic MCC formulation compared to the control. However, this variation remained within a negligible range ($\Delta < \pm 1$), below the threshold for perceptible deviation on the sensory scale used (-3 to $+3$). Importantly, no significant increase in “off-flavours” was observed across all formulations, indicating good sensory compatibility of the probiotic strain with the original organoleptic profile. These findings are consistent with previous research. Dordoni et al. (2019) developed chocolate-based confectionery products enriched with *Bifidobacterium* (BB-12®), which were highly appreciated for their sensory attributes.

These results underscore the potential of chocolate spreads as a vehicle for probiotic delivery, combining indulgence with functional benefits.

4.1.3.3 Chocolate Bars, Bites and Chunks

Three separate experiments were conducted to evaluate the stability and functional properties of six distinct dark and milk chocolate formulations, as detailed in Materials and Methods (Section 3.2.12), incorporating either the I3.1 probiotic blend or AB-KOLICARE. The tests followed the experimental design outlined in Figure 17.

Chocolate Bars, Bites and Chunks

AB-Kolicare
B. longum CECT 7894
P. pentosaceus CECT 8330

i3.1
P. acidilactici CECT 7483
L. plantarum CECT 7484
L. plantarum CECT 7485

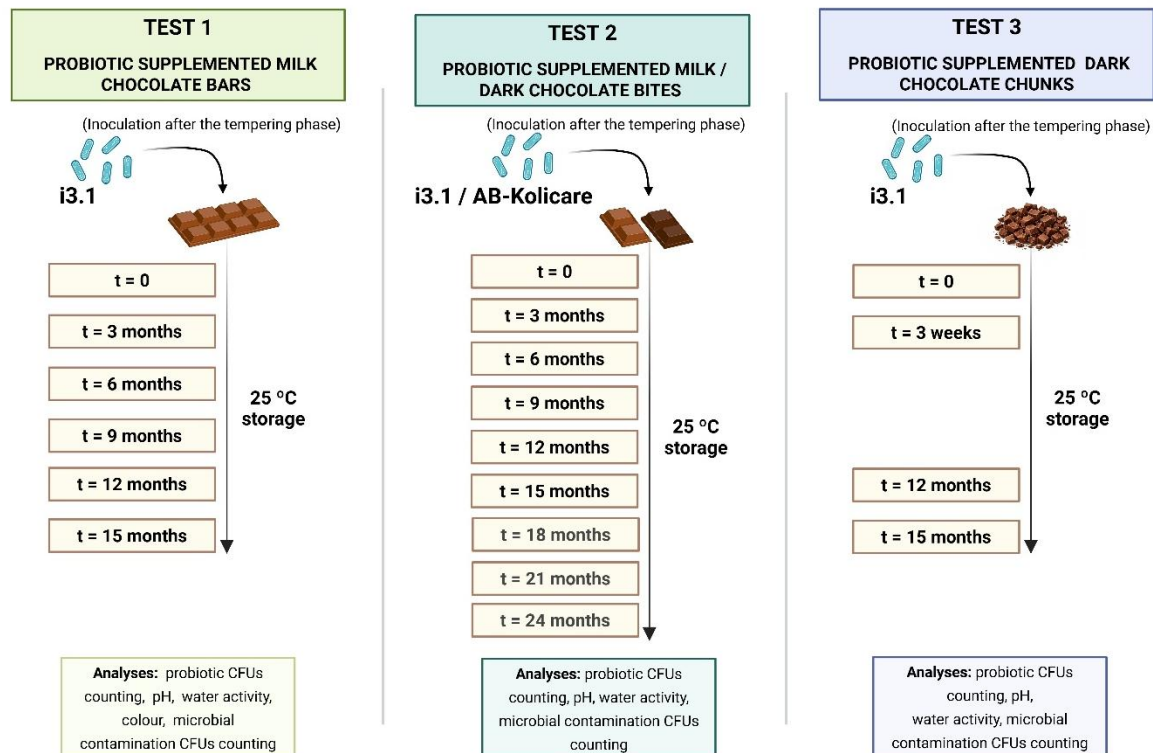


Figure 17. Schematic illustration of the experimental design. Three trials were conducted using different chocolate formats: milk chocolate bars (Test 1), milk/dark chocolate bites (Test 2), and dark chocolate chunks (Test 3). Probiotics were incorporated after the tempering phase, with Test 1 and Test 3 formulated using blend I3.1, and Test 2 using blends I3.1 and AB-KOLICARE. All samples were stored at 25 ± 5 °C and analysed at predefined intervals. Test 1 was evaluated at 0, 3, 6, 9, 12, and 15 months; Test 2 at 0, 3, 6, 9, 12, and 15 months, with extended analyses at 18, 21, and 24 months for the dark chocolate variants; and Test 3 at 0, 3 weeks, 12, and 15 months. Assessed parameters included viable probiotic counts (CFU), pH, aw, colour (Test 1), and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

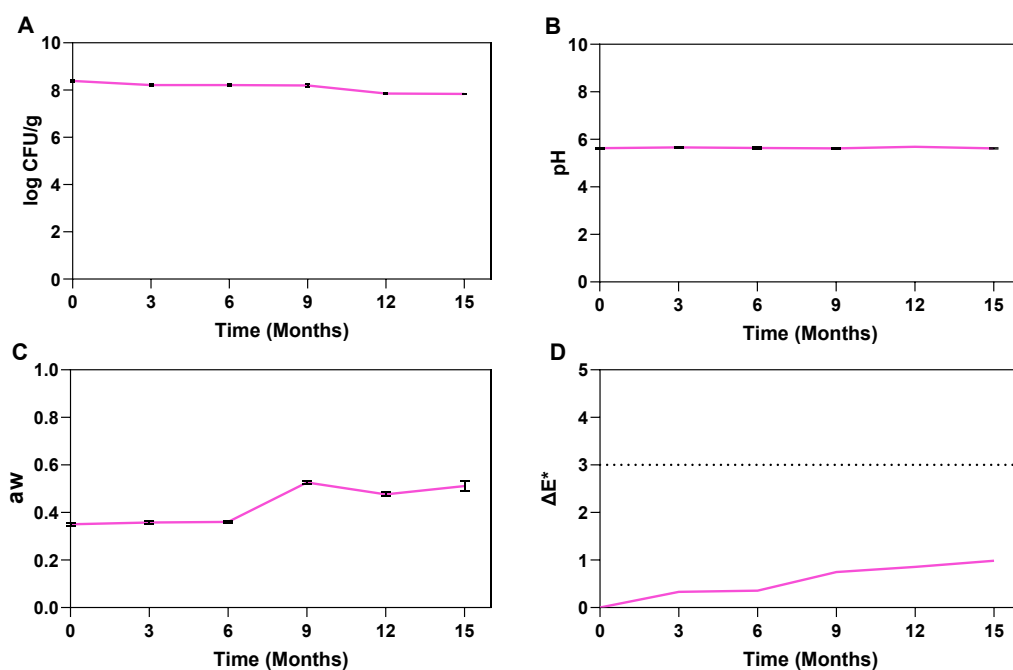


Figure 18. Test 1. Probiotic viability and physicochemical characteristics of MC1 + I3.1 (MC = milk chocolate) during 15 months of storage at room temperature ($25 \pm 5^\circ\text{C}$). A) Viability of probiotic blend I3.1 expressed as log CFU/g. B) pH. C) aw. D) Colour variation expressed as ΔE^* .

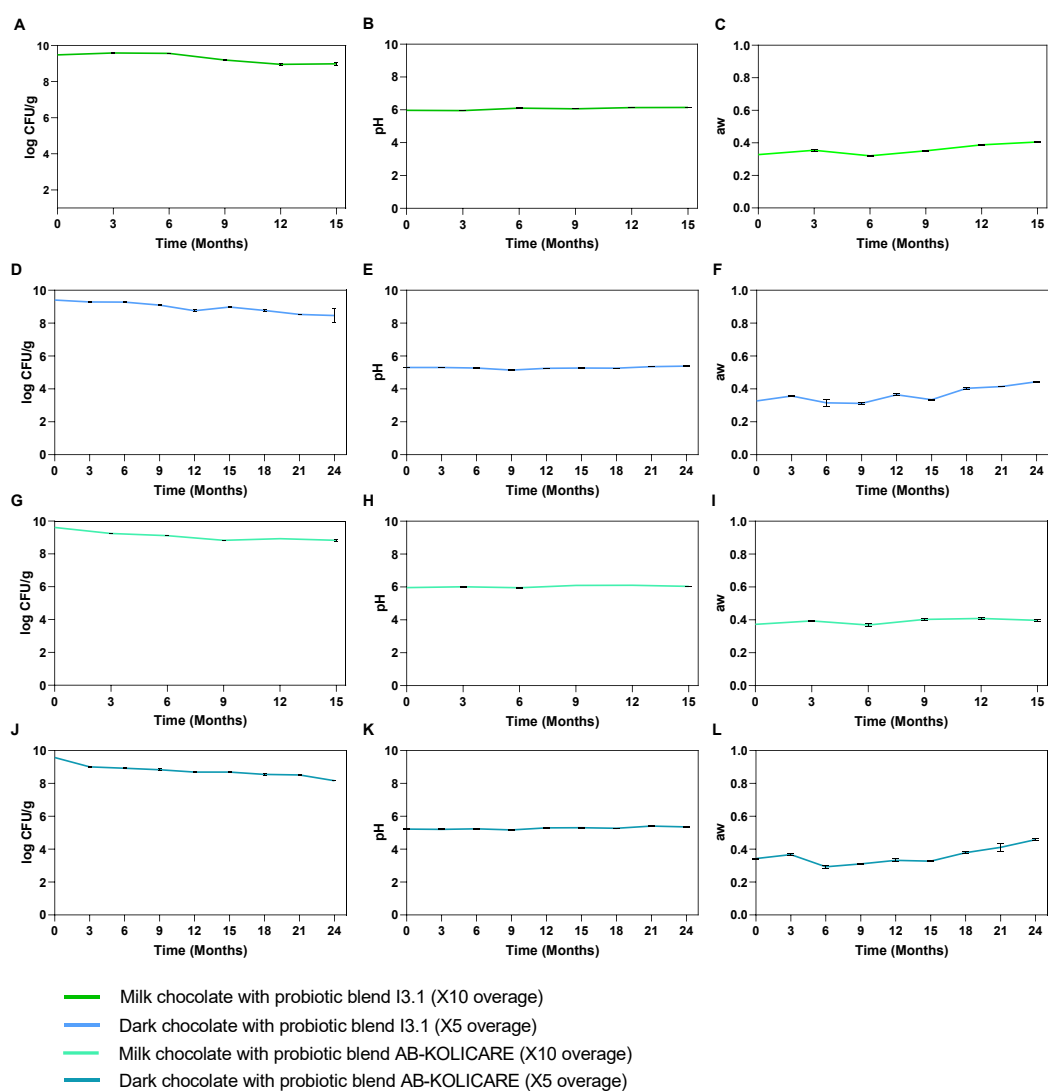


Figure 19. Test 2. Probiotic viability and physicochemical characteristics of MC2 + I3.1 / DC2 + I3.1 / MC2 + AB-KOLICARE / DC2 + AB-KOLICARE (MC = milk chocolate and DC = dark chocolate) bites during 15-24 months of storage at room temperature ($25 \pm 5^\circ\text{C}$). A-D-G-J) Viability of probiotic blends expressed as log CFU/g, B-E-H-K) pH, C-F-I-L) aw.

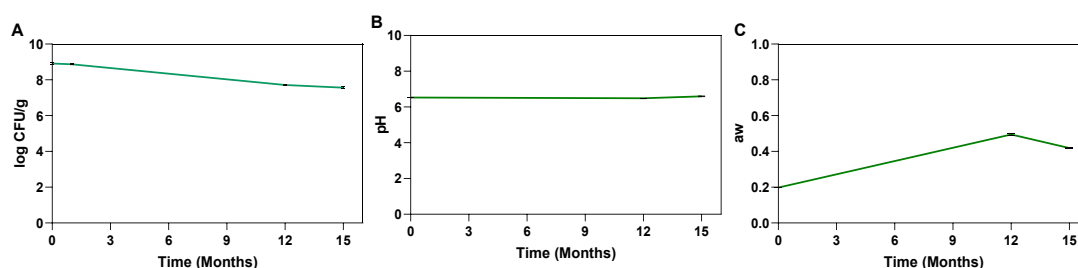


Figure 20. Test 3. Probiotic viability and physicochemical characteristics of DC3 + I3.1 bars during 15 months of storage at room temperature ($25 \pm 5^\circ\text{C}$). A) Viability of probiotic blends expressed as log CFU/g, B) pH, C) aw.

The inoculation of the different probiotic blends, I3.1 and AB-DIGEST, into the chocolate products was designed to ensure a functional probiotic concentration in the final products, as detailed in Section 3.2.12 (Materials and Methods). Target probiotic concentrations were established according to clinically validated daily dosages, and overages were applied to compensate for potential viability loss during extended storage at room temperature and to test the behaviour of different probiotic concentrations.

In Test 1, I3.1 was targeted at 8.08 log CFU/g per 25 g serving and a 2-fold overage was applied, resulting in an initial inoculum of 8.38 log CFU/g.

In Test 2, I3.1 and AB-KOLICARE were targeted at 8.78 and 8.30 log CFU/g per 5 g serving, respectively. A 5-fold overage was applied for DC2 + AB-KOLICARE and DC2 + I3.1 (initial inocula of 9.00 and 9.48 log CFU/g), and a 10-fold overage for MC2 + AB-KOLICARE and MC2 + I3.1 (initial inocula of 9.30 and 9.78 log CFU/g).

In Test 3, I3.1 was targeted at 8.76 log CFU/g per 5.2 g serving. No overage was applied, with the initial inoculum set at 8.76 log CFU/g in the DC3 samples.

The results of Test 1 are resumed in Figure 18. The initial probiotic count was 8.38 ± 0.03 log CFU/g, with a slight decrease of $\Delta \log \text{CFU/g} = -0.54$ observed over the 15-month period. The final count was a little below the target concentration ($\log \text{CFU/g} = -0.24$).

pH remained relatively stable around 5.65 ± 0.02 throughout the storage period.

Water activity of the sample packaged in aluminium foil increased slightly over the storage period, rising from an initial value of 0.357 ± 0.010 to 0.508 ± 0.022 after 15 months.

The ΔE^* values showed a gradual increase, principally due to the parameter b^* , reaching a value close to 1 (0.985) by month 15. A ΔE^* value below 3 is generally considered imperceptible to the human eye (Pathare et al., 2013), indicating that the visual quality of the chocolate remained largely unaffected.

The results of Test 2 are resumed in Figure 19. The initial probiotic count was 9.57 ± 0.04 log CFU/g in DC2 + AB-KOLICARE, 9.61 ± 0.02 log CFU/g in MC2 + AB-KOLICARE, 9.40 ± 0.03 log CFU/g in DC2 + I3.1 and 9.48 ± 0.03 log CFU/g in MC2 + I3.1, respectively. The final probiotic CFU counts decreased respectively $\Delta \log \text{CFU/g} = -1.40$ in DC2 + AB-KOLICARE, $\Delta \log \text{CFU/g} = -0.78$ in MC2 + AB-KOLICARE, $\Delta \log \text{CFU/g} = -0.93$ in DC2 + I3.1 and $\Delta \log \text{CFU/g} = -0.49$ in MC2 + I3.1, over 15 month of storage for milk chocolate and 24 months of storage for dark chocolate. After 15 months of storage, all formulations maintained sufficient probiotic viability to meet the clinical target dose for their respective probiotic blends. However, within 24 months, both dark chocolate formulations, despite their

generally longer shelf life compared to milk chocolate formulations, fell below the target probiotic threshold, indicating a decline in probiotic viability over extended storage.

Throughout the storage period, the pH values across all formulations remained relatively stable, indicating good chemical and microbial stability. Specifically, the pH was maintained at 5.28 ± 0.08 in the dark chocolate formulation DC2 + AB-KOLICARE, and at 6.02 ± 0.07 in milk chocolate formulation MC2 + AB-KOLICARE. Similarly, formulations containing the I3.1 probiotic blend showed consistent pH values of 5.27 ± 0.08 in DC2 and 6.06 ± 0.09 in MC2. These results suggest that both probiotic blends, when incorporated into milk and dark chocolate matrices, do not significantly alter the acidity of the product over time.

Water activity showed a slight increase during storage in the sealed monodose plastic-foil packaging across all samples; however, the values remained well within the acceptable range for shelf-stable chocolate products. The initial values were 0.343 ± 0.031 in DC2 + AB-KOLICARE, 0.373 ± 0.067 in MC2 + AB-KOLICARE, 0.327 ± 0.041 in DC2 + I3.1, and 0.328 ± 0.053 in MC2 + I3.1. At the end of storage, the respective aw was 0.459 ± 0.029 in DC2 + AB-KOLICARE, 0.399 ± 0.038 in MC2 + AB-KOLICARE, 0.444 ± 0.047 in DC2 + I3.1, and 0.404 ± 0.061 in MC2 + I3.1. This phenomenon can be explained by well-documented moisture sorption and migration phenomena in chocolate matrices. Although chocolate is a low moisture system, it is not completely inert to environmental humidity. Water vapour can progressively diffuse into chocolate during storage, particularly through the amorphous regions of sugar, milk solids, and cocoa particles, leading to a gradual increase in aw even when total moisture uptake is minimal (Ghosh et al., 2005). Chocolate is a heterogeneous system composed of a continuous fat phase in which hydrophilic particles (sugars, cocoa solids, and milk components) are dispersed. These solid particles exhibit hygroscopic behaviour, adsorbing small amounts of water at their surfaces and interfaces. Sorption isotherm studies have shown that cocoa powder and sugar particles can adsorb moisture at low aw values, contributing to measurable increases in aw without compromising shelf stability. This phenomenon explains why aw can rise during storage (Ghosh et al., 2005; Labuza & Altunakar, 2020).

Similar probiotic chocolate studies have reported a slight increase in aw during storage while observing no detrimental effects on physicochemical stability or microbial safety, supporting the interpretation that the aw increase reflects physical water redistribution rather than product degradation (Barro et al., 2024; Islam et al., 2022).

The results of Test 3 are summarized in Figure 20. The initial concentration of viable probiotic cells was approximately 8.91 ± 0.05 log CFU/g, which gradually declined to 7.56 ± 0.05 log CFU/g after 15 months of storage at room temperature, corresponding to a reduction of $\Delta \log \text{CFU/g} = -1.35$. Notably, the final count was more than one log unit below the clinical target concentration ($\log \text{CFU/g} = -1.20$), which may be attributed to suboptimal storage conditions affecting probiotic viability.

Throughout the storage period, the pH remained stable at approximately 6.54 ± 0.06 , indicating minimal biochemical changes in the matrix. In contrast, a_w increased from an initial value of 0.198 ± 0.02 to 0.415 ± 0.07 by month 15, a higher increase compared to the observed in Test 1 and Test 2, which could have contributed to the observed higher decline in probiotic viability. This rise in a_w can be plausibly attributed to differences in the packaging conditions applied across tests. In this third experiment, the chocolate was stored as small chunks in a reusable polypropylene container, which is not fully hermetic and typically exhibits higher water vapour permeability. Such conditions may facilitate moisture exchange between the product and the surrounding environment, leading to a gradual increase in a_w during storage.

Overall, the different formulations demonstrated favourable stability in terms of microbial viability physicochemical properties, and sensory appearance.

The observed pH stability along all the formulations suggests minimal acidification or degradation and is typical for chocolate products, which possess low moisture and buffering capacity that help maintain pH over time (Pierezan et al., 2023).

Although a gradual increase in a_w was observed, the values remained below the critical threshold for microbial growth ($a_w < 0.6$), which is typical for shelf-stable chocolate products, further supporting the observed probiotic viability (Islam et al., 2022). Notably, the generally higher initial a_w observed in milk chocolate formulations, compared to dark chocolate, did not appear to compromise probiotic viability, likely due to the greater fat content and buffering capacity of milk chocolate, which offer enhanced protection to probiotic cells (Klindt-Toldam et al., 2016). Although the sensorial properties of the probiotic chocolate formulations were not assessed in this study, previous research has shown that the incorporation of probiotics does not adversely affect the organoleptic characteristics of chocolate (Silva et al., 2017; Succi et al., 2017).

Moreover, multiple studies have confirmed that chocolate serves as an excellent carrier for probiotics due to its protective matrix and extended shelf life. For instance, probiotic-

enriched semisweet chocolate stored at 25 °C maintained viable counts of *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *Lactis* BLC1 without significant reductions over 120 days (Silva et al., 2017). Similarly, *Lactobacillus acidophilus* NCFM® demonstrated high viability (~8 log CFU/g) in both milk and dark chocolate matrices for up to 180 days at 4 °C and 20 °C, with greater stability under refrigerated conditions, while *Bifidobacterium lactis* HN019 showed reduced stability, falling below the therapeutic threshold after approximately 120 days (Laličić-Petronijević et al., 2015).

Our findings further support the robustness of chocolate as a good matrix for maintaining probiotic viability during extended storage.

4.1.3.4 Popping Candy

The stability and properties of popping candies enriched with the probiotic strain *B. coagulans* Unique IS-2 were assessed according to the experimental design outlined in Figure 21.

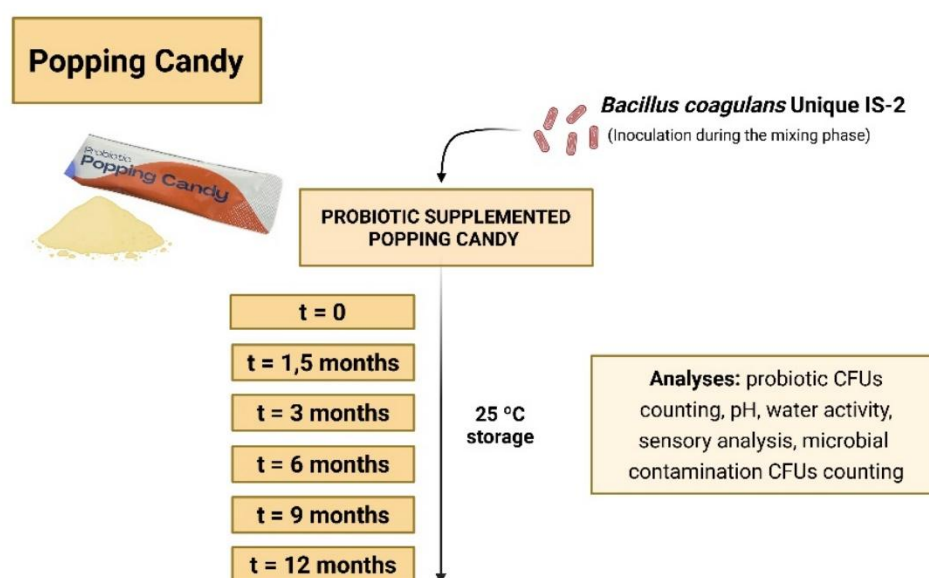


Figure 21. Schematic illustration of the experimental design. Popping candy was formulated with the probiotic strain *B. coagulans* Unique IS-2 and stored at 25 ± 5 °C. Analyses were conducted immediately after inoculation and at 1.5, 3, 6, 9, and 12 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), pH, aw, sensory attributes, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

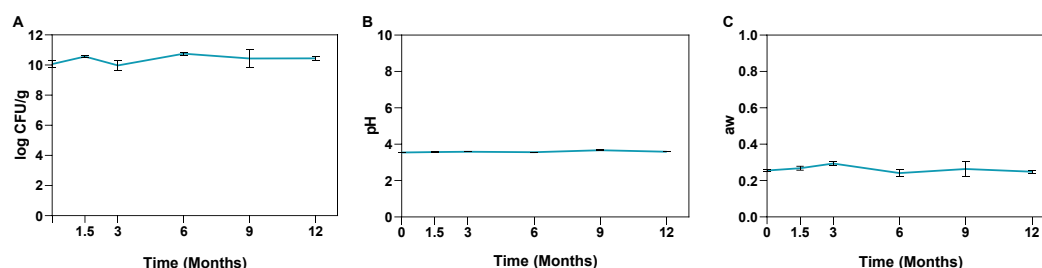


Figure 22. Probiotic viability and physicochemical characteristics of popping candy fortified with *B. coagulans* Unique IS-2 during 12 months of storage at room temperature ($25 \pm 5^\circ\text{C}$). A) Viability of *B. coagulans* spores expressed as log CFU/g. B) pH. C) aw.

The incorporation of *B. coagulans* Unique IS-2 spores into the popping candy was optimized to achieve a functional probiotic concentration in the final product, as detailed in Section 3.2.13. The lyophilized probiotic blend I3.1 was introduced along with all the other dry ingredients in the mixing tank. The target probiotic concentration for the popping candy was set at 9.00 log CFU/g per 2 g serving, which corresponds to the clinically validated daily dosage of the probiotic. A 3-fold overage was applied to counteract possible loss during the long storage, resulting in an initial inoculum of 9.48 log CFU/g.

As shown in Figure 22, the concentration of the probiotic spores in the product remained stable over the entire storage period with an average value of 10.50 ± 0.43 log CFU/g. These results demonstrate that the applied inoculum, 3-fold overage, was highly effective in ensuring a probiotic concentration significantly exceeding the therapeutic threshold throughout the entire evaluated shelf life. For instance, studies have shown that *Bacillus* spores can survive well in confectionery products such as candies. The matrix, characterized by very low moisture content, is known to provide an excellent protective environment for probiotic spores, effectively immobilizing cells and reducing metabolic activity, thereby enhancing long-term survival. For example, Adibpour et al. (2019) incorporated *B. coagulans* and *B. subtilis* spores in a probiotic-coated glassy rock-candy matrix (Nabat) and observed that over 90.39 % and 89.07% of spores, respectively, remained viable through six months of ambient storage, underscoring how the low-moisture, amorphous structure effectively immobilizes cells, suppresses metabolic activity, and protects spores against environmental stresses in hard-candy application.

To the best of our knowledge, no prior studies have investigated the application of probiotics in popping candy formulations, and this exceptional stability underscores the suitability of the popping candy matrix as a protective carrier for the long-term preservation of *B. coagulans* Unique IS-2 spores.

The physicochemical parameters of the probiotic-fortified popping candy also exhibited excellent stability during storage. The pH of the product remained remarkably constant at approximately 3.59 ± 0.04 throughout the 12-month storage period.

This inherent acidic pH contributes significantly to the overall microbiological stability of the product, inhibiting the growth of potential spoilage microorganisms and pathogens. Similarly, the a_w was maintained at a consistently low and stable level, fluctuating approximately between 0.262 ± 0.018 over the entire storage duration. Popping candy is a very low-moisture confection, with reported a_w values typically in the range of 0.20–0.30. At these levels, well below the 0.60 threshold for bacterial growth, microbial proliferation is effectively inhibited, which helps explain both its long shelf life and characteristic crisp “popping” texture (McGill & Hartel, 2020).

The stability, of both pH and a_w values, reinforces the popping candy's suitability as a robust and safe delivery system for probiotics.

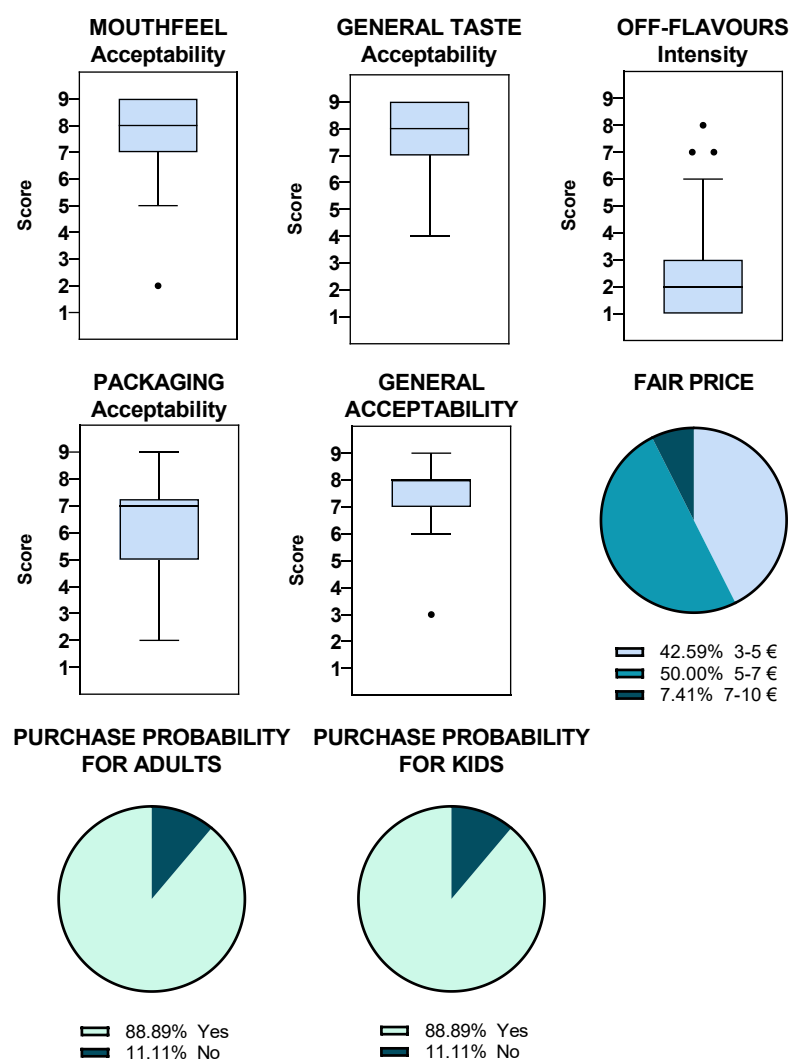


Figure 23. Sensory analysis results of popping candy containing the probiotic strain *B. coagulans* Unique IS-2. The boxplots illustrate the scores for sensory attributes, while the pie charts illustrate the scores about the purchase intent and acceptable price range, as evaluated by 54 untrained panellists.

To assess consumer acceptance, a sensory evaluation was conducted one month after sample production by a panel of 54 untrained panellists (63% female, 37% male, 3.9% other; 19–65 years) and is summarized in Figure 23. As no control samples were included, panellists were informed that they were evaluating popping candies enriched with probiotics. Consequently, their assessments focused on intrinsic product characteristics rather than on comparisons with non-enriched counterparts.

The overall consumer acceptability of the popping candy was notably high, evidenced by a mean score of 7.67 ± 1.06 for 'General Acceptability' on a 9-point hedonic scale. This high score is a critical indicator of the product's market potential, as overall sensory appeal is paramount for the successful adoption of functional food products by consumers.

Key positive attributes contributing significantly to this high overall acceptability included 'Mouthfeel', which received a mean score of 7.72 ± 1.38 , and 'General taste', with a mean score of 7.70 ± 1.22 , suggesting a well-balanced and desirable flavour profile. Crucially, the attribute “off-flavours” registered a very low mean score of 2.63 ± 1.80 , indicating that the incorporation of *B. coagulans* Unique IS-2 spores did not introduce perceptible off-flavours, which is often a significant challenge in the development of probiotic-fortified foods.

Regarding packaging, 'Packaging Acceptability' received a mean score of 6.33 ± 1.61 , and 'Packaging Information' a mean of 6.44 ± 2.30 . While these scores are slightly lower than those for the intrinsic product attributes, they still indicate a reasonable level of acceptance for the packaging design and clarity of information provided.

Panellist’s feedback indicated that the primary reason for the lower score was that the packaging appeared overly formal and serious, particularly for a children target.

Consumer purchase intent further substantiated the strong sensory performance. A substantial majority of panellists, specifically 88.89%, indicated an affirmative 'Yes' for 'Personal Purchase Probability', with an identical percentage observed for 'Purchase Probability for Kids'. This high purchase probabilities underscore the widespread appeal of the probiotic popping candy across different consumer segments and are in line with a clear growing trend toward the consumption of functional foods also targeting specific category like children (Votsi & Koutelidakis, 2024).

Furthermore, the perceived 'Fair Price' analysis revealed that the majority of panellists (92.59%) were willing to pay between 3-7 € per pack (42.59% for 3-5€; 50.00% for 5-7€), suggesting that consumers perceive a good value proposition for the product within the confectionery market.

Overall, these data demonstrate that popping candy can effectively deliver *B. coagulans* Unique IS-2 spores without compromising key sensory qualities. The high scores for sensory parameter, combined with strong purchase intent and clear price expectations, support the commercial feasibility of this “funny” novel functional confectionery format to deliver probiotics.

4.1.3.5 Gummies

The stability and properties of two gummy formulations enriched with the probiotic strain *B. coagulans* Unique IS-2, were assessed according to the experimental design outlined in Figure 24.

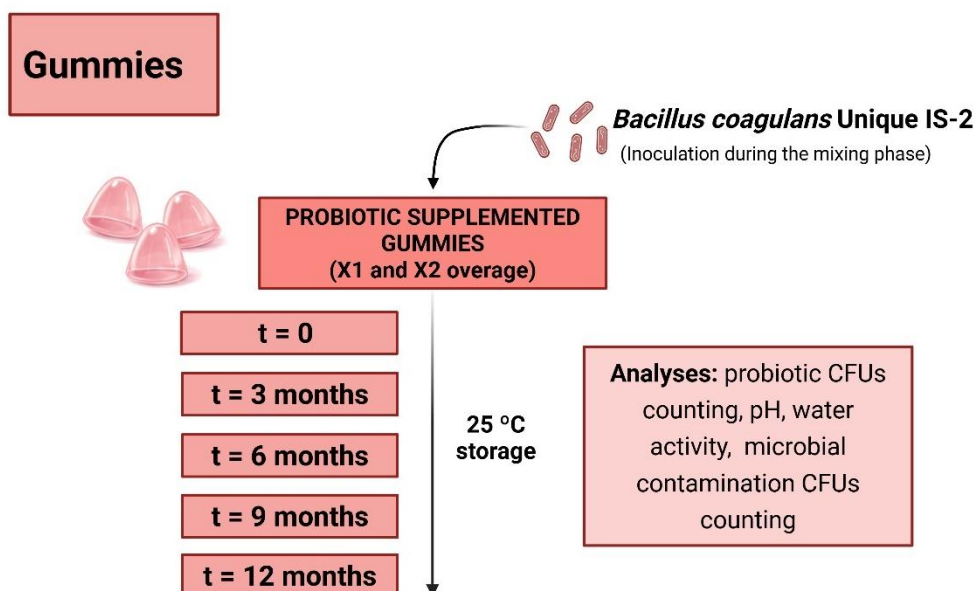


Figure 24. Schematic illustration of the experimental design. Two gummy formulations containing different overages of *B. coagulans* Unique IS-2 were prepared and stored at $25 \pm 5^\circ\text{C}$ for 12 months. Analyses were performed immediately after inoculation and at 3, 6, 9, and 12 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), pH, aw, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

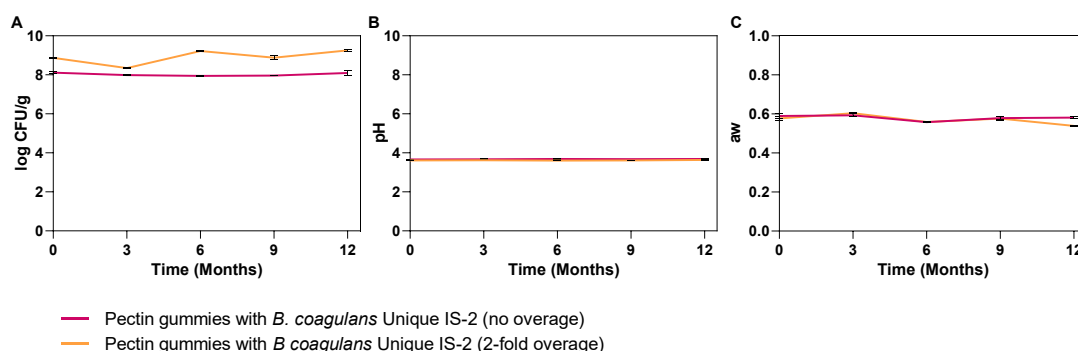


Figure 25. Probiotic viability and physicochemical characteristics of gummy fortified with *B. coagulans* Unique IS-2 during 12 months of storage at room temperature ($25 \pm 5^\circ\text{C}$). A) Viability of *B. coagulans* spores expressed as log CFU/g. B) pH. C) aw.

The incorporation strategy for *B. coagulans* Unique IS-2 spores into the gummy matrix was designed to ensure a functional probiotic concentration in the final product as detailed in Section 3.2.14 (Materials and Methods). The target probiotic load for the gummy was set at 8.82 log CFU/g per 3 g serving, aligning with the clinically validated daily dosage for this

strain. Two batches were produced: one inoculated at the target dose and another with a 2-fold overage (initial load of $9.12 \log \text{ CFU/g}$), to account for potential losses during processing and storage.

As shown in Figure 25, both formulations demonstrated exceptional probiotic viability over a 12-month storage period. The average viable count remained at $8.03 \pm 0.09 \log \text{ CFU/g}$ for the standard formulation and $9.11 \pm 0.22 \log \text{ CFU/g}$ for the 2-fold overage batch. Neither batch exhibited a statistically significant decline in viable cells over time, confirming that the pectin-based gummy matrix serves as a highly protective vehicle for probiotic delivery. Notably, the 2-fold overage proved sufficient, maintaining levels above the minimum therapeutic threshold throughout storage.

The pH values also remained stable, averaging 3.68 ± 0.01 for the standard formulation and 3.62 ± 0.01 for the 2-fold overage version. This stability indicates minimal probiotic metabolic activity and no significant acidification or chemical degradation, which is critical for maintaining product integrity and sensory quality during shelf life.

Likewise, the a_w of the gummies was consistently low and stable, with values of 0.580 ± 0.008 and 0.571 ± 0.004 for the standard and 2-fold overage versions, respectively. This low a_w is a key factor in preserving both microbial viability and physical properties, as it minimizes water-dependent degradation pathways and inhibits the growth of spoilage organisms (Subramaniam, 2016).

The stable physicochemical environment, particularly the combination of low a_w and acidic pH, plays a central role in maintaining high probiotic counts without requiring excessive initial overage. The robust survival of *B. coagulans* Unique IS-2 spores supports the application of this formulation in consumer-ready, shelf-stable probiotic supplements.

This aligns with previous studies highlighting the suitability of gummy and jelly-like matrices for probiotic delivery, especially for spore-forming strains. For example, Kahraman et al. (2023) showed that probiotic jelly candies made with *Bacillus* strains remained stable and effective for 90 days, with high survival rates, unchanged texture and taste, enhanced antioxidant activity, and strong consumer acceptance. Similarly, Miranda et al. (2020) reported that juçara and passion fruit jelly candies enriched with *B. coagulans* GBI-30 6086 stayed microbiologically safe and stable for 90 days, maintaining probiotic counts above $6.4 \log \text{ CFU/g}$ and strong survival under simulated GI conditions. Additionally, Perotti et al., (2024) demonstrated that gummy candies are effective carriers for *Heyndrickxia coagulans*

SNZ1969, with spores successfully germinating in the intestines of all 24 healthy adult volunteers and persisting in the gut for at least two weeks after consumption ceased.

Taken together, these findings confirm that pectin-based gummy formulations are viable and effective carriers for long-term probiotic delivery.

4.1.4 Snack Products

4.1.4.1 Cereal Bar

The stability and properties of probiotic cereal bars enriched with the probiotic strain *B. coagulans* Unique IS-2, were assessed according to the experimental design outlined in Figure 26.

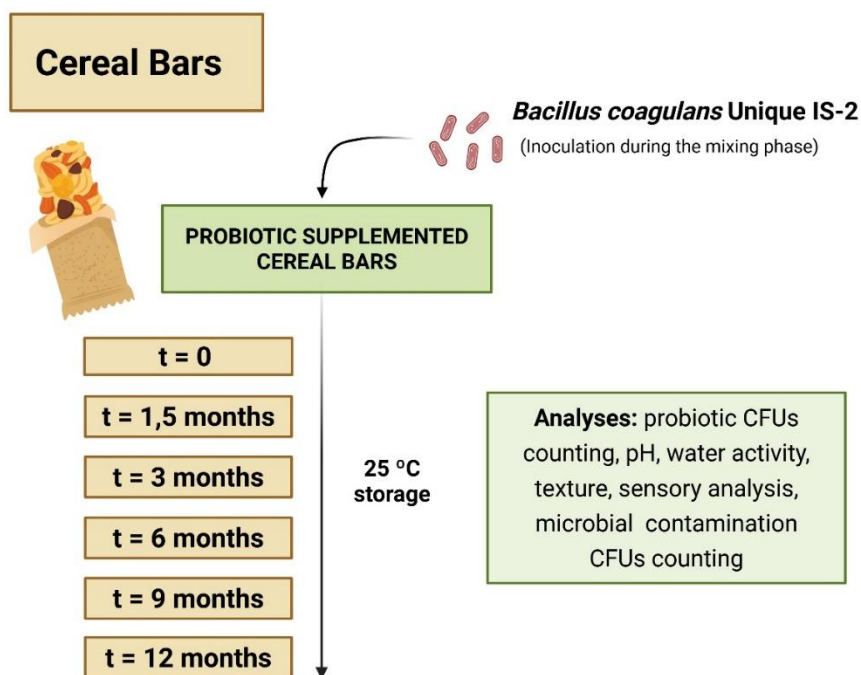


Figure 26. Schematic illustration of the experimental design. Cereal bars were formulated with the probiotic strain *B. coagulans* Unique IS-2 and stored at 25 ± 5 °C for 12 months. Analyses were conducted immediately after sample preparation and at 1.5, 3, 6, 9, and 12 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), pH, aw, texture, sensory attributes, and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

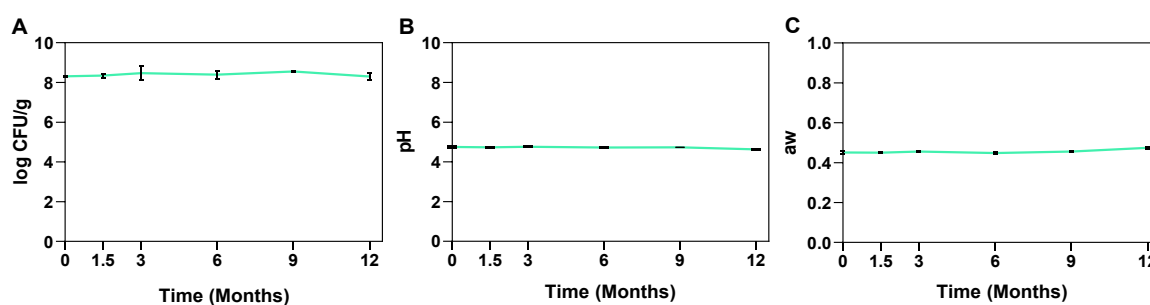


Figure 27. Probiotic viability and physicochemical characteristics of cereal bar fortified with *B. coagulans* Unique IS-2 during 12 months of storage at room temperature (25 ± 5 °C). A) Viability of *B. coagulans* spores expressed as log CFU/g. B) pH. C) aw.

The incorporation of *B. coagulans* Unique IS-2 spores into the cereal bars was designed to ensure the delivery of a functional probiotic concentration in the final baked product as detailed in Section 3.2.8 (Materials and Methods). The spores were introduced into the bar mix prior to syrup incorporation. The target probiotic concentration was established at 7.90 log CFU/g per 25 g serving, corresponding to the clinically validated daily dosage of the strain. To effectively counteract a possible viability reduction during processing and storage, a 2-fold overage was applied, aiming for an initial inoculum of 8.20 log CFU/g.

As depicted in Figure 27, the probiotic demonstrated remarkable stability over the 12-month storage period with an average value of 8.39 ± 0.10 log CFU/g. This result confirms the high survival rate of the spores, demonstrating that both the matrix and processing conditions effectively preserved their viability. The absence of significant viability loss over time suggests that the low-moisture bars environment provided protective conditions, aligning with findings from similar food systems (Payne et al., 2024). For example, de Paula Antunes et al. (2025) showed that *Bacillus clausii* spores were able to maintain CFUs count higher than 6.5 log CFU/g over 60 days of storage in cereal bars.

Throughout 12 months of ambient storage, the cereal bars showed high physicochemical stability. The pH of the probiotic formulations remained constant (4.72 ± 0.05), indicating that the addition of *B. coagulans* spores did not alter the acid–base balance of the matrix, consistent with their metabolic inactivity prior to germination. Water activity also remained stable (0.456 ± 0.009), well below the 0.6 threshold associated with microbial spoilage in low-moisture products (Allai et al., 2022).

Comparable behaviour has been reported for other spore-forming probiotic cereal bars: Vasilean et al. (2022) observed that *Bacillus subtilis*–enriched cereal bars maintained aw

values between 0.42 and 0.47, which ensured microbiological stability while maintaining probiotic viability throughout storage.

Table 26. Texture analysis of cereal bars with or without *B. coagulans* Unique IS-2 throughout the storage period.

Time (Months)	Sample	Compression Force $\pm$ SD (N)	Adhesivity Work $\pm$ SD (N·mm)
T0	Control	95.22 $\pm$ 13.11	38.14 $\pm$ 8.97
	Inoculated	115.30 $\pm$ 17.89	47.42 $\pm$ 12.44
T1.5	Control	150.08 $\pm$ 35.37	63.95 $\pm$ 9.17
	Inoculated	174.84 $\pm$ 31.04	55.49 $\pm$ 19.23
T3	Control	147.19 $\pm$ 13.54	75.20 $\pm$ 2.32
	Inoculated	137.9 $\pm$ 14.75	53.43 $\pm$ 18.28
T6	Control	169.85 $\pm$ 17.49	70.40 $\pm$ 23.67
	Inoculated	126.07 $\pm$ 14.90	58.14 $\pm$ 24.81
T9	Control	133.26 $\pm$ 28.19	47.44 $\pm$ 7.63
	Inoculated	158.04 $\pm$ 10.76	53.30 $\pm$ 24.9
T12	Control	138.97 $\pm$ 21.97	68.56 $\pm$ 6.14
	Inoculated	144.17 $\pm$ 29.84	64.46 $\pm$ 28.09
No statistically significant differences were observed ($p > 0.05$) between Control and Inoculated samples at any time point (Student's t-test), nor over time within each group (One-way ANOVA).			

Texture parameters of the cereal bar formulations, control and inoculated with *B. coagulans* Unique IS-2 spores, were monitored over a 12-month storage period. The analysis focused on two mechanical properties: compression force and adhesivity work, as summarized in Table 26. While both formulations maintained acceptable texture profiles throughout storage, some fluctuations were observed across time points.

The fluctuations are likely attributable to the heterogeneous composition of the bar matrix, which included large-particle ingredients such as seeds, cranberries, and other inclusions. The granulometry and distribution of these components can significantly influence mechanical resistance and adhesion during texture analysis. Variability in ingredient placement and moisture migration over time may also contribute to inconsistencies in force measurements. Although the absolute texture values cannot be directly compared with those reported by de Paula Antunes et al. (2025), due to differences in formulation and in the specific texture parameters assessed, both studies show a pattern of stability along storage.

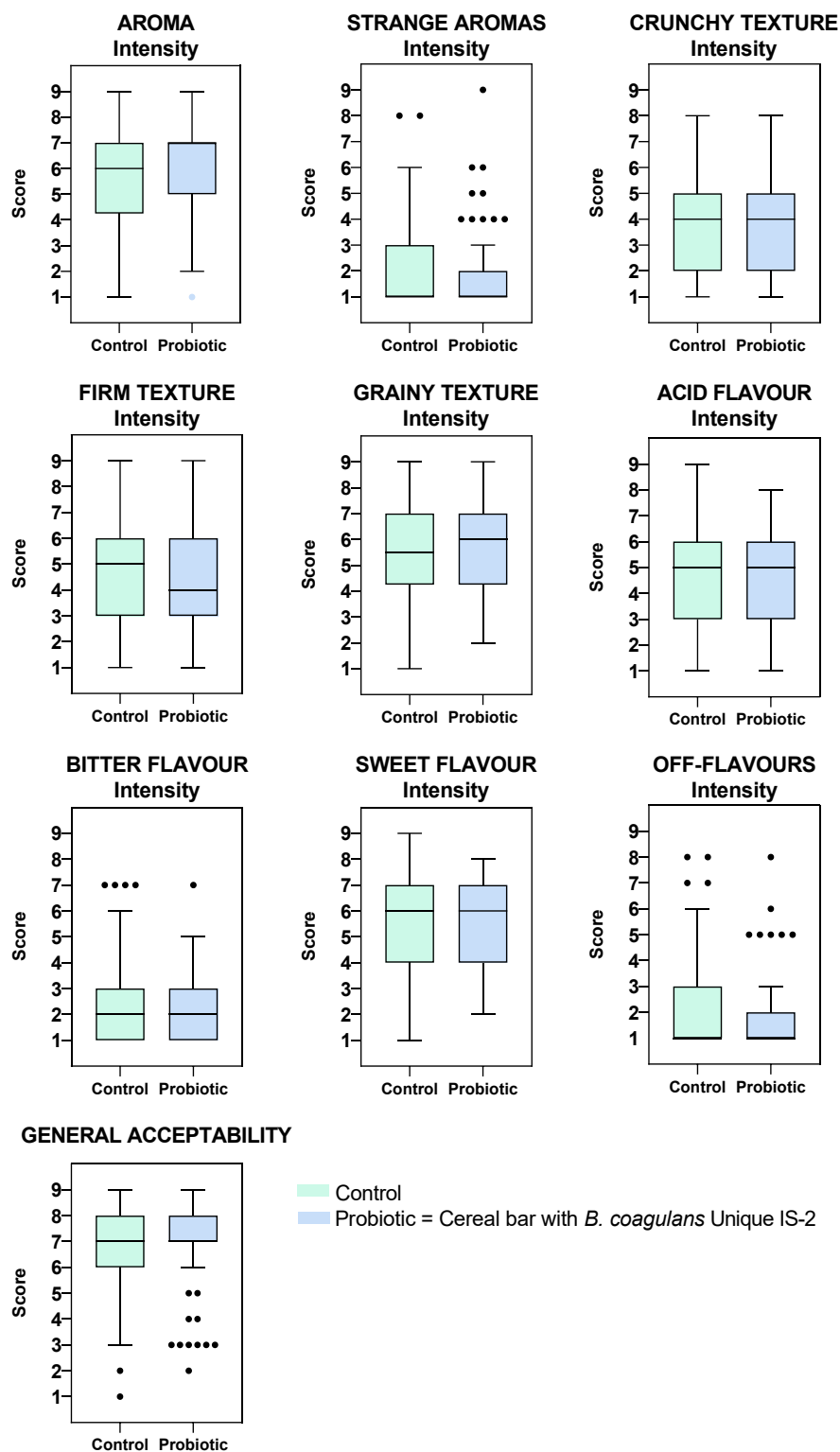


Figure 28. Sensory analysis results of cereal bar formulations without (Control) and with the probiotic strain *B. coagulans* Unique IS-2 (Probiotic). Box plots illustrate the sensory attribute scores for control bars (green) and probiotic-enriched bars (light blue), as evaluated by N=64 untrained panellists. Scores represent intensity or acceptability on a 0-9 scale.

To evaluate consumer acceptance, a sensory analysis was conducted one day after sample production with a panel of 64 untrained panellists (62.5% female, 37.5% male; 18–65 years).

The outcomes, summarised in Figure 28, revealed no statistically significant differences across any of the assessed sensory attributes ($p > 0.05$).

The probiotic bars scored slightly higher in aroma intensity (6.17 ± 1.92 vs. 5.61 ± 1.79 in the control), suggesting that the addition of *B. coagulans* spores may have enhanced the olfactory profile, though the difference was not statistically significant. Importantly, strange aromas and off-flavours were reported with low intensity in the probiotic version (1.95 ± 1.61 and 1.83 ± 1.52 , respectively), which also showed fewer outliers and greater consistency in panellists' responses. In contrast, the control samples scored 2.14 ± 1.82 and 2.31 ± 1.93 , respectively, indicating that the incorporation of the probiotic did not introduce off-notes or other undesirable aromatic features.

The perception of acidic (4.47 ± 2.17 in the probiotic version and 4.52 ± 1.87 flavours remained), bitter (2.16 ± 1.43 in the probiotic version and 2.52 ± 1.82 in the control), and sweet (5.59 ± 1.77 in the probiotic version and 5.59 ± 1.80 in the control) flavours remained relatively unchanged between the control and probiotic bars, with overlapping interquartile ranges and medians, indicating that the addition of *B. coagulans* spores did not significantly impact these key taste attributes.

In terms of texture, no notable differences were observed between the two groups in crunchiness (3.89 ± 1.96 in the probiotic version and 3.86 ± 1.93 in the control), firmness (4.48 ± 1.94 in the probiotic version and 4.73 ± 1.99 in the control), or granularity (5.66 ± 1.62 in the probiotic version and 5.63 ± 1.79 in the control). Both formulations maintained consistent textural properties, suggesting that the integration of *B. coagulans* spores did not interfere with ingredient binding or matrix structure during bar formation.

One of the most relevant outcomes is reflected in the general acceptability scores, which were slightly but not significantly higher in the probiotic bars (6.98 ± 1.86 in the probiotic version and 6.75 ± 1.94 in the control). The distribution of responses also showed fewer low scores and less variability in the probiotic group, suggesting that the functional version was at least as well accepted, if not more, than the control.

Sensory evaluation confirmed that *B. coagulans* Unique IS-2 spores can be effectively incorporated into shelf-stable cereal bars without compromising sensory attributes. These results are consistent with existing literature indicating that probiotics can be added to cereal bars with minimal sensory impact (Bampi et al., 2016; de Paula Antunes et al., 2025; Pereira et al., 2019).

In addition to sensory compatibility, the formulation delivers a clinically validated dose of *B. coagulans* Unique IS-2, while preserving product quality over 12 months of storage, highlighting the feasibility of using spore-forming probiotics in shelf-stable, non-refrigerated functional foods.

4.1.5 Bakery Products

4.1.5.1 Butter Cookies

Three separate experiments were conducted to evaluate the stability and functional properties of probiotic cookies formulations enriched with the probiotic strain *B. coagulans* Unique IS-2. The tests followed the experimental design outlined in Figure 29.

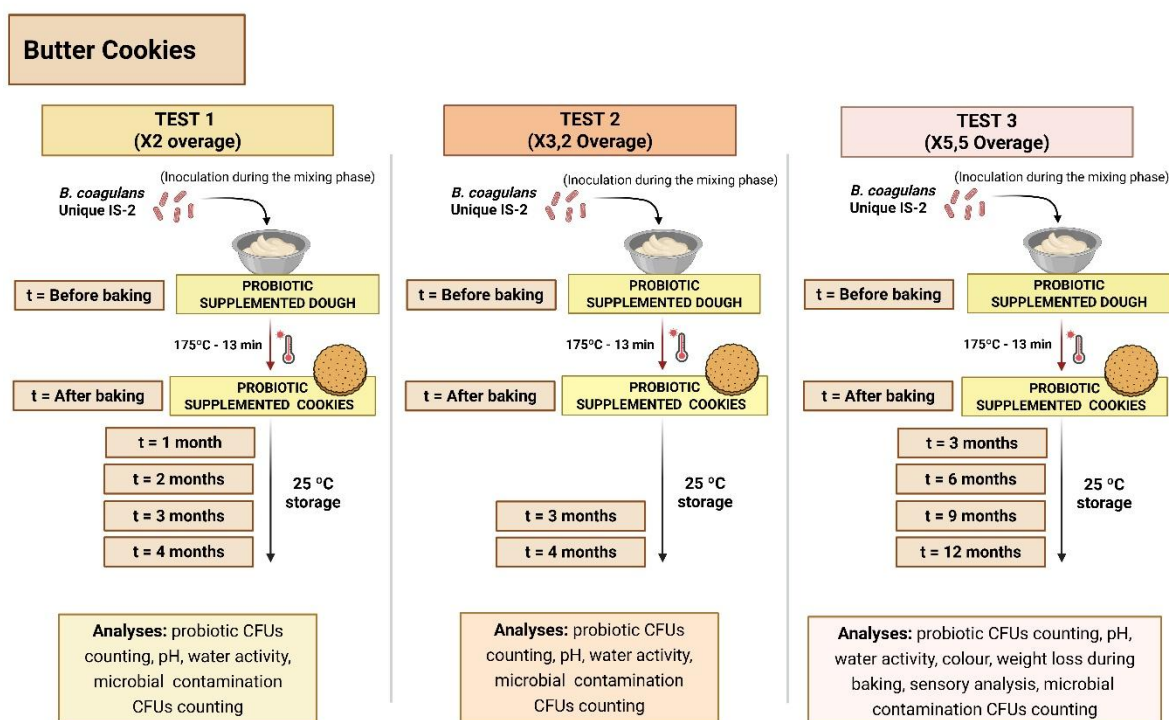


Figure 29. Schematic illustration of the experimental design. Three butter cookie trials were formulated with increasing overage levels of the clinical dose of *B. coagulans* Unique IS-2 spores (2×10^9 CFU), incorporated during the mixing phase: Test 1 (2-fold overage), Test 2 (3.2-fold overage), and Test 3 (5.5-fold overage). Dough samples were collected both before and after baking (175 °C for 13 min), and the baked cookies were subsequently stored in airtight plastic containers at 25 ± 5 °C. Analyses were performed at predefined intervals: in Test 1 before and after baking and at 1, 2, 3, and 4 months; in Test 2 before and after baking and at 3 and 4 months; and in Test 3 before and after baking and at 3, 6, 9, and 12 months. Viable probiotic spore counts (CFU) were assessed both pre- and post-baking and throughout storage, whereas all additional parameters were evaluated only after the baking step and monitored during storage. Evaluated parameters included probiotic viability (CFU), pH, aw, colour (Test 3), and microbiological contamination (at the beginning and at the end of storage). Additionally, weight loss during baking was calculated, and sensory analysis was carried out on freshly produced samples (Test 3 formulation). The figure was created in <https://BioRender.com>.

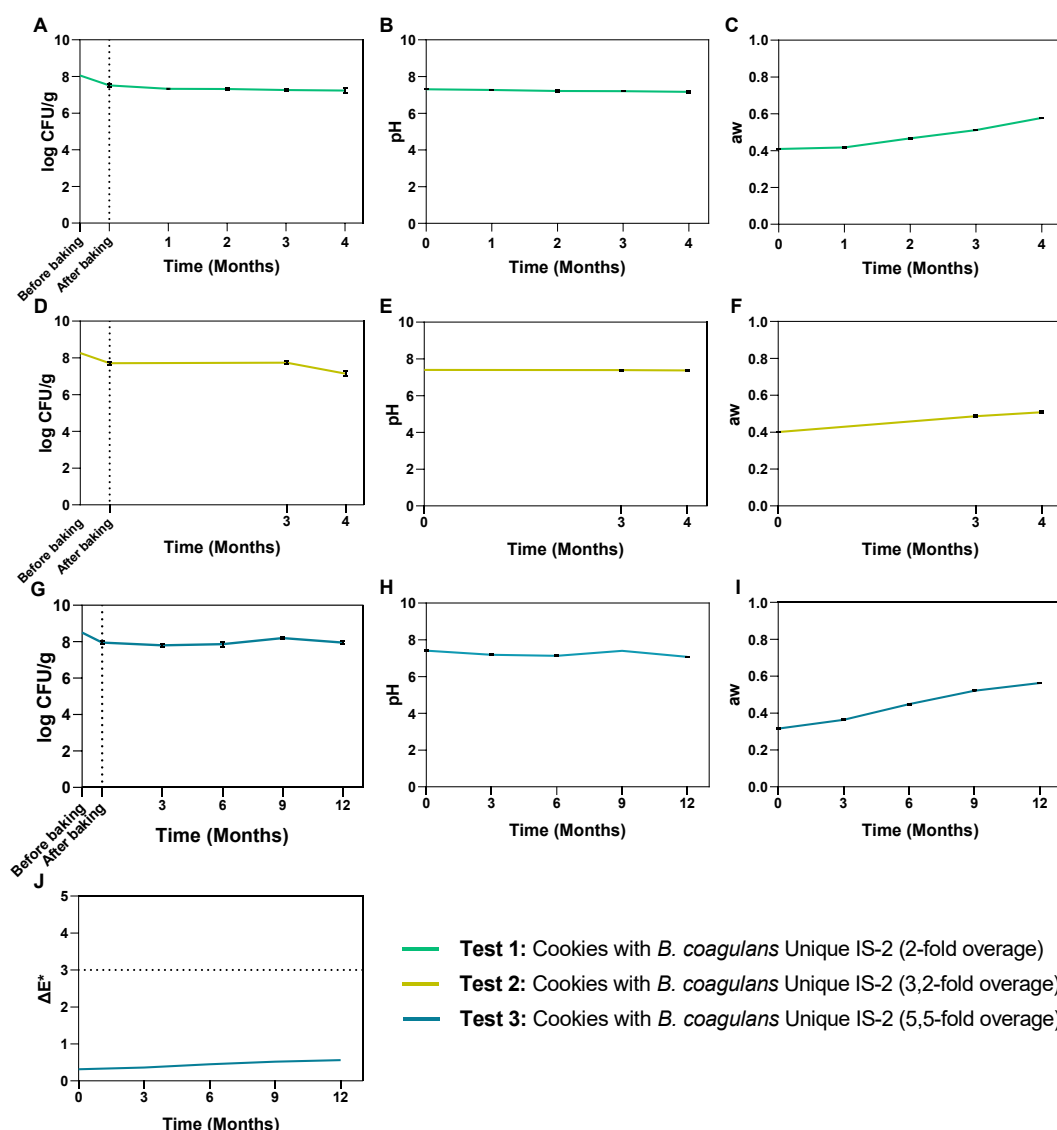


Figure 30. Probiotic viability and physicochemical characteristics of butter cookies formulations, fortified with *B. coagulans* Unique IS-2 spores over 4 months-storage (Test 1 and Test 2) or 12 months-storage (Test 3) at room temperature (25 ± 5 °C). A-D-G) Viability of *B. coagulans* spores expressed as log CFU/g. B-E-H) pH. C-F-I) aw. J) Colour variation expressed as ΔE^* (only Test 3).

The incorporation strategy for *B. coagulans* Unique IS-2 spores into the butter cookie dough was developed, as detailed in Section 3.2.9 (Materials and Methods), to ensure a viable probiotic concentration in the final baked product.

Three tests were conducted using different initial concentrations of the probiotic strain. In all cases, the target was to achieve a final probiotic level of 7.76 log CFU/g per 35 g serving, corresponding to the clinically validated daily dose of *B. coagulans* Unique IS-2. To compensate for potential viability loss during storage, different overages were applied: a 2-fold overage in Test 1, a 3.2-fold overage in Test 2, and a 5.5-fold overage in Test 3. These

adjustments resulted in initial inoculum levels of approximately 8.06 CFU/g, 8.26 CFU/g, 8.50 CFU/g, respectively.

As shown in Figure 30 (panels A, D, G), the survival of *B. coagulans* Unique IS-2 spores in butter cookies was evaluated before and after the baking process and subsequent storage. During baking, a significant average logarithmic reduction of 0.55 ± 0.01 log CFU/g was observed across the three different probiotic overage formulations (Test 1: X2, Test 2: X3.2, Test 3: X5.5). Post-baking, the initial probiotic concentrations in the cookies were determined to be 7.51 ± 0.12 log CFU/g for Test 1, 7.71 ± 0.09 log CFU/g for Test 2, and 7.96 ± 0.10 log CFU/g for Test 3. During the storage period the probiotic spores maintained good viability. The *B. coagulans* CFU counts remained highly stable throughout the entire storage duration of up to 4 and 12 months, with minimal reduction observed across all tested overage levels. Specifically, at the end of the storage period, the probiotic concentrations were maintained at 7.23 ± 0.16 log CFU/g for Test 1, 7.14 ± 0.14 log CFU/g for Test 2, and 7.95 ± 0.09 log CFU/g for Test 3. Despite the effects of baking and subsequent decline during storage, the viable counts in Test 3, formulated with a 5.5-fold overage, remained above the target level of 7.76 log CFU/g per 35 g serving. This confirms that the applied overage is sufficient to deliver the intended probiotic dose through baked goods.

The superior heat resistance observed in butter cookies, compared with the larger reductions typically reported in other baked goods like bread, can be attributed to the unique characteristics of the matrix, such as its lower moisture and higher fat content (Payne et al., 2025). Also, factors such as lower initial aw, use of chemical leavening, smaller product size, and potentially faster drying during baking likely contribute to better protection of the *B. coagulans* spores during thermal processing.

Key physicochemical parameters (Figure 30, panels B, E, H and C, F, I) of the cookies remained stable throughout the 4 and 12-month storage periods at room temperature, across all tested probiotic overage levels, especially the pH, that remained within a range of 7.08–7.41 in all formulations. This sustained pH stability is a critical indicator of the product physicochemical integrity. The average of initial aw values across all tests was 0.375 ± 0.051 . Over the course of storage, a gradual increase in aw was observed. Specifically, Test 1 showed an increase of 0.169 units, Test 2 increased by 0.108 units, and Test 3 by 0.247 units, and reached values of 0.578 ± 0.002 (Test 1), 0.508 ± 0.005 (Test 2), and 0.563 ± 0.002 (Test 3), by the end of the monitoring periods. Despite these increases, all

values remained below 0.6, the critical threshold for microbial spoilage in low-moisture baked goods.

Furthermore, the colour of the cookies (Figure 30-J) exhibited excellent stability throughout the 12-month storage period. The total colour difference (ΔE^*), relative to the initial time point (T0), ranged between 0.270 and 0.977, well below the commonly accepted threshold of 3 for visually perceptible changes (Mokrzycki & Tatol, 2011; Pathare et al., 2013). This indicates that neither the incorporation of the probiotic nor the storage conditions led to noticeable discoloration or oxidative degradation.

During processing, an average weight loss of 13.38% was observed as a result of the baking step. This moisture reduction contributed to the decrease in a_w and the development of the desired crunchy texture typical of low-moisture baked goods (Budžaki et al., 2014). Importantly, these results indicate that the incorporation of *B. coagulans* Unique IS-2 spores did not adversely affect the key physicochemical properties that determine the safety, shelf-life stability, and overall quality of the butter cookies.

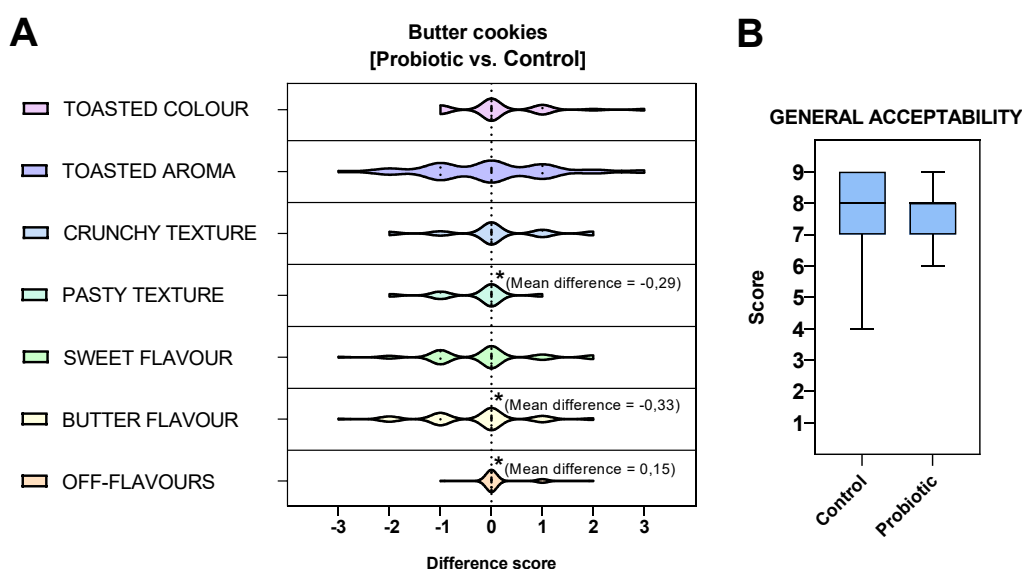


Figure 31. Sensory analysis results of butter cookies (Test 3 formulation) without (Control) and with the probiotic strain *B. coagulans* Unique IS-2 (Probiotic). A) Violin plot represents the distribution of scores reflecting the perceived differences for various sensory attributes. B) Boxplot summarizes general acceptability scores, as rated by 55 untrained panellists. (Significance: * $p < 0.05$).

To assess consumer acceptance, a sensory analysis was performed one day after sample production, by a group of 55 untrained panellists (81.8% female, 18.2% male; 18–65 years), and is summarized in Figure 31.

The results demonstrated high consumer acceptance for the butter cookies fortified with *B. coagulans* Unique IS-2, with minimal differences between probiotic and control cookies in most sensory attributes. Only minor statistically significant differences were observed in pasty texture (mean difference = -0.29), butter flavour (mean difference = -0.33), and off-flavours (mean difference = 0.15). However, these deviations remained within a negligible $\Delta < \pm 1$ score, which is below the “minimum deviation from the control” threshold on the sensory scale used, ranging from -3 to $+3$. The high scores for “general acceptability”, 7.67 ± 1.44 for the control and 7.42 ± 1.51 for the probiotic version, suggest that any subtle differences in specific sensory attributes did not significantly impact the consumers’ overall perception of the product. These findings are consistent with previous studies indicating that spore-forming probiotics, such as *B. coagulans*, typically do not cause noticeable changes in sensory characteristics when incorporated into low-moisture baked matrices (Almada-Érix et al., 2022; Mani-López et al., 2023; Payne et al., 2024).

The successful integration of *B. coagulans* Unique IS-2 spores into butter cookies, characterized by minimal processing-induced viability loss, prolonged shelf-life stability, and excellent sensory acceptance, positions butter cookies as an outstanding vehicle for delivering gut health benefits in a widely consumed and convenient format.

4.1.6 Beverage Products

4.1.6.1 Orange Juice

Three separate experiments were conducted to evaluate the stability and properties of different probiotic-enriched orange juice formulations. The tests followed the experimental design outlined in Figure 32.

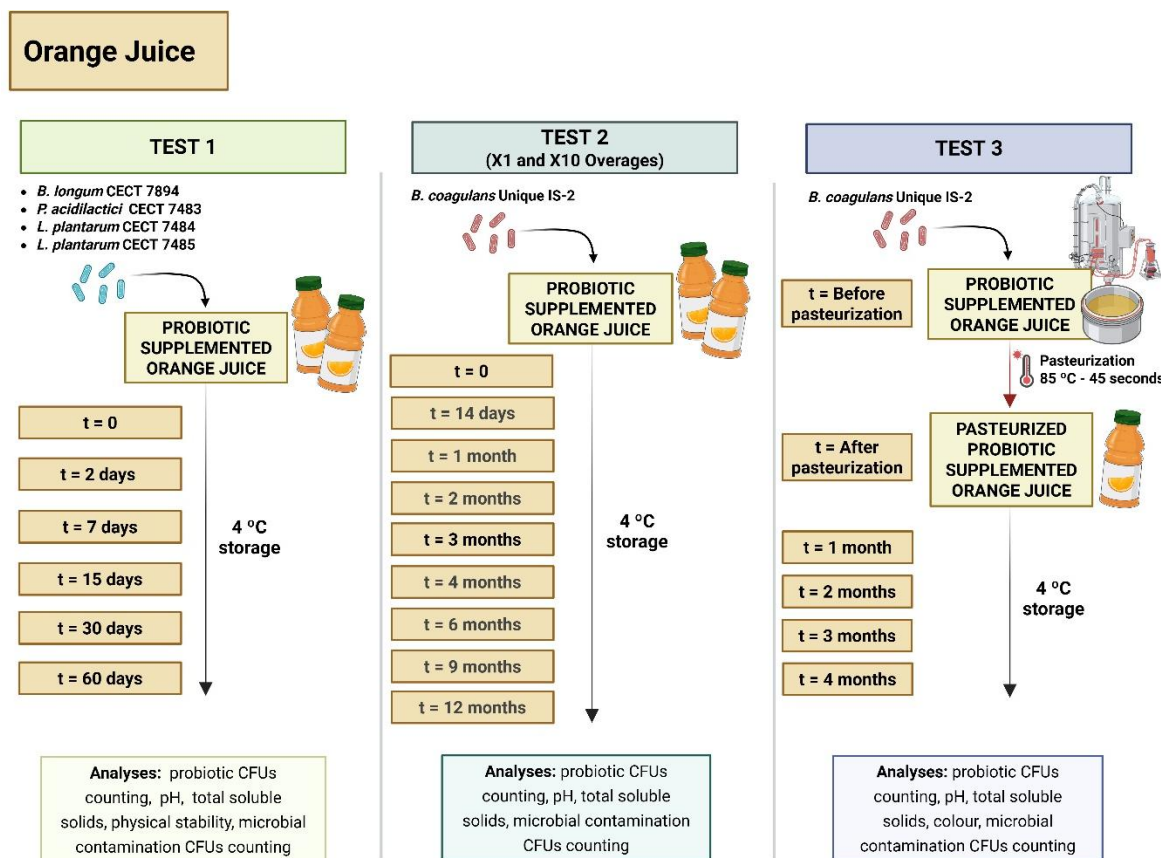


Figure 32. Schematic illustration of the experimental design. The study comprised three distinct supplementation strategies. In Test 1, orange juice was fortified with individual vegetative probiotic strains (*B. longum* CECT 7894, *P. acidilactici* CECT 7483, and *L. plantarum* CECT 7484 and CECT 7485) and samples were stored at 4 ± 2 °C and analysed at days 0, 2, 7, 15, 30, and 60 (stopped at 15 days for *B. longum* CECT 7894) at. Test 2 consisted of orange juice inoculated with *B. coagulans* Unique IS-2 spores at two inclusion levels corresponding to 1-fold and 10-fold of the clinical daily dose (2×10^9 CFU). The X10 samples were evaluated at 0 and 14 days and at 1, 2, 3, and 4 months, whereas X1 samples were assessed at 0, 3, 6, 9, and 12 months, both with storage at 4 ± 2 °C. Test 3 involved orange juice supplemented with *B. coagulans* Unique IS-2 spores followed by a post-inoculation pasteurization step (85 °C for 45 s), with analyses performed immediately before and after pasteurization and at 1, 2, 3, and 4 months at 4 ± 2 °C. Evaluated parameters included viable probiotic spore count (CFU), pH, TSS, colour (Test 3), physical stability (Test 1), and microbiological contamination (at the beginning and at the end of storage). The figure was created in <https://BioRender.com>.

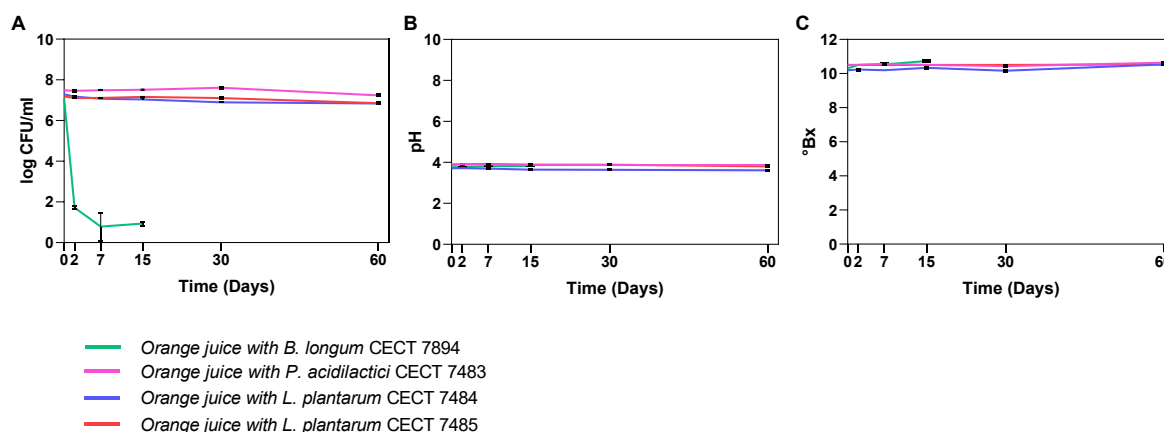


Figure 33. Test 1. Probiotic viability and physicochemical characteristics of orange juice fortified with four different probiotic strains, during 60 days (15 days for *B. longum* CECT 7894) at 4 ± 2 °C. A) Viability of probiotic expressed as log CFU/ml. B) pH. C) Total soluble solids expressed as °Brix.

The inoculation of the different probiotic strains was designed to ensure a target probiotic concentration in the final products, as detailed in Section 3.2.3 (Materials and Methods), and overages were applied to compensate for potential viability loss during extended storage.

In Test 1, *B. longum* CECT 7894, *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, *L. plantarum* CECT 7585 were respectively targeted at 6.96 log CFU/ml per 330 ml serving and a 10-fold overage was applied, resulting in an initial inoculum of 7.96 log CFU/ml.

The results of Test 1 are presented in Figure 33. Among the tested strains, *B. longum* CECT 7894 exhibited poor survival, with a sharp decline in viable counts from 7.07 ± 0.04 log CFU/ml to 1.72 ± 0.08 log CFU/ml within the first two days. By day seven, the count dropped further to 0.78 ± 0.68 log CFU/ml, prompting early termination of the experiment for this strain. This rapid loss of viability is consistent with the known sensitivity of bifidobacteria to acidic and oxygen-rich environments, as they are strictly anaerobic and acid-labile (Averina et al., 2023; Tsakalidou & Papadimitriou, 2011).

In contrast, *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, and *L. plantarum* CECT 7485 demonstrated high stability, maintaining viable counts of 7.47 ± 0.13 , 7.05 ± 0.17 , and 7.09 ± 0.12 log CFU/ml, respectively, with only slight reductions over the 60-day period. These results align with previous studies highlighting the acid tolerance and robustness of *L. plantarum* and *P. acidilactici* in non-dairy matrices (Cristiny De Oliveira Vieira et al., 2020; Nualkaekul & Charalampopoulos, 2011).

The pH values remained stable across all samples, with averages of 3.79 ± 0.03 (*B. longum*), 3.89 ± 0.02 (*P. acidilactici*), 3.67 ± 0.04 (*L. plantarum* CECT 7484), and 3.88 ± 0.04 (*L. plantarum* CECT 7485). These results suggest that probiotic supplementation did not

significantly alter the acidity of the juice and are in line with previous results (Nualkaekul & Charalampopoulos, 2011). Similarly, TSS remained stable throughout the storage period, with values of 10.5 ± 0.2 °Brix (*B. longum*), 10.5 ± 0.1 °Brix (*P. acidilactici*), 10.3 ± 0.1 °Brix (*L. plantarum* CECT 7484), and 10.5 ± 0.0 °Brix (*L. plantarum* CECT 7485). These findings indicate that the probiotic strains did not significantly metabolize sugars or alter the original profile of the juice (Wibowo et al., 2015a). Based on these results, *L. plantarum* CECT 7484 and CECT 7485, along with *P. acidilactici* CECT 7483, emerge as promising candidates for probiotic fortification in orange juice, due to their high viability, acid tolerance, and negligible impact on juice quality. This observation is consistent with the work of Kardooni et al. (2023), who demonstrated that *Lactobacillus acidophilus* B103 retained high viability and did not significantly affect the pH of non-fermented orange juice during 21 days of refrigerated storage, underscoring the compatibility of certain probiotic strains with fruit juice matrices. Conversely, *B. longum* CECT 7894, despite its recognized health benefits, may require encapsulation or formulation adjustments to enhance its survival in acidic and oxygenated environments.

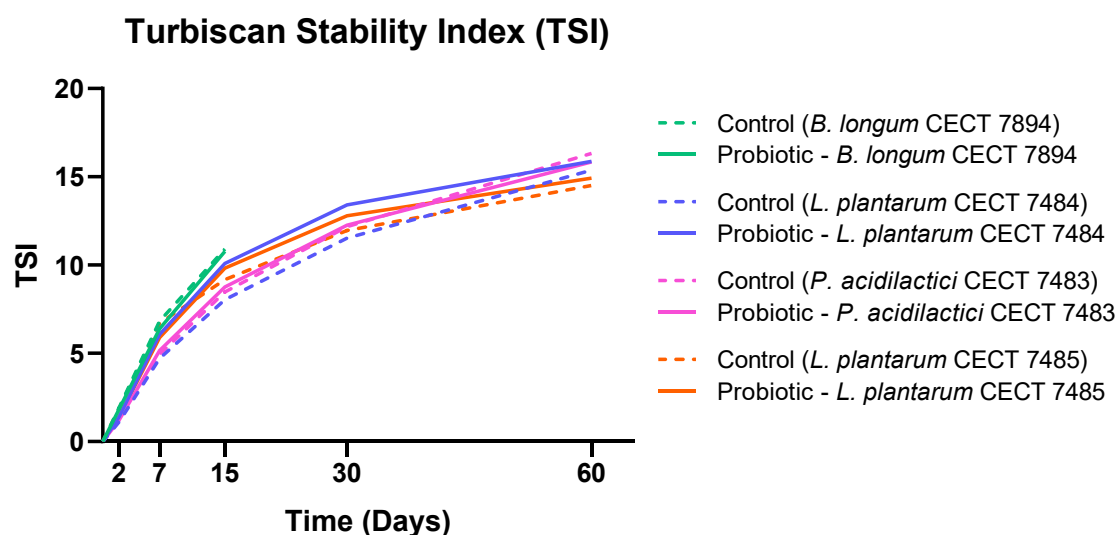


Figure 34. Test 1. Physical stability (Evolution of Turbiscan Stability Index) of orange juice formulations without probiotics (Control) and supplemented with different strains (Probiotic): *B. longum* CECT 7894, *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, and *L. plantarum* CECT 7485, during 60 days (15 days for *B. longum* CECT 7894) at 4 ± 2 °C.

Table 27. Variation in backscattering (Δ BS) between initial (Day 0) and final (Day 60, or day 15 in the case of *B. longum* CECT 7894) measurements across sedimentation (1.5–15 mm), flocculation (10–30 mm), and creaming (30–40 mm) zones.

Strain	Treatment	Sedimentation Zone* (1.5-15 mm)	Flocculation Zone* (15-30 mm)	Creaming Zone* (30-40 mm)
<i>B. longum</i> CECT 7894	C	6.78 ± 2.10^{aAB}	-5.54 ± 0.11^{aA}	-5.64 ± 0.15^{aA}
	P	7.13 ± 0.64^{aA}	-6.73 ± 0.10^{bB}	-7.08 ± 0.13^{bB}
<i>L. plantarum</i> CECT 7484	C	6.35 ± 0.82^{aA}	-6.81 ± 0.51^{aB}	-6.56 ± 0.52^{aB}
	P	8.17 ± 0.86^{bB}	-6.85 ± 0.43^{aB}	-6.87 ± 0.55^{aB}
<i>P. acidilactici</i> CECT 7483	C	7.48 ± 1.79^{aB}	-6.84 ± 0.52^{aB}	-6.93 ± 0.48^{aB}
	P	8.49 ± 1.36^{bB}	-6.78 ± 0.48^{aB}	-6.91 ± 0.59^{aB}
<i>L. plantarum</i> CECT 7485	C	6.08 ± 0.09^{aA}	-7.39 ± 0.06^{aC}	-7.45 ± 0.06^{aC}
	P	6.17 ± 0.53^{aA}	-7.41 ± 0.15^{aC}	-7.49 ± 0.19^{aC}

* Δ BS $\pm$ SD

C = Control

P = probiotic-supplemented

Two-way ANOVA (strain $\times$ treatment) followed by LDS post hoc test was applied; different superscript letters indicate significant differences ($p < 0.05$).

Different lowercase letters within a column indicate significant differences between treatments (control vs. Probiotic) within the same strain.

Different uppercase letters indicate significant differences between microorganisms within the same treatment.

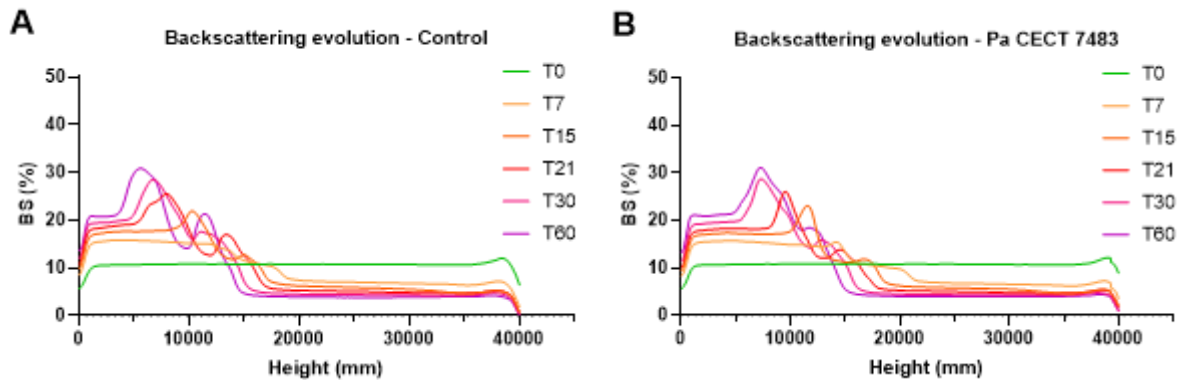


Figure 35. Test 1. Evolution of backscattering (BS, %) profiles (mean values) of orange juice during storage at $4 \pm 2^\circ\text{C}$ for 60 days. (A) Control orange juice without probiotic supplementation. (B) Orange juice supplemented with *P. acidilactici* CECT 7483.

The impact of probiotic supplementation on the physical stability of orange juice suspensions was evaluated over 60 (of 15 in the case of *B. longum* CECT 7894) days of refrigerated storage ($4 \pm 2^\circ\text{C}$) using SMLS, expressed as the Turbiscan Stability Index (TSI), and by analysing backscattering variations (Δ BS) in specific height zones associated with sedimentation, flocculation, and creaming (Figure 34 and Table 27, respectively).

As shown in Figure 34, all formulations, both probiotic-inoculated samples and their respective controls, exhibited a progressive increase in TSI over storage time, reflecting the natural evolution of a pulp-containing juice matrix. Importantly, the TSI profiles of inoculated samples closely overlapped with those of their controls throughout the 60-day period (15-days for *B. longum* CECT 7894 samples), with final TSI values remaining within a comparable range (approximately 14–16). This behaviour indicates that the incorporation of *B. longum* CECT 7894, *P. acidilactici* CECT 7483, and *L. plantarum* CECT 7484 and CECT 7485 did not accelerate or exacerbate global destabilisation phenomena relative to the non-inoculated juices.

According to the Turbiscan Stability Scale, TSI is a dimensionless cumulative parameter integrating all detectable migration and particle size variation events across the sample height. In food dispersions, TSI values > 3 are generally associated with visually perceptible destabilisation (Batista et al., 2026).

The zone-specific Δ BS analysis (Table 27) further corroborated the global TSI results. Among the evaluated instability parameters, sedimentation exhibited the highest positive Δ BS values, indicating that gravitational settling of suspended particles was the predominant mechanism contributing to physical instability during storage. This behaviour is consistent with the well-documented characteristics of orange juice systems, in which pulp particles progressively settle at the bottom, leading to clarification of the supernatant phase.

The overall magnitude of sedimentation-related changes was comparable across treatments and microbial strains. Nevertheless, small but statistically significant differences were observed. Specifically, P samples containing *L. plantarum* CECT 7484 and *Pediococcus acidilactici* CECT 7483 showed significantly higher Δ BS values than their respective controls. Within the control (C) samples, CECT 7483 exhibited significantly higher sedimentation compared with CECT 7484 and CECT 7485. In the P treatment, strains *L. plantarum* CECT 7484 and *P. acidilactici* CECT 7483 resulted in significantly higher sedimentation than CECT 7485 and *B. longum* CECT 7894.

Δ BS values in both the flocculation and creaming zones were negative, further corroborating the increase in sedimentation. Comparable trends were observed across the different treatments. The only exception was the P sample supplemented with *B. longum* CECT 7894, that showed a significant difference, indicating enhanced particle aggregation, compared to the control.

Significant strain-dependent differences were also observed. Specifically, both C and P samples containing *L. plantarum* CECT 7485 showed significantly different behaviour in terms of flocculation and creaming compared with all other strains. In addition, C samples containing *B. longum* CECT 7894 exhibited significant differences in both flocculation and creaming relative to the remaining strains.

An illustrative example of the backscattering signal evolution recorded for *P. acidilactici* CECT 7483-supplemented samples is shown in Figure 35.

It is also important to acknowledge that minor differences in TSI values and Δ BS profiles observed among formulations are likely influenced by batch-to-batch variability of the commercial orange juice used for each strain. Variations in pulp content, particle size distribution, and colloidal composition are known to significantly affect light scattering behaviour and sedimentation kinetics in fruit juices. Such matrix dependent effects can act as confounding factors when comparing absolute stability parameters across different experimental series and should therefore be considered when interpreting strain specific differences.

Overall, the combined TSI evolution and zone resolved Δ BS analysis indicate that the few detected differences were low in magnitude and that the destabilization processes observed over storage time are primarily governed by the intrinsic characteristics of the juice matrix rather than by the presence of probiotic microorganisms. confirming their physical compatibility with orange juice under refrigerated storage.

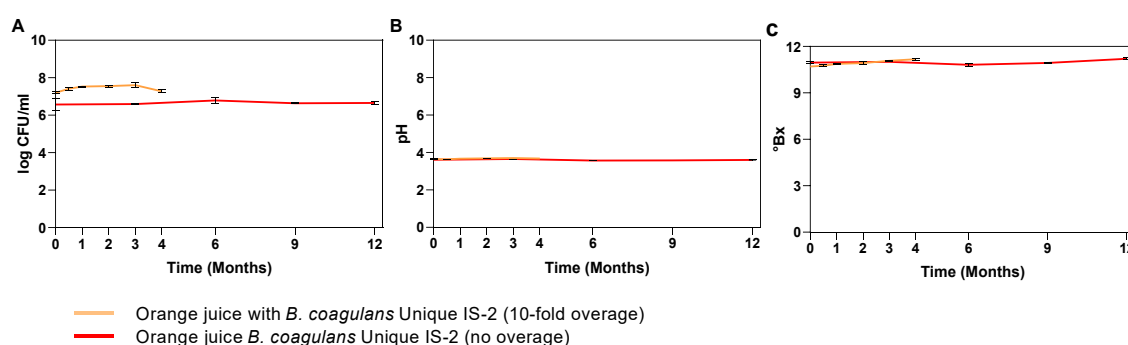


Figure 36. Test 2. Probiotic viability and physicochemical characteristics of orange juice fortified with *B. coagulans* Unique IS-2 over 4 months (10-fold overage) and 12 months (no overage) of 4 ± 2 °C storage. A) Viability of *B. coagulans* spores expressed as log CFU/ml. B) pH. C) TSS expressed as °Bx.

In Tests 2, *B. coagulans* Unique IS-2 was targeted at 6.78 log CFU/mL per 330 mL serving and both 1-fold (no overage) and a 10-fold overage were evaluated, corresponding to initial inoculum levels of 6.78 and 7.78 log CFU/mL, respectively.

The results of Test 2 are presented in Figure 36. This study aimed to assess the long-term stability of *B. coagulans* Unique IS-2 spores in probiotic-enriched orange juice over two storage durations: the first (10-fold overage), lasting four months, and second (1-fold overage), extending to twelve months.

Across both inoculum levels, the strain exhibited consistently high viability, with mean recoveries of 7.10 ± 0.52 log CFU/mL after four months and 6.65 ± 0.09 log CFU/mL after twelve months. Despite the inherently low pH of orange juice, the overall physicochemical composition of the matrix, known to influence the extent of sublethal injury depending on the surrounding medium, and the use of refrigeration likely created conditions that minimized acid-induced membrane disruption and dipicolinic acid loss, thereby preserving the structural integrity of *B. coagulans* Unique IS-2 spores and supporting their maintenance in a culturable state during storage (Bevilacqua et al., 2020; Majeed et al., 2018).

The pH values remained stable throughout both tests, averaging 3.67 ± 0.02 and 3.61 ± 0.03 , respectively. This indicates that inoculation with *B. coagulans* Unique IS-2 spores did not significantly alter the acidity of the juice. Such stability is desirable in commercial applications, as it preserves the sensory profile and microbial safety of the product. The observed results align with previous findings that, *B. coagulans*, due to its sporulated form and low metabolic activity under refrigerated conditions, does not actively ferment sugars or produce organic acids in fruit-based matrices. This finding is consistent with the results reported by Majeed et al. (2016), who observed no significant change in the pH of apple juice fortified with *B. coagulans*, along with remarkable stability in probiotic viability (CFU counts) over a six-month period of refrigerated storage. Similarly, the TSS remained consistent, between 10.9 ± 0.2 °Brix and 11.0 ± 0.1 °Brix over the storage periods.

Overall, these findings underscore the excellent stability and suitability of *B. coagulans* Unique IS-2 spores for use in orange juice. Its spore-forming nature offers a distinct advantage over more sensitive strains such as bifidobacteria and lactobacilli, which often exhibit rapid viability loss in acidic environments. The maintenance of viable counts, pH, and °Brix over a twelve-month period supports the feasibility of developing shelf-stable, functional fruit-based products using this strain.

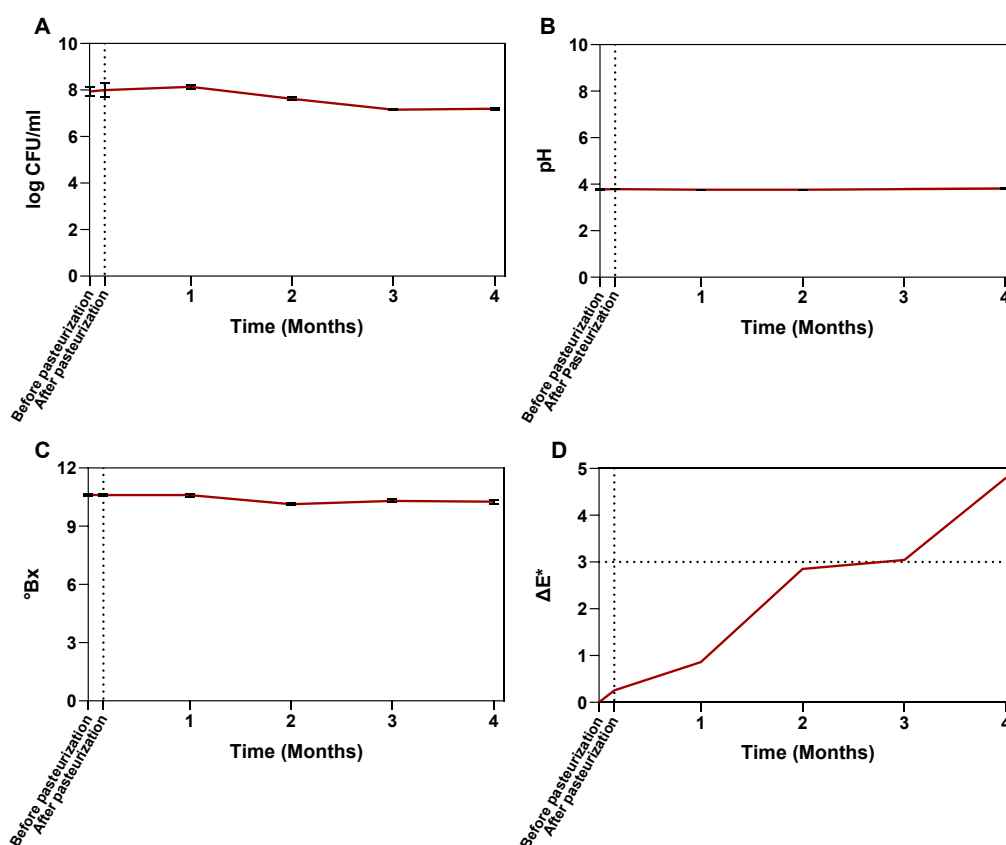


Figure 37. Test 3. Probiotic viability and physicochemical characteristics of orange juice fortified with *B. coagulans* Unique IS-2 and subjected to pasteurization, during 4 months at 4 ± 2 °C. A) Viability of *B. coagulans* spores expressed as log CFU/ml. B) pH. C) TSS expressed as °Bx. D) Colour variation expressed as ΔE^* .

In Tests 3, *B. coagulans* Unique IS-2 was targeted at 6.78 log CFU/mL per 330 mL serving and a 10-fold overage was applied, corresponding to initial inoculum levels of 7.78 log CFU/mL

Test 3 was designed to complement the previous stability tests and evaluate the impact of a pasteurization treatment on the probiotic's viability and the physicochemical properties of the matrix. In this test, following inoculation, the probiotic-enriched orange juice underwent an intensive HTST thermal treatment (82 °C for 45 s), and was then stored under refrigerated conditions. The results of Test 3 are presented in Figure 37 and provide additional insight into the thermal resilience of *B. coagulans* spores and the overall stability of the juice formulation under realistic processing conditions. The pasteurization treatment did not significantly reduce the viable spore count, confirming the thermal resistance of *B. coagulans* spores, which are known to withstand conditions that typically inactivate vegetative cells (Maresca et al., 2024; Žvirdauskienė et al., 2025). Over the 4-month storage period, the viable cell counts exhibited only a modest but statistically significant decline (p

= 0.0001), decreasing from an initial average of 7.94 ± 0.19 log CFU/mL to 7.20 ± 0.04 log CFU/mL. This reduction suggests that while some spores may have experienced sublethal damage or delayed germination, the majority remained viable and stable throughout the storage duration.

The pH of the juice remained consistently low, averaging 3.78 ± 0.02 , which reflects the inherent acidity of orange juice and indicates that the probiotic strain did not significantly alter the acid-base profile of the matrix. Similarly, the TSS (°Brix) remained stable at 10.5 ± 0.2 , suggesting that the metabolic activity of the spores under refrigerated conditions was minimal and did not affect the sugar content or nutritional quality of the juice.

In terms of colour stability, the ΔE^* values showed a progressive increase, reaching approximately +4.80 by the fourth month. Notably, the samples exceeded a ΔE^* of 3 as early as the third month of storage and this change was visually evident when comparing the sample to freshly opened juice. The increase of ΔE was primarily associated with a decrease in all L^* , a^* and b^* values, indicating that the juice became darker and less orange over time, which may be attributed to matrix oxidation. While *B. coagulans* itself is not known to produce pigments, thermal processing and prolonged storage can accelerate oxidative degradation in carotenoids and flavonoids, leading to visible colour shifts (Kardooni et al., 2023; Wibowo, et al., 2015b). It is also important to consider the sampling methodology employed in this study. A single bottle was used for each biologic replicate and was repeatedly opened at various time points to collect aliquots for analysis. This approach likely led to progressive oxygen ingress, which may have accelerated oxidative reactions even under refrigerated storage, protected from light. Consequently, the observed darkening may reflect not only natural aging processes but also the cumulative impact of oxygen exposure introduced during sampling. For future studies, the use of individual containers for each time point or oxygen-reducing storage techniques is recommended to better preserve the physicochemical integrity of the juice.

These findings overall confirm that pasteurized orange juice is a suitable carrier for *B. coagulans* Unique IS-2 spores, enabling the development of long shelf-life probiotic products and support the feasibility of non-dairy probiotic beverages, in line with consumer trends toward plant-based functional foods. Moreover, it is noteworthy that in a study from Almada-Érix et al. (2021), *Bacillus coagulans* GBI-30 6086 demonstrated robust survival (≥ 7 log CFU/mL) throughout simulated GI digestion when incorporated into non-fermented

orange juice, reinforcing the suitability of this matrix for delivering spore-forming probiotics under gastric and intestinal conditions.

4.1.6.2 Isotonic Drinks

Three separate experiments were conducted to evaluate the stability and properties of different isotonic drinks formulations enriched with *B. coagulans* Unique IS-2. The tests followed the experimental design outlined in Figure 38.

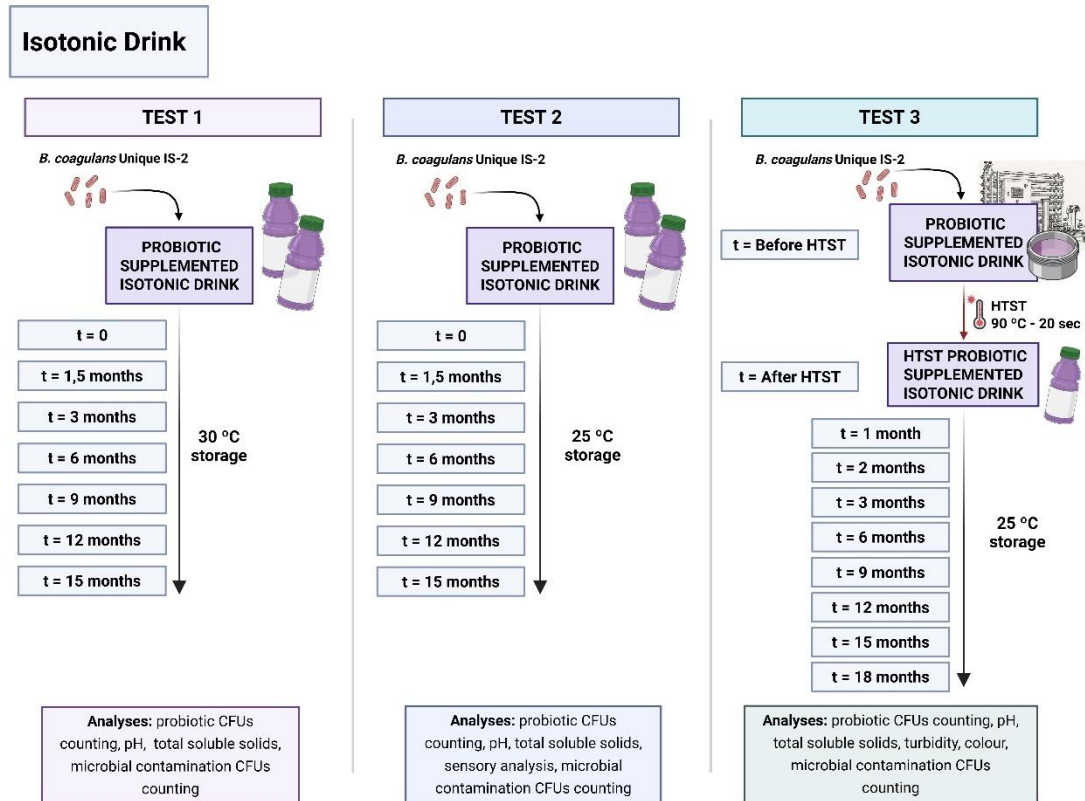
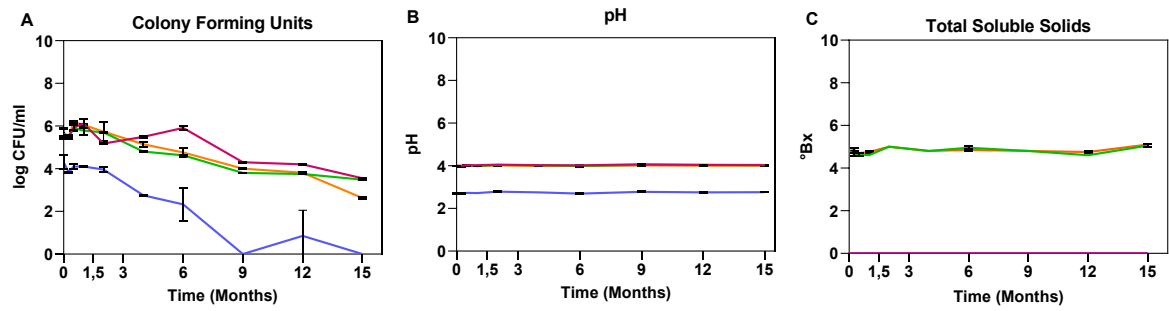
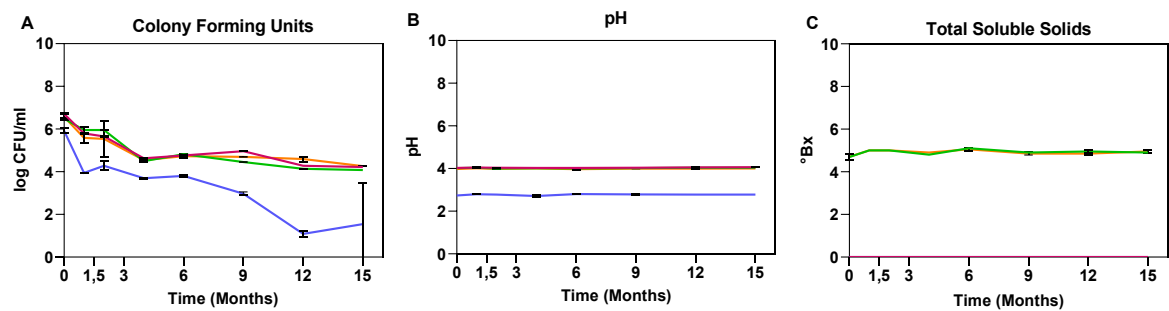


Figure 38. Schematic illustration of the experimental design. Three experimental trials were carried out using isotonic beverages supplemented with *B. coagulans* Unique IS-2 spores. In Test 1, inoculated drinks were stored at 30 ± 1 °C under dark conditions, whereas Test 2 involved storage at 25 ± 5 °C under light exposure to simulate ambient commercial distribution environments. Test 3 incorporated a high-temperature short-time (HTST) pasteurization step (90 °C for 20 s) immediately after probiotic inoculation, followed by storage at 25 ± 5 °C in the dark. For Tests 1 and 2, samples were analysed at 0, 1.5, 3, 6, 9, 12, and 15 months, while Test 3 was evaluated pre- and post-pasteurization and subsequently at 1, 2, 3, 6, 9, 12, 15, and 18 months of storage. Evaluated parameters included viable spore counts (CFU), pH, TSS, microbiological contamination (at the beginning and at the end of storage), turbidity (Test 3), and colour (Test 3). Additionally, a sensory analysis using freshly reconstituted F3 samples (Test 2) was performed. The figure was created in <https://BioRender.com>.

Test 1 - Isotonic drink with *Bacillus coagulans* Unique IS-2 (30°C, dark storage)



Test 2 - Isotonic drink with *Bacillus coagulans* Unique IS-2 (25°C, light-exposed storage)



- F1 - NO Sugar-Natural Flavour
- F2 -NO Sugar-Artificial Flavour
- F3 - Sugar-Natural Flavour
- F4 - Sugar-Artificial Flavour

Figure 39. Test 1 and Test 2 results including probiotic viability and physicochemical characteristics of different isotonic drink formulations (F1, F2, F3, F4) fortified with *B. coagulans* Unique IS-2 during 15 months of storage. A) Viability of *B. coagulans* spores expressed as log CFU/ml. B) pH. C) TSS expressed as °Bx.

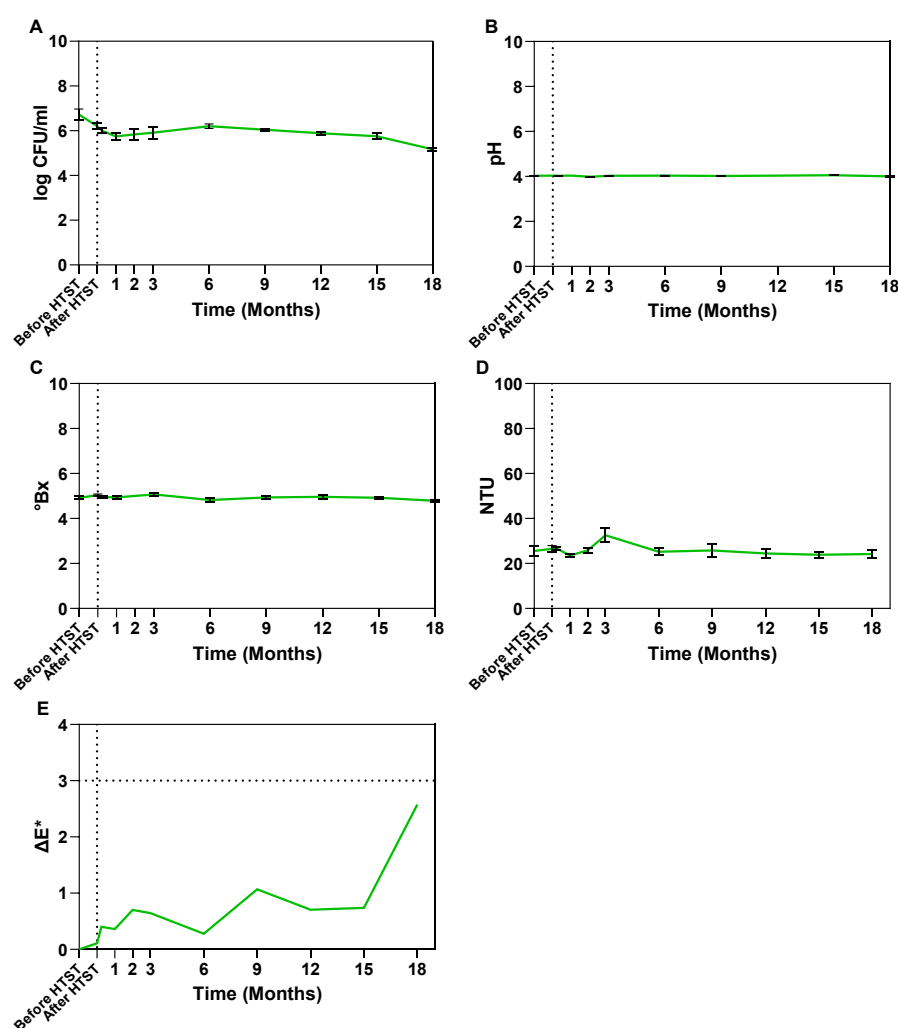


Figure 40. Test 3 results including probiotic viability and physicochemical characteristics of isotonic drink formulation F3 (Sugar – Natural Flavour) fortified with *B. coagulans* Unique IS-2 and subjected to HTST treatment, during 18 months of room temperature storage (25 ± 5 °C). A) Viability of *B. coagulans* spores expressed as log CFU/ml. B) pH. C) TSS expressed as °Bx. D) Turbidity expressed as nephelometric turbidity units (NTU). E) Colour variation expressed as ΔE^* .

The incorporation of *B. coagulans* Unique IS-2 spores into isotonic drinks was designed as detailed in Section 3.2.15 (Materials and Methods) to ensure the delivery of a functional probiotic concentration in the final heat-treated product. No overage was applied in any of the three tests. The target concentration was set at 6.78 log CFU/ml per 330 ml serving.

The results of Test 1 and Test 2 are presented in Figure 39.

The probiotic formulations varied in sugar content, flavour type (natural vs. artificial), and pH and were named as follows: F1 (No Sugar, Natural Flavour), F2 (No Sugar, Artificial Flavour), F3 (Sugar, Natural Flavour), and F4 (Sugar, Artificial Flavour).

Across the two storage conditions evaluated, all formulations exhibited a gradual decline in cell counts. Under 30 °C dark storage, average CFU values decreased from 5.48 ± 0.08 (F1),

4.30 ± 0.34 (F2), 5.65 ± 0.24 (F3), and 5.69 ± 0.22 log CFU/mL (F4) at inoculation, to approximately 3.53 ± 0.07 (F1), 0 (F2), 3.48 ± 0.05 (F3), and 2.63 ± 0.03 log CFU/mL (F4) after 15 months. Similarly, under 25 °C light storage, counts decreased from 6.70 ± 0.01 (F1), 5.92 ± 0.13 (F2), 6.49 ± 0.05 (F3), and 6.57 ± 0.16 log CFU/mL (F4) to approximately 4.22 ± 0.06 (F1), 1.54 ± 1.94 (F2), 4.07 ± 0.03 (F3), and 4.26 ± 0.01 log CFU/mL (F4).

Among the formulations, F3 (Sugar–Natural Flavour) exhibited the most pronounced decrease of countable cells since inoculation (Δ log CFU/mL = −4.30 in Test 1 and Δ log CFU/mL = −4.37 in Test 2). Notably, F3 exhibited a consistently markedly lower pH (2.7–2.8), a condition capable of imposing substantial acid stress on *Bacillus coagulans* spores. Such extreme acidity is known to perturb the inner spore membrane and trigger premature dipicolinic acid release, thereby diminishing resistance and impairing the initiation of germination. Under these conditions, spores may remain metabolically viable yet incur sublethal injury, including leakage of core components and loss of membrane homeostasis, which prevents successful germination and renders them non-culturable despite retained viability (Bevilacqua et al., 2020; Bora, 2009; Luu & Setlow, 2014; Majeed et al., 2018). This response is consistent with documented transitions into a viable-but-non-culturable (VBNC) state in *B. coagulans* MTCC 5856 under acidic stress, where spores fail to form colonies on agar although viable cells can be detected by alternative methods such as flow cytometry (Majeed et al., 2018).

Total soluble solids also remained consistent, with F1 and F2 (sugar-free) maintaining ~0 °Brix, while F3 and F4 (sugar-containing) fluctuated only slightly between 4.6 and 5.1 °Brix across both storage conditions. This stability suggests minimal metabolic activity or degradation of sugars and other soluble components, which is essential for preserving sensory attributes and shelf life.

The results of Test 3 are presented in Figure 40. The HTST process resulted in an immediate reduction in cell counts, with a Δ log CFU/mL of −0.53 (from 6.73 ± 0.24 to 6.20 ± 0.15), indicating a moderate thermal impact on the spore-forming probiotic.

Following heat treatment, the colony-forming units (CFU) declined gradually over the storage period, from 6.20 ± 0.15 to 5.17 ± 0.07 log CFU/mL (Δ log CFU/mL = −1.03). This trend reflects a slow but consistent reduction in viability, which is expected in long-term storage of functional beverages. Despite this decline, the strain maintained good viable levels, suggesting good resilience and compatibility with the beverage matrix.

The pH of the beverage remained stable throughout the storage period, with an average value of 4.03 ± 0.02 . This stability contributes positively to product consistency and shelf-life. However, despite the enhanced resistance of *B. coagulans* Unique IS-2 in its spore form, prolonged exposure to acidic conditions can still negatively impact spore viability. As observed in the two previous tests, although spores are metabolically dormant and significantly more robust than vegetative cells, extended storage at pH values near or below 4.0 may compromise spore integrity or delay germination, ultimately reducing recoverable CFU counts.

Total soluble solids, measured in °Brix, remained constant at approximately 4.9 ± 0.1 , indicating no significant fermentation or degradation of sugars during the 18-month storage period. This stability confirms that the probiotic spores did not germinate or metabolize the sugars present. The lack of fermentative activity further supports the suitability of this strain for shelf-stable functional beverages, where preservation of sweetness and sensory attributes is essential.

Turbidity remained stable throughout the 18-month storage period, with values averaging 25.87 ± 2.58 NTU, measured after homogenization of the product by shaking. This indicates that although spore sedimentation occurred during storage, the beverage was able to recover its colloidal uniformity upon mixing, reflecting consistent re-dispersion of suspended ingredients and probiotic cells. Such behaviour is typical in shelf-stable functional beverages, where physical separation during rest is expected but reversible. The ability to maintain turbidity post-mixing is critical for preserving the visual and textural appeal of the product, as it ensures a uniform appearance and mouthfeel upon consumption.

Colour variation (ΔE^*) remained relatively stable throughout the 18-month storage period, consistently below the perceptibility threshold of 3 units for distinctly noticeable colour changes (Mokrzycki & Tatol, 2011; Pathare et al., 2013). However, the upward trend near the end of storage suggests the onset of oxidative or compositional changes that may begin to influence the visual characteristics of the product. Given that the beverage exhibited a translucent violet hue, the simultaneous increase in L^* , a^* , and b^* values indicate a shift toward a lighter, redder, and more yellow appearance. This chromatic drift implies a gradual fading of the original violet tone, potentially due to pigment degradation or interactions between flavour compounds and light or oxygen. Although not yet perceptible, such changes could become relevant in extended shelf-life scenarios and should be considered in formulation and packaging strategies.

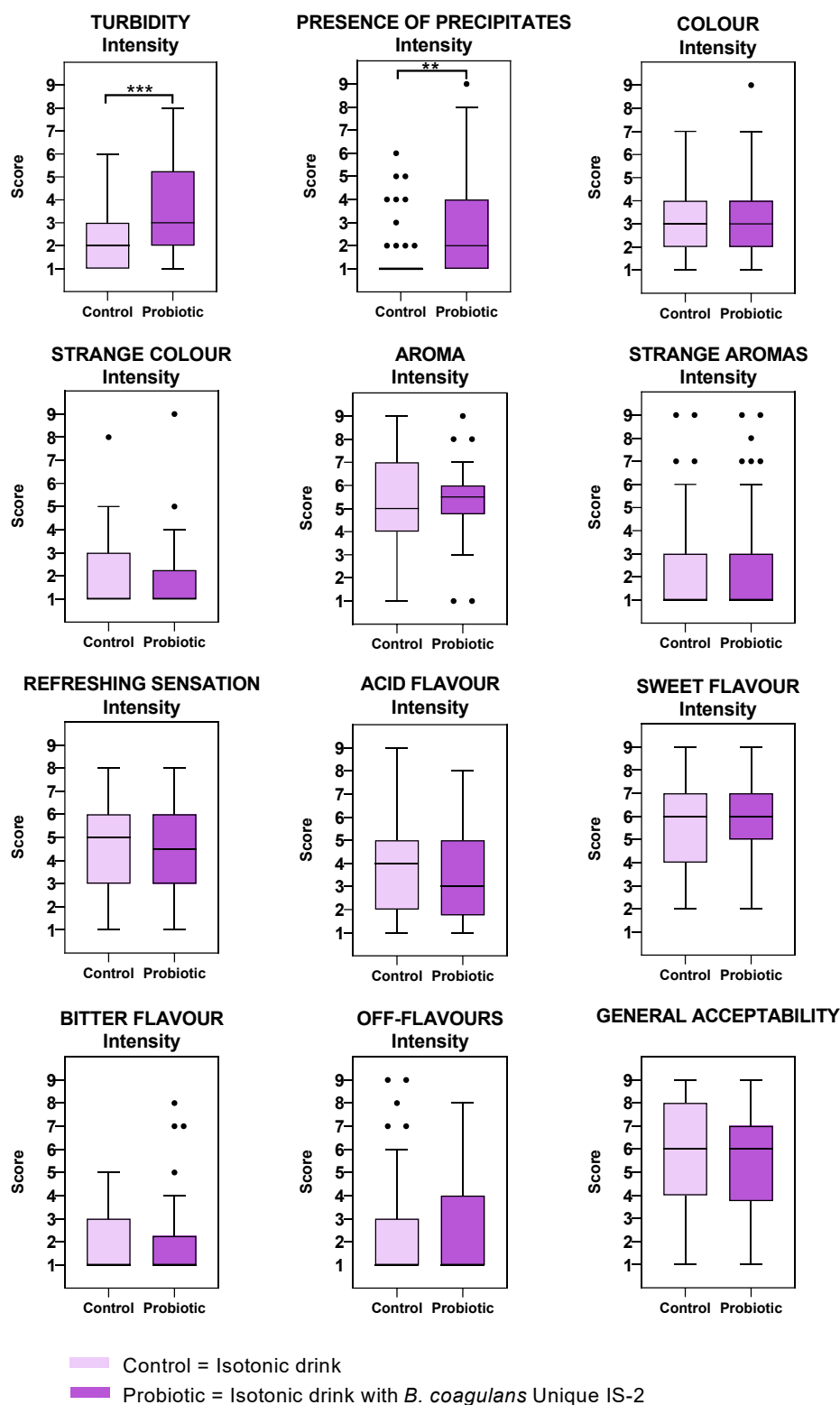


Figure 41. Sensory analysis of F3 formulation of isotonic drinks with (Probiotic) and without (Control) *B. coagulans* Unique IS-2. Box plots display the distribution of scores for key sensory attributes, as assessed by 50 untrained panellists. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Based on its superior storage stability under both Test 1 and Test 2 conditions, formulation F3 fortified with *B. coagulans* Unique IS-2 spores, was selected for sensory evaluation. Consumer acceptance was assessed through a sensory test conducted one day after sample production with 50 untrained panellists (68% female, 32% male; 18–71 years), and the results are summarized in Figure 41.

In terms of appearance, the probiotic formulation exhibited slightly but significant higher turbidity (3.72 ± 2.27) compared to the control (2.26 ± 1.54), which is consistent with the expected increase in suspended solids due to the presence of microbial biomass in a clear drink. Similarly, the presence of precipitates was rated significantly higher in the probiotic sample (2.80 ± 2.27) than in the control (1.56 ± 1.25), although both values remained within acceptable sensory thresholds. These differences, did not appear to negatively influence the overall visual appeal of the product. Colour intensity was comparable between the two samples (3.40 ± 1.88 for the probiotic vs. 3.14 ± 1.43 for the control), and no unusual coloration was reported, as reflected in the similar scores for strange colour intensity (1.98 ± 1.49 vs. 2.04 ± 1.56).

Aroma-related attributes also showed no variation ($p > 0.05$). The probiotic drink scored 5.36 ± 1.61 for aroma intensity, closely matching the control at 5.44 ± 1.72 . The presence of strange aromas was similarly low in both samples (2.40 ± 2.38 for the probiotic and 2.38 ± 2.18 for the control), indicating that the inclusion of *B. coagulans* spores did not introduce off-flavours or undesirable aromatic notes.

Taste attributes followed a similar trend. The refreshing sensation, a key functional characteristic of isotonic beverages, was rated nearly identically in both formulations (4.56 ± 2.01 for the probiotic and 4.54 ± 1.97 for the control). Acidic flavour was slightly more pronounced in the control (3.90 ± 2.13) than in the probiotic version (3.32 ± 1.98), while sweetness levels were consistent (5.92 ± 1.75 vs. 5.82 ± 1.65). Bitterness remained low in both samples (1.92 ± 1.68 for the probiotic and 1.94 ± 1.43 for the control), and off-flavours were similarly rated (2.66 ± 2.26 vs. 2.50 ± 2.23), suggesting that the probiotic strain did not introduce any off-flavours.

Importantly, general acceptability scores were nearly identical between the two formulations, with the probiotic sample rated at 5.60 ± 2.09 and the control at 5.68 ± 2.25 . This indicates that the inclusion of *B. coagulans* Unique IS-2 spores did not compromise the overall sensory appeal of the product.

Taken together, these results demonstrate that the probiotic formulation maintained a sensory profile comparable to the control across all evaluated attributes. The slight increases in turbidity and precipitate perception are consistent with the physical presence of the probiotic but did not negatively affect consumer perception.

The results of this study collectively support the feasibility of incorporating *B. coagulans* Unique IS-2 spores into isotonic beverage formulations without compromising sensory quality. The probiotic strain demonstrated strong stability under long-term storage and thermal processing, reinforcing its suitability for shelf-stable functional drinks. Sensory evaluation confirmed that the inclusion of *B. coagulans* did not negatively affect the organoleptic properties of the beverage, with consumer acceptability remaining consistent across formulations.

However, the viability of the probiotic was significantly influenced by formulation parameters, particularly pH. Beverages with lower pH values (~2.7–2.8) exhibited a marked reduction in viable cell counts, despite the inherent resilience of *B. coagulans* as a spore-forming microorganism. In contrast, formulations maintaining a pH closer to 4.0 preserved microbial viability more effectively over time. These findings underscore the critical role of matrix composition, especially pH, in determining probiotic performance in isotonic systems. Therefore, while *B. coagulans* Unique IS-2 spores offers promising functional and technological advantages, optimizing beverage formulation is essential to balance probiotic efficacy with product stability and consumer acceptance. This highlights the importance of a tailored approach in the development of probiotic-enriched isotonic drinks, where both microbiological and sensory parameters must be carefully considered.

4.1.6.3 Reconstitutable Isotonic Drink Powder

Two separate experiments were conducted to evaluate the stability and properties of the probiotic blend AB-DIGEST or the probiotic strain *B. coagulans* Unique IS-2 in different reconstitutable isotonic drink powders. The tests followed the experimental design outlined in Figure 42.

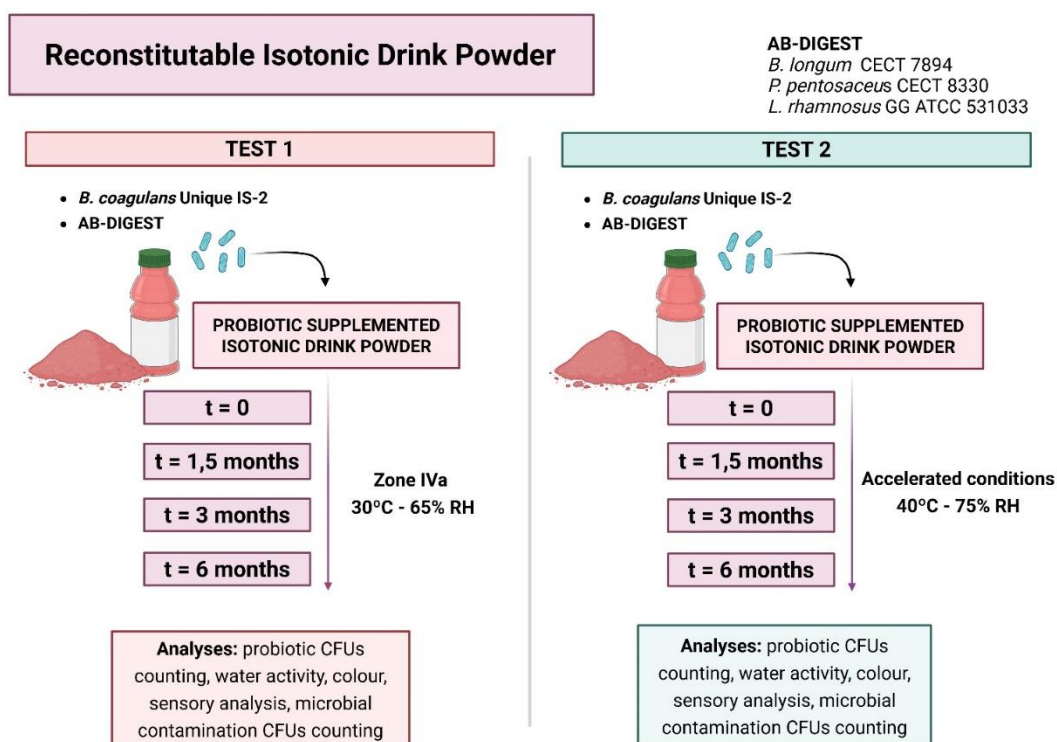
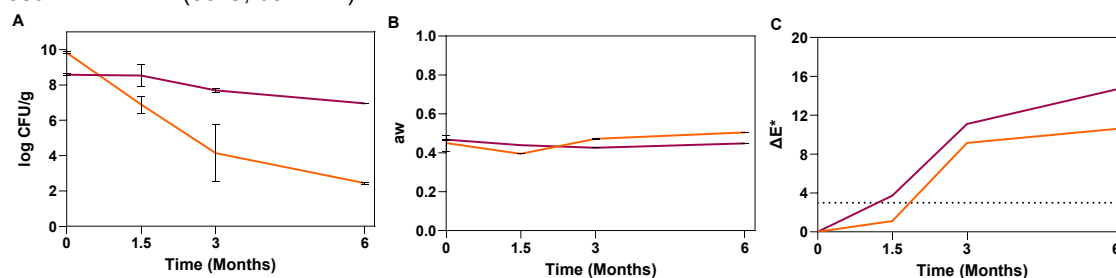
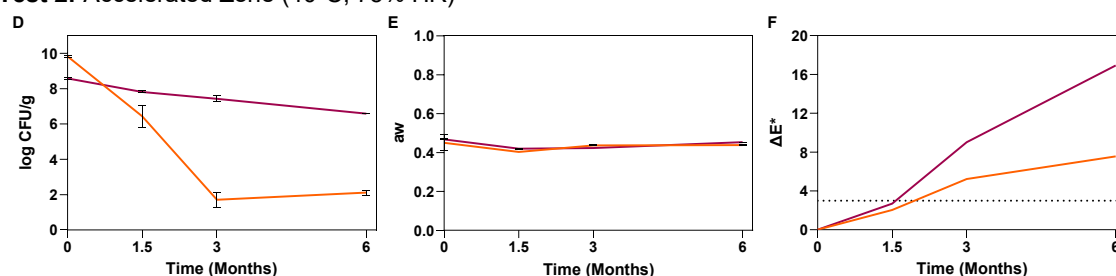


Figure 42. Schematic illustration of the experimental design. Two trials were conducted using isotonic drink powders supplemented with *B. coagulans* Unique IS-2 or with the probiotic blend AB-DIGEST. Test 1 involved storage under Zone IVa conditions (30 °C, 65 % RH), while Test 2 was stored under accelerated conditions (40 °C, 75 % RH). Analyses were performed at 0, 1.5, 3, and 6 months of storage. Evaluated parameters included viable probiotic spore counts (CFU), aw, colour, and microbiological contamination (at T0 and T6 months). Additionally, a sensory analysis using freshly reconstituted samples was performed. The figure was created in <https://BioRender.com>.

Test 1: Zone IVa (30°C, 65% HR)



Test 2: Accelerated Zone (40°C, 75% HR)



— Isotonic beverage powder formula with probiotic blend AB-DIGEST
 — Isotonic beverage powder formula with probiotic strain *B. coagulans* Unique IS-2

Figure 43. Probiotic viability and physicochemical characteristics of reconstitutable isotonic drinks fortified either with the probiotic blend AB-DIGEST or with the probiotic strain *B. coagulans* Unique IS-2 were evaluated over 6 months of storage under two conditions: 30 °C / 65% RH (Test 1) and 40 °C / 75% RH (accelerated, Test 2). A–D) Probiotic spores viability expressed as log CFU/g. B–E) aw. C–F) Colour variation expressed as ΔE*.

The probiotic blend AB-DIGEST and the single strain *B. coagulans* Unique IS-2 spores were inoculated in reconstitutable isotonic drinks, as detailed in Section 3.2.16 (Materials and Methods). To account for potential viability loss during processing and storage, an overage of 7.5-fold was applied for AB-DIGEST and 3-fold for *B. coagulans* Unique IS-2. Target concentrations were set at 8.12 log CFU/mL for AB-DIGEST and 8.60 log CFU for *B. coagulans* Unique IS-2, per 15 g serving.

As shown in Figure 43, in Test 1 (storage 30 °C, 65% RH), both formulations showed a gradual reduction in viable counts over six months. The probiotic blend AB-DIGEST exhibited a sharper decline in viability ($\Delta \log \text{CFU/g} = -7.38$) compared with *B. coagulans* Unique IS-2 ($\Delta \log \text{CFU/g} = -1.63$). The latter maintained substantially higher viability throughout the study, with levels exceeding those observed in the ready-to-drink formulations of Test 1 and Test 2 (Figure 39). For this matrix, pH measurements were not performed. This methodological omission limited the interpretation and comparability of the results with those obtained from the ready-to-drink formulation.

Under the accelerated conditions of Test 2 (40 °C, 75% RH), the viability loss was more pronounced for both formulations. After three months, *B. coagulans* Unique IS-2 retained substantially more CFU than the multi-strain blend. At the end of the six-months storage period, AB-DIGEST exhibited a total viability loss of $\Delta \log \text{ CFU/g} = -7.70$, while *B. coagulans* Unique IS-2 showed a comparatively modest decline of $\Delta \log \text{ CFU/g} = -1.99$. These results are consistent with previous studies demonstrating the superior resilience of *B. coagulans*, attributed to its spore-form, which enhances survival under adverse environmental conditions (Cutting, 2011). Spore-forming *Bacillus* species are generally more stable than non-spore-forming genera such as *Lactobacillus* and *Bifidobacterium*, which often require encapsulation or protective matrices to maintain viability during storage (Payne et al., 2024).

Initial a_w was 0.451 ± 0.042 for AB-DIGEST and 0.468 ± 0.003 for *B. coagulans* Unique IS-2. In all cases, a_w remained stable across storage conditions and formulations, indicating that the packaging materials provided an effective moisture barrier.

Colour change, measured as ΔE^* , increased over time in both formulations, with a more pronounced shift observed in the *B. coagulans* Unique IS-2 version. At the end of the six-month real-time storage (Test 1), ΔE^* values reached 10.60 for AB-DIGEST and 7.56 for *B. coagulans* Unique IS-2. Under accelerated conditions (Test 2), ΔE^* values were 14.66 and 16.93, respectively. This may be attributed to oxidative processes accelerated by high temperature (García-Baños et al., 2005). Although colour change does not directly affect probiotic efficacy, it is a key factor in consumer acceptance. The trade-off between microbial stability and aesthetic quality should be carefully considered during product development, particularly for products targeting health-conscious consumers.

Collectively, these findings underscore the critical influence of storage conditions on probiotic viability in isotonic powdered beverages. While both formulations experienced viability loss, *B. coagulans* Unique IS-2 consistently retained higher CFU counts under both real-time and accelerated conditions, confirming its robustness and suitability for applications in tropical and subtropical markets. Water activity remained stable, indicating formulation integrity, while colour change emerged as a secondary quality parameter sensitive to environmental stress. Further investigation into formulation strategies may help optimize sensory attributes without compromising functional performance.

To assess consumer acceptance, a sensory evaluation was conducted one day after sample production with a panel of 50 untrained panellists (68% female, 32% male; 18–71 years), and is summarized in Figure 44.

The results revealed comparable sensory profiles between the probiotic and control formulations. Although some visual differences in score distributions were for specific parameters, none of these differences were statistically significant ($p > 0.05$). This indicates that the inclusion of *B. coagulans* Unique IS-2 spores did not produce perceptible changes in sensory quality beyond natural variation. These findings are promising for the development of functional beverages, where maintaining sensory acceptability is essential for consumer uptake and market success (Granato et al., 2010).

Appearance-related attributes, including turbidity and presence of precipitates, did not differ significantly between the two formulations. The probiotic version showed an average turbidity score of 5.76 ± 2.24 , compared to 5.58 ± 2.11 for the control. Similarly, the presence of precipitates was rated 1.84 ± 1.42 for the probiotic and 1.98 ± 1.67 for the control. Colour intensity was also similar between the two samples, with scores of 7.22 ± 1.72 for the probiotic and 7.16 ± 1.69 for the control. "Strange colour intensity" was rated 2.92 ± 2.71 for the probiotic and 2.98 ± 2.72 for the control, suggesting that the probiotic strain did not introduce unusual visual characteristics.

Several studies have reported that the addition of *Bacillus* probiotics to beverages and other food products does not significantly alter sensory characteristics, supporting their suitability for consumer products (Payne et al., 2024). Aroma intensity was rated 5.62 ± 2.19 for the probiotic and 5.96 ± 2.12 for the control, while "strange aromas" received scores of 3.62 ± 2.58 and 3.88 ± 2.76 , respectively. These results suggest that the inclusion of *B. coagulans* spores did not introduce off-flavours, a common challenge in probiotic product development.

Flavour descriptors, including acidity (4.34 ± 1.88 for probiotic vs. 4.20 ± 1.96 for control), sweetness (4.94 ± 1.67 vs. 4.82 ± 1.95), and bitterness (3.34 ± 2.25 vs. 3.32 ± 2.14), were also statistically indistinguishable.

Scores for refreshing sensation were 3.84 ± 1.81 for the probiotic and 4.26 ± 1.99 for the control, while general acceptability was rated 4.22 ± 2.14 and 4.38 ± 2.17 , respectively. These results indicate that the addition of *B. coagulans* Unique IS-2 spores did not compromise the product's core functional attribute, refreshment, and may have even slightly enhanced it. The absence of statistically significant differences across all sensory parameters

supports the feasibility of incorporating *B. coagulans* Unique IS-2 spores into isotonic powdered drinks without negatively impacting consumer perception.

The consistent sensory performance of the probiotic formulation across all evaluated attributes reinforces the potential of spore-forming probiotics in beverage applications. Minor trends may be addressed through formulation optimization, but do not currently pose barriers to product development.

4.2 CHAPTER 2: ASSESSMENT OF AB-BIOTICS PROBIOTIC STRAINS STABILITY AND IMPACT IN DIFFERENT FERMENTED FOOD AND BEVERAGE MATRICES

4.2.1 Dairy Products

4.2.1.1 Fermented Milk

Within the scope of applying probiotics in different fermented milk formats, three trials were conducted:

4.2.1.1.1 TEST 1: Drinkable fermented milk with probiotic blend I3.1

The stability and properties of fermented milk (drinkable) enriched with the probiotic blend I3.1, were assessed according to the experimental design outlined in Figure 45.

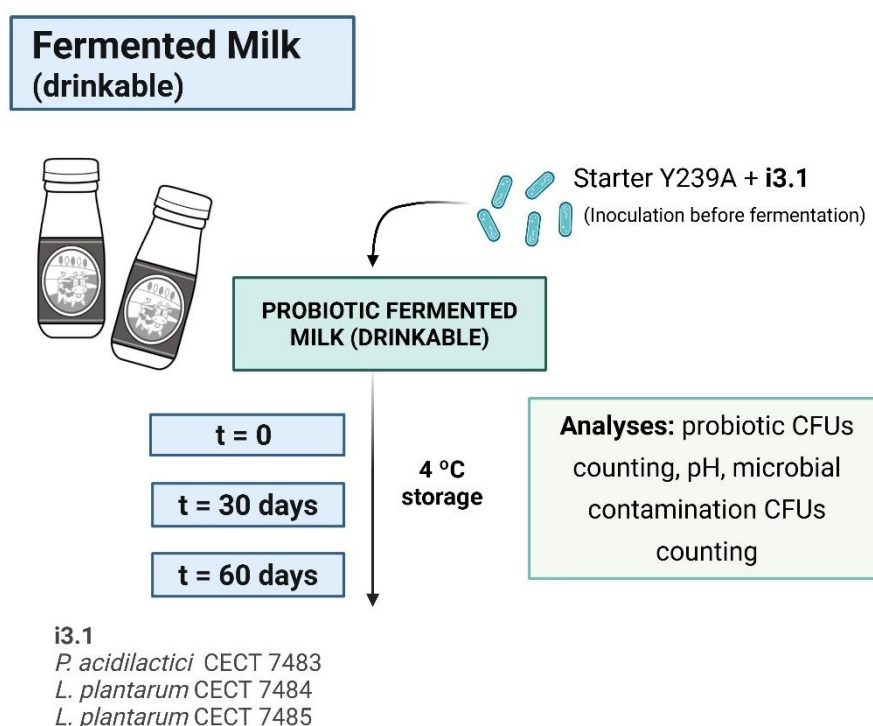


Figure 45. Schematic illustration of the experimental design. Drinkable fermented milk was prepared by co-inoculating UHT milk with the yogurt starter culture Y239A and the probiotic blend I3.1 prior to fermentation. Samples were stored at 4 ± 2 °C and analysed at 0, 30, and 60 days. Evaluated parameters included probiotic viability (CFU counts), pH, and microbiological contamination (post-fermentation and at day 60). The figure was created in <https://BioRender.com>.

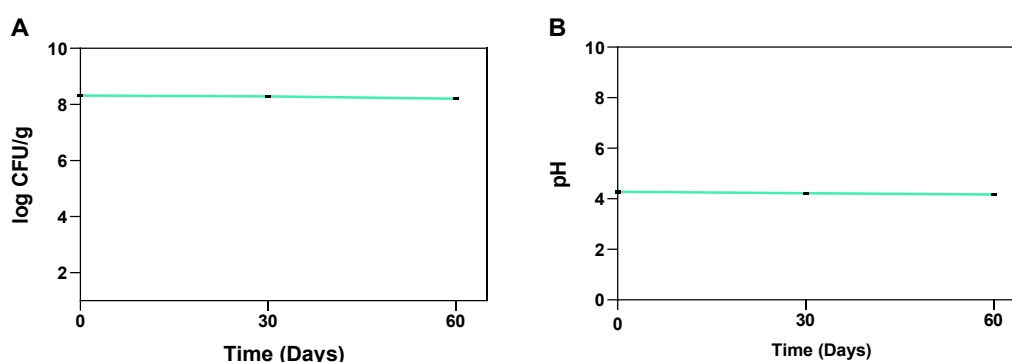


Figure 46. Probiotic viability and pH of drinkable fermented milk with starter Y239A and the I3.1 probiotic blend, during 60 at 4 ± 2 °C. A) Probiotic viability expressed as log CFU/g. B) pH.

The incorporation approach for the I3.1 probiotic blend into milk was designed, as detailed in Section 3.2.17 (Materials and Methods), to ensure a functional probiotic concentration in the final product. The target probiotic concentration for the fermented milk was set at 7.48 log CFU/g per 100 ml serving, corresponding to the clinically validated daily dosage of the blend. To compensate for potential viability losses during storage, a 1.5-fold overage was applied, resulting in an initial inoculum of 7.65 log CFU/g.

As shown in Figure 46, the drinkable fermented milk inoculated with a yogurt starter plus the i3.1 probiotic blend showed high initial viability (8.32 ± 0.03 log CFU/g) which declined minimally to 8.28 ± 0.01 and 8.21 ± 0.01 log CFU/g at 30 and 60 days, respectively.

Concurrently, pH decreased from 4.28 ± 0.03 (day 0) to 4.17 ± 0.01 (day 30) and 3.90 ± 0.07 (day 60), indicating controlled post-acidification under 4 ± 2 °C storage.

These trajectories are consistent with previous reports on probiotic fermented milks. For instance, Mani-López et al. (2014) found that commercially formulated probiotic yogurts maintained viable counts $\geq 10^7$ CFU/mL over 35 days of refrigerated storage, with only modest pH declines (~ 0.17 – 0.50 units) and increased acidity (~ 0.09 – 0.29%). Similarly, Li et al. (2017) demonstrated stable viability and minimal pH changes in yogurt co-fermented with *L. plantarum* over 28 days at 4 °C.

Overall, these findings confirm that the incorporation strategy for I3.1 successfully delivered a clinically validated probiotic dose while preserving viability and maintaining pH within acceptable limits throughout storage, aligning with best practices for functional dairy product development.

4.2.1.1.2 TEST 2: Spoonable fermented milk with probiotic blend I3.1

The stability and properties of fermented milk (spoonable) enriched with the probiotic blend I3.1, were assessed according to the experimental design outlined in Figure 47.

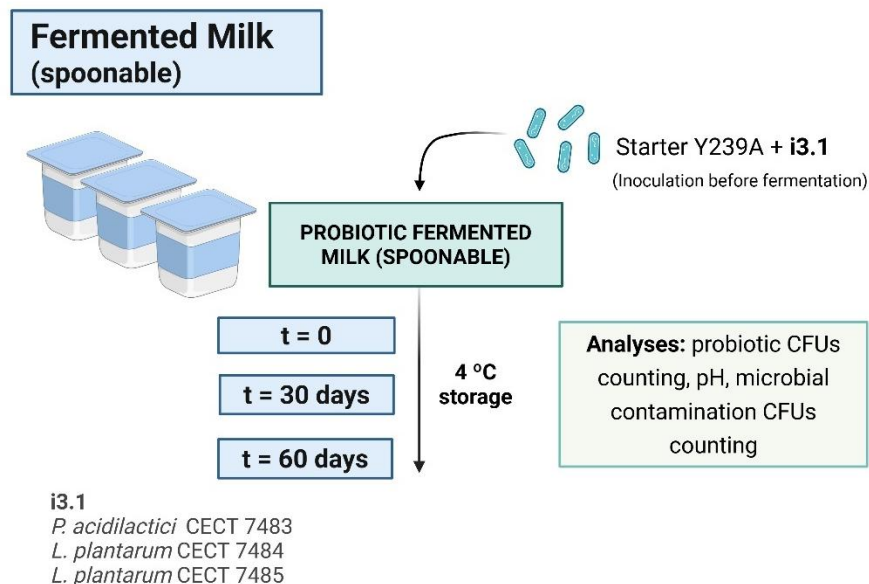


Figure 47. Schematic illustration of the experimental design. Spoonable fermented milk was prepared by co-inoculating UHT milk with the yogurt starter culture Y239A and the probiotic blend I3.1 prior to fermentation. Samples were stored at 4 ± 2 °C and analysed at 0, 30, and 60 days. Evaluated parameters included probiotic viability (CFU counts), pH, and microbiological contamination (post-fermentation and at day 60). The figure was created in <https://BioRender.com>.

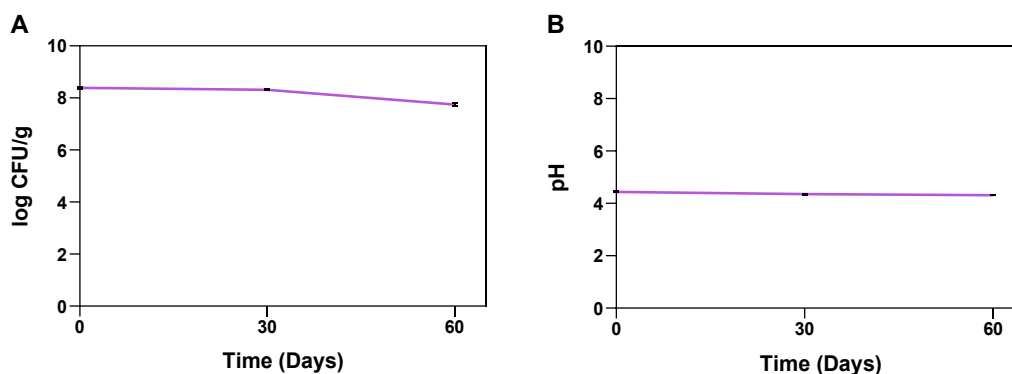


Figure 48. Probiotic viability and pH of spoonable fermented milk with starter Y239A and I3.1 probiotic blend, during 60 days at 4 ± 2 °C. A) Probiotic viability expressed as log CFU/g. B) pH.

The incorporation approach for the I3.1 probiotic blend into milk was designed as detailed in the Section 3.2.17 (Materials and Methods). The target probiotic concentration for the fermented milk was set at 7.48 log CFU/g per 100 ml serving, which corresponds to the clinically validated daily dosage of the blend. A 1.5-fold overage was applied to counteract possible loss during storage, resulting in an initial inoculum of 7.65 log CFU/g.

As shown in Figure 48, the spoonable-style fermented milk formulated with starter Y239A and the i3.1 probiotic blend exhibited a slight but significant (p value $< 0,05$) decline in probiotic viability during refrigerated storage ($4\text{ }^{\circ}\text{C}$) over 60 days. Initial counts averaged approximately 8.39 ± 0.03 log CFU/g, decreasing to 8.31 ± 0.02 log CFU/g at day 30 and 7.75 ± 0.05 log CFU/g at day 60.

The observed decline of ~ 0.64 log units is consistent with previous reports on set-style probiotic yogurts stored for 35 days at $5\text{ }^{\circ}\text{C}$ (Mani-López et al., 2014). Concurrently, pH values remained relatively stable, shifting from 4.44 ± 0.02 at day 0 to 4.31 ± 0.01 at day 60, indicating controlled post-acidification typical of thermophilic starter cultures under cold storage (Donkor et al., 2006). These findings confirm that the spoonable-style formulation provides an adequate environment for probiotic persistence while maintaining physicochemical integrity throughout shelf life, aligning with industry standards and literature benchmarks for functional fermented dairy products.

4.2.1.1.3 TEST 3: Set fermented milk with probiotic blend I3.1

The stability and properties of fermented milk (set) enriched with several probiotic individual strains and a probiotic blend, were assessed according to the experimental design outlined in Figure 49.

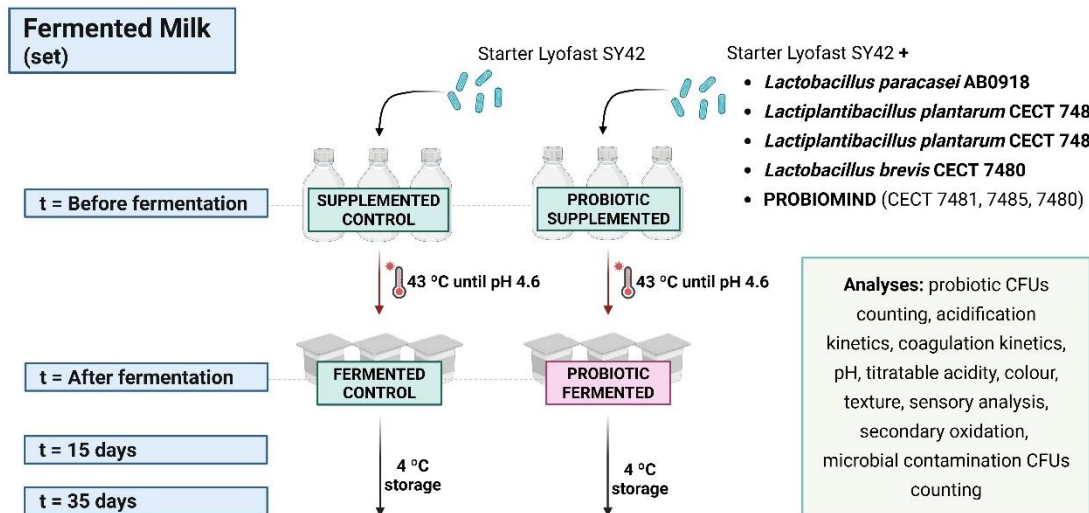


Figure 49. Schematic illustration of the experimental design. Set-type fermented milks were manufactured using the starter culture Lyofast SY42, either alone (control) or supplemented with one of the following probiotic strains or blends: *L. paracasei* AB0918, *L. plantarum* CECT 7481 or CECT 7485, *L. brevis* CECT 7480, PROBIOMIND (1:1:1 mixture of strains CECT 7481/7485/7480). Probiotics were inoculated prior to fermentation, and samples were incubated at $43\text{ }^{\circ}\text{C}$ until reaching pH 4.6 and stored at $4 \pm 2\text{ }^{\circ}\text{C}$ for 35 days. Samples were collected before and after fermentation and at 15 and 35 days of storage. Evaluated parameters included viable probiotic counts (CFU), pH, TA, and microbiological contamination (post-fermentation and at day 35). Colour and texture were assessed after fermentation and at 15 and 35 days. Acidification and

coagulation kinetics were continuously monitored during fermentation. For the AB0918-supplemented formulation, additional analyses included secondary lipid oxidation (MDA–TBARS) and sensory acceptability. The figure was created in <https://BioRender.com>.

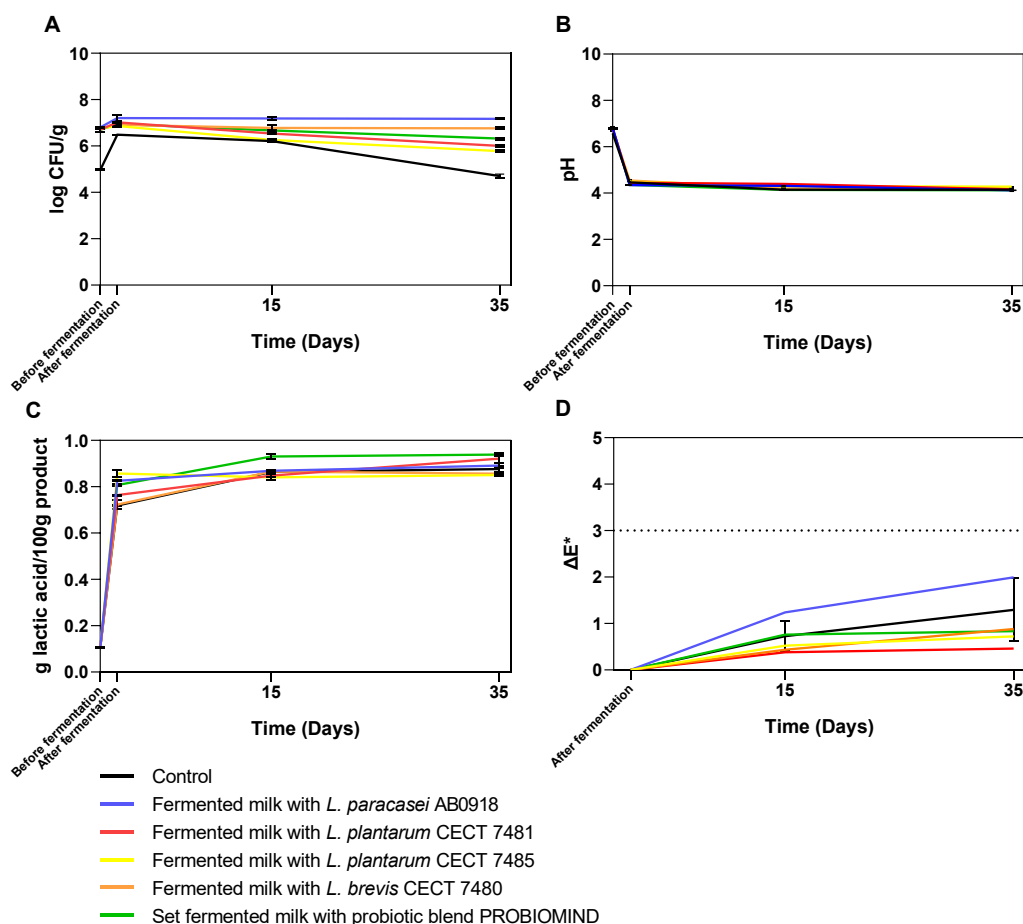


Figure 50. Probiotic viability and physicochemical properties of set fermented milk produced with an industrial yogurt starter (Control) or in co-fermentation with various probiotics (individual strains and a multi-strain blend). Measurements were taken before fermentation, immediately after fermentation, and during storage at 4 ± 2 °C on days 15 and 35. A) Probiotic viability expressed as log CFU/g. B) pH. C) TA expressed as g of lactic acid / 100g of product. D) Colour variation expressed as ΔE^* .

Probiotic Viability During Fermentation and Storage

The incorporation strategy for individual probiotic strains and the multi-strain blend is detailed in Section 3.2.17 (Materials and Methods) and aimed to achieve a target concentration of approximately 7 log CFU/g per 200 mL serving of the final product, without applying any overage.

As shown in Figure 50, initial counts after inoculation ranged from 4.97 ± 0.02 log CFU/mL (starter-only control) to 6.75 ± 0.04 log CFU/mL (probiotic-supplemented formulations). Post-fermentation counts increased to 6.49 ± 0.01 log CFU/mL for the control and 6.99 ± 0.13 log CFU/mL for probiotic treatments. The greatest increase was observed for the

starter culture ($\Delta\log \text{CFU/mL} = +1.52$), followed by *L. paracasei* AB0918 ($\Delta\log \text{CFU/mL} = +0.40$).

During refrigerated storage (4 °C, 35 days), *L. paracasei* AB0918 was the strain exhibiting the smallest decrease in viability ($\Delta\log \text{CFU/mL} = -0.033$), whereas the starter culture showed the most pronounced decline ($\Delta\log \text{CFU/mL} = -1.79$). Among probiotics, *L. plantarum* CECT 7485 displayed the largest reduction ($\Delta\log \text{CFU/mL} = -1.08$), while other strains exhibited intermediate decreases.

This variability highlights the critical role of strain selection in yogurt formulations. Robust strains such as *L. paracasei* AB0918 demonstrate excellent resilience under cold storage, making them strong candidates for extended shelf-life applications. Conversely, less stable strains may require protective strategies including microencapsulation, optimized storage conditions, or initial overdosage, to ensure delivery of functional counts throughout shelf life (Afzaal et al., 2019; Hussein et al., 2021; Radu et al., 2021).

All formulations exhibited the expected acidification pattern: pH successfully decreased from ~ 6.75 to the point where fermentation was stopped (~ 4.5). Only slight post-acidification occurred during storage. Specifically, pH declined from an average of 6.75 ± 0.09 to 4.44 ± 0.08 post-fermentation and further to 4.18 ± 0.06 by Day 35 (Fig. 1B). The starter-only product showed the greatest pH decrease during storage ($\Delta\text{pH} = -0.32$), followed by *Levilactobacillus brevis* CECT 7480 ($\Delta\text{pH} = -0.31$), whereas *L. plantarum* CECT 7485 exhibited the smallest change ($\Delta\text{pH} = -0.22$). These findings confirm that co-fermentation with probiotics did not impair the fermentative performance of the starter culture. The slight acidification observed during storage is typical for yogurt products and is primarily attributed to residual metabolic activity of lactic acid bacteria.

Titrate acidity mirrored pH trends. Initial values were 0.105 ± 0.000 g lactic acid/100 g milk, increasing post-fermentation to 0.795 ± 0.056 g/100 g, and reaching 0.892 ± 0.035 g/100 g by Day 35 (Fig. 1C). Among treatments, during the storage, the product containing the starter alone and the one with *L. plantarum* CECT 7481 exhibited the highest increase ($\Delta\text{TA} = +0.157$ g/100 g), while CECT 7485 showed minimal change ($\Delta\text{TA} = -0.006$ g/100 g). These results align with typical yogurt acidification dynamics and confirm that probiotic addition did not compromise starter-driven fermentation (An et al., 2025).

Colour changes during fermentation and refrigerated storage were minimal across all formulations, indicating that the incorporation of individual probiotic strains or the multi-strain blend did not significantly affect the visual appearance of the fermented milk

compared with the starter-only control. During 35 days of storage at 4 °C, ΔE^* values increased slightly in all treatments but remained well below the threshold of visual perception ($\Delta E^* \approx 3$), confirming good colour stability. Overall, these results indicate that probiotic fermentation and subsequent storage did not promote colour altering reactions in the product. Similar findings have been reported previously; for example, Mani-López et al. (2014) demonstrated that probiotic yogurts and fermented milks exhibit high colour stability during refrigerated storage, with no detectable changes in CIELAB parameters throughout 35 days at 5 °C.

Table 28. Acidification kinetics of milk fermented with the yogurt starter Lyofast SY42, either alone (Control) or in combination with individual probiotic strains or a probiotic blend.

Sample	Te $\pm$ SD (min)	Vm $\pm$ SD (pHu/min)	Tm $\pm$ SD (min)	Ta $\pm$ SD (min)
Starter + <i>L. paracasei</i> AB0918	232.00 $\pm$ 20.88	-0.017 $\pm$ 0.001	96.00 $\pm$ 10.39	36.67 $\pm$ 1.15 ^a
Starter + <i>L. plantarum</i> CECT 7481	238.00 $\pm$ 2.00	-0.017 $\pm$ 0.000	102.00 $\pm$ 4.00	36.67 $\pm$ 1.15 ^a
Starter + <i>L. plantarum</i> CECT 7485	225.33 $\pm$ 6.43	-0.017 $\pm$ 0.001	82.67 $\pm$ 13.32 ^a	30.67 $\pm$ 1.15 ^b
Starter + <i>L. brevis</i> CECT 7480	242.67 $\pm$ 6.11	-0.016 $\pm$ 0.001	113.33 $\pm$ 3.06 ^b	37.33 $\pm$ 2.31 ^a
Starter + PROBIOMIND	234.67 $\pm$ 2.31	-0.016 $\pm$ 0.000	90.67 $\pm$ 18.48	36.67 $\pm$ 1.15 ^a
Starter Lyofast SY42 (Control)	235.33 $\pm$ 24.19	-0.017 $\pm$ 0.000	104.67 $\pm$ 4.62	37.33 $\pm$ 1.15 ^a

One-way ANOVA followed by Tukey's post hoc test was applied; different superscript letters indicate significant differences ($p < 0.05$).

Acidification kinetics parameters:

Te (min): time at which pH 4.6 was reached (isoelectric point of milk proteins).

Vm (pHu·min⁻¹): local slope around the first-derivative minimum (maximum acidification rate).

Tm (min): time to first-derivative minimum (time at which the maximum acidification rate is observed).

Ta (min): time needed for $\Delta pH = 0.08$ from the start (acidification onset speed).

The results of the acidification kinetics analysis are shown in Table 28. The time required to reach pH 4.6 (Te) showed that all treatments followed a comparable acidification trajectory, with values clustering between 225 and 243 min. No statistically significant differences were detected among samples; therefore, none of the formulations, whether containing individual strains, the multi-strain blend, or the starter alone, acidified faster or slower within the resolution of the assay. This uniformity in Te is consistent with previous reports describing

3.5–4 h fermentation windows for thermophilic set-style fermented milk systems under standard incubation conditions (Medeiros et al., 2015; Sanusi et al., 2022).

The maximum acidification rate (V_m) was likewise statistically indistinguishable across formulations, with all samples exhibiting slopes around $-0.016 \pm 0.002 \text{ pHu} \cdot \text{min}^{-1}$. The absence of detectable differences indicates that co-inoculation with probiotic strains does not perturb the peak acidification rate defined by the yogurt starter culture.

In contrast, significant differences emerged in the timing of the acceleration phase (T_m). The formulation containing *L. plantarum* CECT 7485 reached this phase earlier than the sample containing *L. brevis* CECT 7480, while the remaining formulations displayed intermediate behaviour. These T_m variations reflect strain-specific metabolic activation patterns that may modulate early casein destabilization and gel network development, aligning with mechanistic descriptions of acid-induced aggregation (Lucey, 2016).

The acidification onset speed (T_a ; $\Delta\text{pH} = 0.08$) also differed significantly for the formulation containing *L. plantarum* CECT 7485, which showed a faster onset (30.67 min) than all other treatments. Faster or earlier acidification promotes earlier isoelectric precipitation, producing denser protein networks and higher viscoelastic moduli, as established in acid-induced gelation studies (Wang & Zhao, 2022). Reviews of yogurt microstructure further confirm that fermentation kinetics, particularly maximum acidification rate and time to reach target pH, are primary determinants of gel density and mechanical behaviour (Ba et al., 2025).

Overall, these results demonstrate that co-fermentation with different probiotic strains does not alter the main acidification profile driven by the industrial starter, as evidenced by the lack of significant differences in T_e and V_m . However, strain-dependent divergences in T_m and T_a indicate early kinetic modulations that could influence gelation pathways and, consequently, the microstructural and rheological attributes of the final product.

Table 29. Coagulation kinetics of milk during fermentation with yogurt starter Lyofast SY42 with or without different probiotic single strains or blend.

Sample	CT $\pm$ SD (min)	AR $\pm$ SD (mA/min)	GH $\pm$ SD (mA)
Starter + <i>L. paracasei</i> AB0918	134.68 $\pm$ 3.06	0.43 $\pm$ 0.02 ^a	30.96 $\pm$ 1.28 ^{ab}
Starter + <i>L. plantarum</i> CECT 7481	128.02 $\pm$ 4.00	0.43 $\pm$ 0.03 ^a	28.73 $\pm$ 1.36 ^b
Starter + <i>L. plantarum</i> CECT 7485	128.02 $\pm$ 2.00	0.64 $\pm$ 0.04 ^b	35.49 $\pm$ 2.10 ^a
Starter + <i>L. brevis</i> CECT 7480	128.02 $\pm$ 0.00	0.60 $\pm$ 0.03 ^b	33.69 $\pm$ 2.07 ^a
Starter + PROBIOMIND	132.02 $\pm$ 2.00	0.60 $\pm$ 0.06 ^b	34.77 $\pm$ 1.51 ^a
Starter Lyofast SY42 (Control)	129.68 $\pm$ 2.08	0.57 $\pm$ 0.09	35.04 $\pm$ 2.06 ^a

One-way ANOVA followed by Tukey's post hoc test was applied. Different superscript letters indicate significant differences ($p < 0.05$).

Coagulation parameters (Optigraph):
CT (min): Time at which the first derivative of the coagulation curve reaches its maximum (coagulation time).
AR (mA/min): Aggregation rate, slope of the coagulation curve with respect to time.
GH (mA): Gel hardness, first value when the coagulation curve becomes straight (indicative of curd firmness).

The results of the coagulation kinetics analysis are shown in Table 29. The coagulation time (CT) values recorded in this study ranged from 128.02 to 134.68 min, indicating that the onset of gel formation was only marginally affected by either the individual probiotic strains or the multistrain blend. The narrow CT interval suggests that the tested adjunct cultures did not induce substantial alterations in the early destabilization of casein micelles. In contrast, the aggregation rate (AR) exhibited more pronounced differences among treatments, with values ranging between 0.43 and 0.64 mA/min. The highest AR values were measured in formulations containing *L. plantarum* CECT 7485 (0.64 $\pm$ 0.04 mA/min), PROBIOMIND, *L. brevis* CECT 7480. Conversely, the lowest AR values were observed in samples containing *L. paracasei* AB0918 (0.43 $\pm$ 0.02 mA/min) and *L. plantarum* CECT 7481. Compared with CT, the broader variation in AR suggests that acidification kinetics and associated microstructural rearrangements are more sensitive to the auxiliary metabolic pathways of the adjunct cultures.

Gel hardness (GH), measured at signal stabilization, ranged from 28.73 to 35.49 mA and followed trends similar to those observed for AR. The firmest gels were produced by formulations containing *L. plantarum* CECT 7485 (35.49 $\pm$ 2.10 mA), the control, PROBIOMIND, and *L. brevis* CECT 7480, whereas the softest gels were obtained with *L. plantarum* CECT 7481 (28.73 $\pm$ 1.36 mA). The positive association between AR and GH aligns with established links between acidification rate, protein network density, and mechanical gel properties, whereby faster aggregation promotes the formation of tighter and

stronger protein matrices. Nevertheless, strain-dependent factors such as exopolysaccharide (EPS) biosynthesis, proteolytic activity, and fermentation profiles can modulate this relationship, contributing to variability in gel firmness (An et al., 2025; Arab et al., 2023). Overall, the findings indicate that CT was not influenced by the adjunct cultures, while AR and GH exhibited clear strain-associated differences. These results highlight that specific probiotic strains can exert measurable effects on the structural development and mechanical attributes of acid milk gels (Kuerman et al., 2024).

Table 30. Textural properties of fermented milk using yogurt starter Lyofast SY42 with and without different probiotic strains or blend.

Time (days)	Treatment	Deformability* (N/mm)	Hardness* (N)	Work* (mJ)
1	<i>L. paracasei</i> AB0918	0.243 ± 0.018 ^{bB}	0.140 ± 0.013	1.264 ± 0.128
	<i>L. plantarum</i> CECT 7481	0.311 ± 0.011 ^{aB}	0.176 ± 0.013 ^A	1.589 ± 0.110 ^A
	<i>L. plantarum</i> CECT 7485	0.267 ± 0.008 ^b	0.137 ± 0.007	1.260 ± 0.050
	<i>L. brevis</i> CECT 7480	0.249 ± 0.033 ^{ab}	0.135 ± 0.017	1.223 ± 0.129
	PROBIOMIND	0.223 ± 0.015 ^b	0.130 ± 0.023	1.118 ± 0.258
	Starter Lyofast SY42 (control)	0.241 ± 0.021 ^{ab}	0.142 ± 0.011	1.205 ± 0.042
15	<i>L. paracasei</i> AB0918	0.281 ± 0.026 ^A	0.182 ± 0.019	1.678 ± 0.165
	<i>L. plantarum</i> CECT 7481	0.252 ± 0.004 ^A	0.139 ± 0.009 ^B	1.327 ± 0.055 ^B
	<i>L. plantarum</i> CECT 7485	0.268 ± 0.021	0.145 ± 0.004	1.331 ± 0.005
	<i>L. brevis</i> CECT 7480	0.250 ± 0.017	0.120 ± 0.009	1.094 ± 0.077
	PROBIOMIND	0.228 ± 0.047	0.139 ± 0.014	1.274 ± 0.162
	Starter Lyofast SY42 (control)	0.236 ± 0.034	0.145 ± 0.033	1.149 ± 0.055
35	<i>L. paracasei</i> AB0918	0.245 ± 0.023 ^{AB}	0.171 ± 0.003 ^a	1.569 ± 0.053 ^a
	<i>L. plantarum</i> CECT 7481	0.234 ± 0.023 ^{AB}	0.124 ± 0.004 ^{cB}	1.185 ± 0.037 ^{bc}
	<i>L. plantarum</i> CECT 7485	0.243 ± 0.028	0.154 ± 0.005 ^b	1.355 ± 0.057 ^b
	<i>L. brevis</i> CECT 7480	0.227 ± 0.023	0.134 ± 0.004 ^c	1.238 ± 0.088 ^b
	PROBIOMIND	0.243 ± 0.067	0.138 ± 0.013 ^{abc}	1.251 ± 0.141 ^{ab}
	Starter Lyofast SY42 (control)	0.224 ± 0.038	0.143 ± 0.016 ^{abc}	1.287 ± 0.004 ^b

Mean ± SD.

Two-way ANOVA (strain × time) followed by Tukey post hoc test was applied; different superscript letters indicate significant differences ($p < 0.05$).

Different lowercase letters indicate significant differences between treatments within the same time point, for each different texture parameter.

Different uppercase letters indicate significant differences between different timepoint within the same treatment, for each different texture parameter.

Deformability (N/mm): force per unit displacement; higher values indicate a less deformable gel.

Hardness (N): peak force at compression; a proxy for curd firmness under small strain.

Work (μJ): energy required to deform the sample; integrates firmness and structural cohesion.

Texture profile analysis revealed a dynamic evolution of gel structure during refrigerated storage, with clear straindependent effects that were most pronounced at early fermentation and late shelf life (Table 30).

At the beginning of storage (Day 1), significant differences in deformability were detected among formulations, with samples containing *Lactiplantibacillus plantarum* CECT 7481 differing from those containing *L. plantarum* CECT 7485 and the PROBIOMIND blend. No significant differences were observed for hardness or work at this time point, indicating that early textural variability was primarily related to gel deformability rather than overall gel strength or cohesiveness. By midstorage (Day 15), textural differences among samples were no longer significant for deformability, hardness, or work. This convergence suggests that postacidification structural rearrangements and moisture redistribution within the protein matrix led to a homogenization of gel mechanical properties, regardless of the probiotic strain or blend employed.

At the end of shelf life (Day 35), deformability values remained statistically comparable across all formulations, confirming the absence of further gel stiffening beyond midstorage. Despite this convergence in deformability, significant differences emerged in hardness and work. The formulation containing *Lactocaseibacillus paracasei* AB0918 exhibited the highest gel strength and energy requirement at this stage, indicating sustained network integrity throughout storage. In contrast, formulations containing *L. plantarum* CECT 7481, which exhibited greater resistance to deformation at early storage, displayed reduced hardness and work at the end of shelf life. This evolution is consistent with straindependent proteolytic activity and increased water mobility, both of which can progressively weaken the protein network during prolonged refrigerated storage (Lucey & Singh, 1997; Sodini et al., 2004).

Throughout storage, only subtle and nonvisible syneresis was observed across all formulations, indicating that moisture redistribution occurred primarily within the gel matrix without resulting in macroscopic whey separation. This behaviour aligns with the stable deformability observed after Day 15 and suggests adequate water holding capacity regardless of probiotic supplementation.

Statistical analysis confirmed that significant, albeit limited, differences in texture parameters were mainly detected at Day 1 and Day 35 among probiotic containing samples, while no systematic differences were observed between the starter control and inoculated formulations at any storage time point. These results support previous findings

demonstrating that carefully selected adjunct probiotic cultures can be incorporated into yogurt without adversely affecting gel structure or consumer relevant textural attributes (Ilić et al., 2024; L. Wang et al., 2022).

Overall, the data highlight the importance of targeted strain selection to modulate gel firmness and cohesiveness at specific stages of shelf life, while preserving overall textural stability and process robustness in fermented milk products.

Table 31. Comparative analysis of secondary lipid oxidation (MDA-TBARS) in milk beverages: impact of yogurt starter and *L. paracasei* AB0918 fermentation.

Sample	Average $\pm$ SD (MDA $\mu\text{mol/L}$)	Oxidation Reduction vs. Control (%)
Milk (Blank)	5.13 ± 2.58^a	
Starter Lyofast SY42	1.36 ± 0.73^b	73.55
Starter + <i>L. paracasei</i> AB0918	1.65 ± 1.10^b	67.89
Both pairwise t-tests and one-way ANOVA with multiple comparisons were applied across the three groups, yielding consistent results. Different superscript letters indicate statistically significant differences ($p < 0.05$).		

Lactocaseibacillus paracasei AB0918 was selected for further investigation based on both its commercial relevance and its high potential for technological and functional innovation. Additional analyses were carried out, in dairy and plant-based matrices, to determine whether this strain exhibits unique traits, that could support its patentability and justify its incorporation into innovative foods.

The evaluation of secondary lipid oxidation through MDA–TBARS analysis under forced oxidation conditions (50 °C for 48 h) revealed a marked protective effect of *L. paracasei* AB0918-fermentation against oxidative deterioration (Table 31). Raw milk exhibited the highest TBARS value (5.13 ± 2.58 MDA $\mu\text{mol/L}$), confirming its susceptibility to lipid peroxidation when exposed to thermal stress. In contrast, fermented samples showed a substantial reduction in MDA-reactive compounds, with the starter-only yogurt presenting a value of 1.36 ± 0.73 $\mu\text{mol MDA/L}$ and the co-fermented product with *L. paracasei* AB0918 registering 1.65 ± 1.10 $\mu\text{mol MDA/L}$. These values fall within the lower range typically reported for fresh milk, suggesting limited lipid deterioration in the fermented products. Although the control milk reached 5.13 $\mu\text{mol MDA/L}$ (≈ 0.37 mg/kg), this level remains below the threshold associated with perceptible sensory oxidation in milk (> 1.3 mg/L, ≈ 18 $\mu\text{mol MDA/L}$), as reported by Johnson et al. (2015). These reductions correspond to

oxidation decreases of approximately 73.55 % and 67.89 % compared to the control, respectively. Statistical analysis indicated that both fermented treatments were significantly different from milk ($p < 0.05$) but not from each other, suggesting that the antioxidative effect is primarily attributable to the fermentation process itself rather than to the presence of AB0918.

This outcome aligns with previous reports demonstrating that lactic acid fermentation enhances oxidative stability by lowering pH and reducing pro-oxidant activity, while generating peptides and metabolites with radical-scavenging properties (Habib et al., 2025; I. T. Khan et al., 2019; Lin & Yen, 1999; Park & Nam, 2015). Interestingly, the similarity in TBARS values between the starter-only and AB0918 formulations contrasts with the greater colour variation (ΔE^*) observed for AB0918 at the end of shelf life, implying that the chromatic changes may be driven by non-lipid reactions such as protein carbonylation or Maillard-derived compounds rather than lipid oxidation (Fan et al., 2023). Overall, these results confirm that yogurt fermentation significantly enhances oxidative stability under severe stress conditions, supporting its role in prolonging product quality during storage.

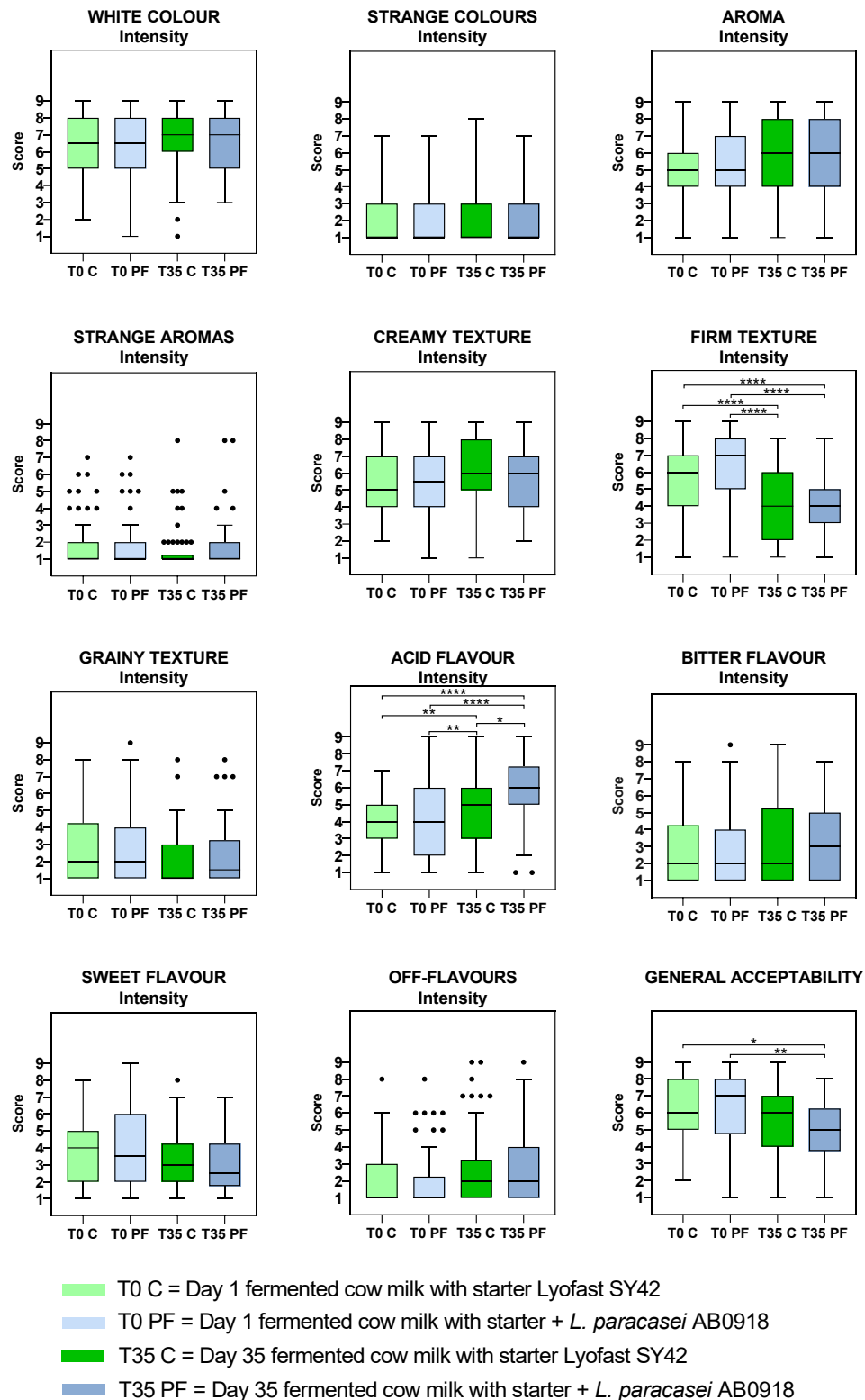


Figure 51. Sensory analyses of milk fermented with starter (C, control) and with starter plus *L. paracasei* AB0918 (PF, probiotic fermented), assessed at the beginning (T0) and end (T35) of 35 days of storage at 4 ± 2 °C. Box plots display the distribution of scores for key sensory attributes, as evaluated by 58 untrained panellists. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To assess consumer acceptance, a sensory evaluation was performed using four samples: freshly prepared control and probiotic-fermented formulations (T0), and their counterparts after 35 days of storage (T35). A total of 58 untrained panellists (70.69% female, 29.31% male; 19–65 years) participated, and results are summarized in Figure 51.

At T0, no significant differences were detected between the control and probiotic-fermented samples for appearance or aroma attributes. White colour, presence of strange colours, aroma intensity, milky aroma, and strange aromas all received comparable scores between formulations, indicating that co-fermentation with *L. paracasei* AB0918 did not introduce detectable visual or olfactory deviations. This observation aligns with previous reports showing that *L. paracasei* strains generally do not generate perceptible off-aromas in fermented dairy matrices (Gu et al., 2020).

Firm texture scores at T0 did not differ significantly between formulations (C: 6.26 ± 2.16 ; PF: 5.93 ± 1.97). A significant decrease in firmness from T0 to T35 was observed for both products, reaching values of approximately. Creamy texture remained stable throughout storage, similar to grainy texture, which consistently received low scores and did not differ significantly between formulations or timepoints.

Regarding flavour, acidity increased significantly from T0 to T35 in both formulations, reaching 5.09 ± 2.01 in the control and 6.00 ± 1.94 in the probiotic-fermented sample. These time-dependent changes align with the progressive post-acidification typically observed during refrigerated storage of fermented milk products (Mani-López et al., 2014). Bitterness, sweetness, and off-flavours remained stable over storage and did not differ between formulations, supporting the absence of oxidative rancidity, an interpretation consistent with the lipid oxidative stability observed in the TBARS analysis (Table 31).

General acceptability decreased significantly for PF T35 compared with T0 for both formulations, consistent with the higher acidity reached by the probiotic product at the end of storage and in line with previous reports indicating that excessive acidity and texture softening can reduce consumer liking (Deshwal et al., 2021). The lack of divergence between control and probiotic products at T35 suggests that *L. paracasei* AB0918 did not negatively affect overall consumer acceptance under the tested conditions.

Overall, the sensory analysis demonstrates that co-fermentation with *L. paracasei* AB0918 preserved sensory attributes comparable to the control at T0, and that both products exhibited similar time-dependent changes during storage, despite the higher post-acidification observed in the PF sample at T35. These findings indicate that the strain can be incorporated

without compromising initial consumer acceptance, while underscoring the importance of controlling post-acidification to maintain sensory quality throughout shelf life.

4.2.2 Bakery Products

4.2.2.1 Sandwich Bread

The stability and properties of sandwich bread enriched with the probiotic strain *B. coagulans* Unique IS-2 were assessed according to the experimental design outlined in Figure 52.

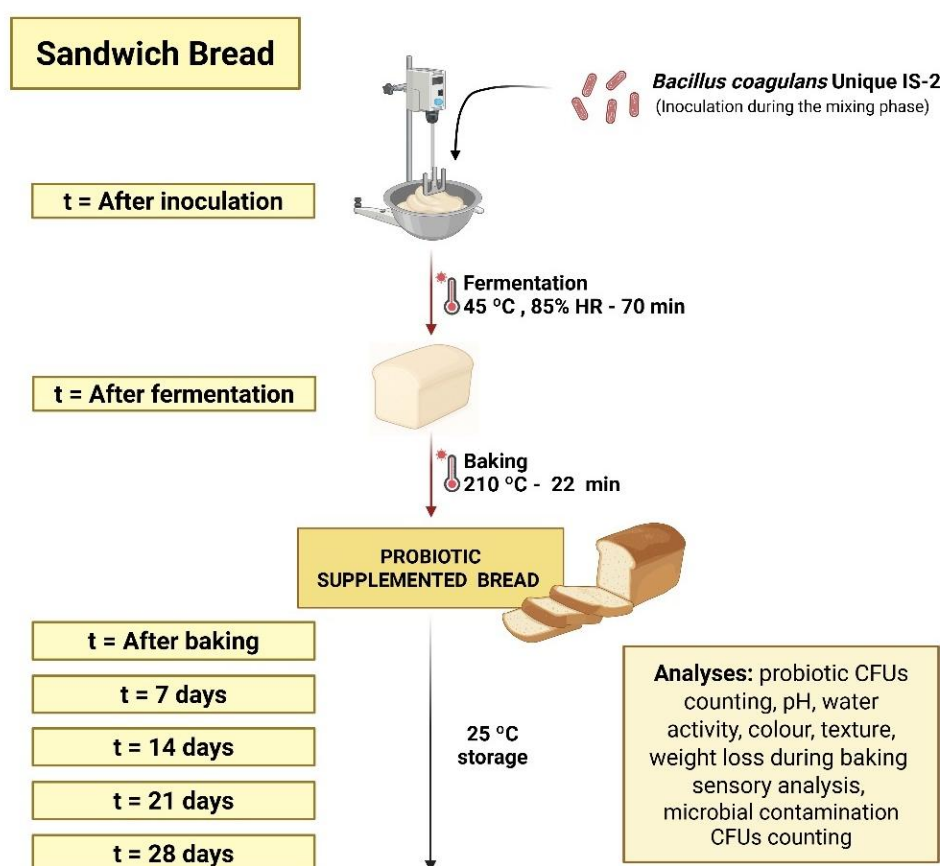


Figure 52. Schematic illustration of the experimental design. Sandwich bread was formulated with the probiotic strain *B. coagulans* Unique IS-2. After inoculation, dough underwent fermentation at 45 °C and 85 % RH for 70 minutes, followed by baking at 210 °C for 22 minutes. The probiotic-supplemented bread was packaged in sterile plastic bags and stored at 25 ± 5°C. Analyses were performed before and immediately after baking and at 7, 14, 21, and 28 days of storage. Evaluated parameters included viable probiotic spore count (CFU), pH, aw, colour, texture, weight loss during baking, and microbiological contamination (post-baking and at day 28). Additionally, a sensory analysis was performed using freshly produced samples. The figure was created in <https://BioRender.com>.

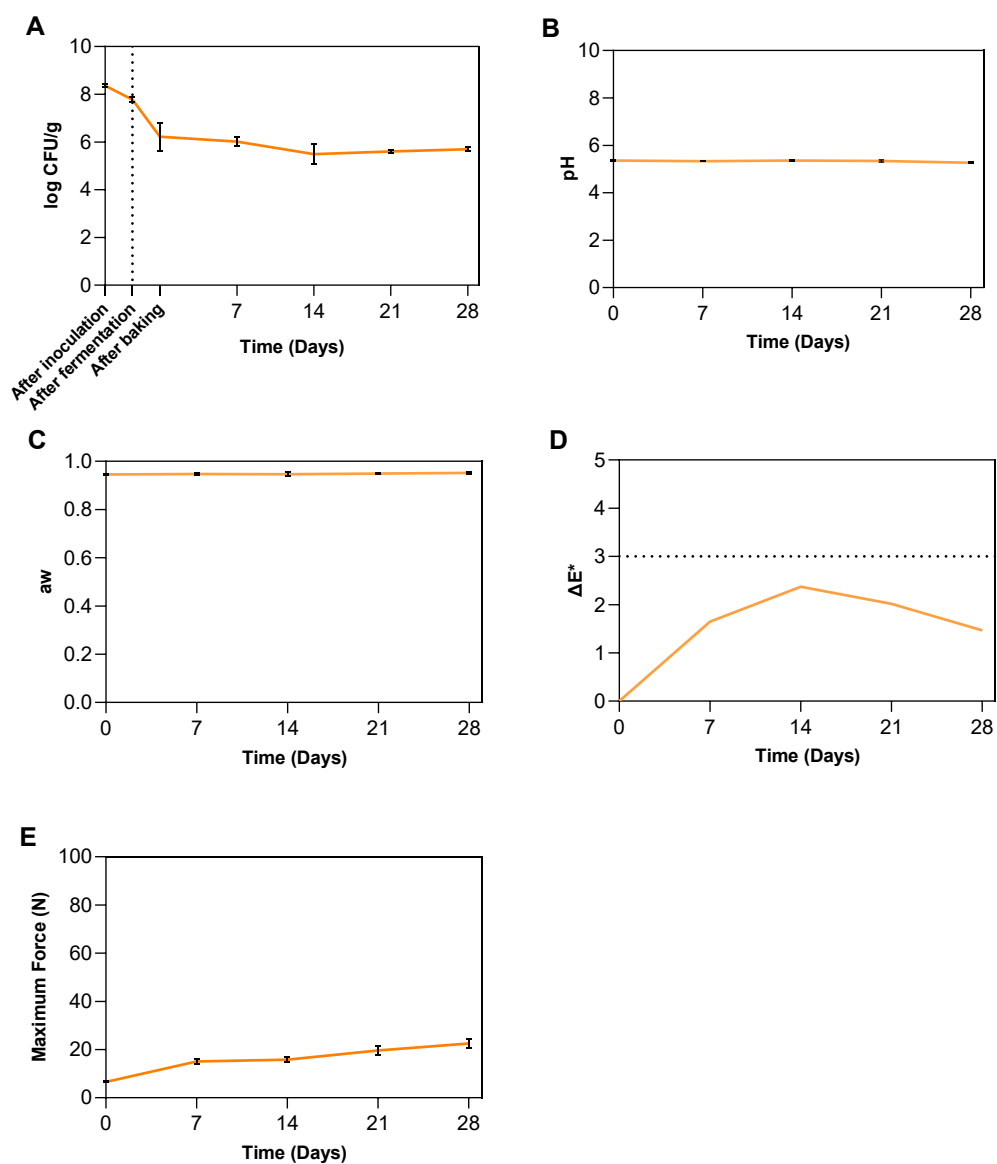


Figure 53. Probiotic viability and physicochemical characteristics of sandwich bread fortified with *B. coagulans* Unique IS-2 during 28 days of storage at room temperature (25 ± 5 °C). A) Viability of *B. coagulans* spores expressed as log CFU/g. B) pH. C) aw. D) Colour variation expressed as ΔE^* . E) Hardness measured as maximum force (N).

Table 32. Enumeration of viable *B. coagulans* Unique IS-2 (CFU) in sandwich bread crust and crumb at post-baking (T0) and following 28 days of storage (T28).

	log CFU/g* T0	log CFU/g* T28
Crumb	6.41 ± 0.56 ^a	5.51 ± 0.05 ^a
Crust	5.10 ± 0.62 ^b	4.05 ± 0.11 ^b

*Mean ± SD.

For each time point, a t-test was performed to compare crust and crumb results. Different superscript letters indicate statistically significant differences ($p < 0.05$).

The incorporation strategy for *B. coagulans* Unique IS-2 spores into the bread dough was designed to ensure a functional probiotic concentration in the final baked product, as detailed in Section 3.2.7 (Materials and Methods). The spores were introduced prior to the kneading phase. The target probiotic concentration for the sandwich bread was set at 7.46 log CFU/g per 70 g serving, which corresponds to the clinically validated daily dosage of the strain. To effectively counteract the anticipated viability reduction inherent in processing steps such as fermentation and the high thermal load of baking, a 10-fold overage was applied, resulting in an initial inoculum of 8.46 log CFU/g.

As shown in Figure 53, the initial probiotic concentration after kneading was 8.36 ± 0.07 log CFU/g, which decreased to 7.70 ± 0.09 log CFU/g after fermentation and further to 6.23 ± 0.59 log CFU/g following baking. Notably, the post-baking concentration did not meet the predefined target dose (7.46 log CFU/g). The overall reduction of 2.13 log CFU/g observed during fermentation and baking is attributed to the partial germination of spores followed by their subsequent inactivation during the thermal processing phase.

This decline is likely attributed to the partial germination of *B. coagulans* spores during fermentation, increasing their sensitivity to heat exposure during baking (Jao et al., 2011). Nonetheless, after the initial post-baking decline, the probiotic demonstrated remarkable stability within the bread matrix throughout the study, indicating that the remaining probiotic population can survive well in the matrix during storage conditions. The CFU counts after baking decreased from 6.23 ± 0.59 to 5.69 ± 0.10 CFU/g at the end of the study (T28).

These results align with findings from probiotic bakery research. For instance, *B. coagulans* GBI-30 6086 has demonstrated excellent resilience through both processing and storage stages, showing an average of 1.5 CFU/g reduction during baking, and maintaining viability with minimal losses under standard storage conditions (Almada-Érix et al., 2022).

Spatial analysis of probiotic distribution revealed significantly higher viable counts in the crumb compared to the crust, with approximately a 1-log difference at both sampling times (T0 and T28 days) (Table 32).

This observation supports the hypothesis that the internal bread matrix provides a more favourable microenvironment, likely due to reduced heat exposure during baking.

Nevertheless, a major challenge persists: the final probiotic concentration did not meet the intended therapeutic threshold. The observed loss in viability indicates that the initial 10-fold overage was insufficient. Back-calculations reveal that achieving the target post-baking dose would necessitate a substantially higher initial inoculum, approximately a 97-fold overage, to compensate for the viability losses incurred during processing.

To address this, future studies may explore process-optimization strategies aimed at reducing thermal impact, such as lowering baking temperature, reducing bread size, or adjusting fermentation parameters (Cinbaş et al., 2024). Additionally, advanced protective technologies like microencapsulation represent a promising alternative to enhance thermal stability and ensure delivery of viable probiotics to the consumer (Longoria-García et al., 2018). Finally, screening and evaluating alternative *Bacillus* spp. strains with inherently higher heat tolerance could further improve post-baking viability and broaden the repertoire of spore-forming probiotics suitable for baked goods.

Key physicochemical parameters of the sandwich bread remained largely stable over the 28-day storage period.

During processing, an average weight loss of 12.69% was observed as a result of the baking step, which is consistent with the moisture evaporation and water vapor migration phenomena that occur during bread baking, as water is progressively lost from the dough matrix through evaporation at the surface and diffusion from the crumb to the crust under thermal gradients. Comparable baking-induced weight losses have been widely reported in bread systems and are primarily attributed to water vaporization during thermal processing, with typical values ranging from approximately 10–25% depending on formulation and baking conditions (Papasidero et al., 2015; Purlis & Salvadori, 2009; Waldert et al., 2025).

The pH and aw values measured throughout the study are critical physicochemical parameters that directly influence microbial safety, probiotic viability, and shelf life of the bread. In this formulation, both parameters remained within expected ranges for similar baked products, aligning with established benchmarks for microbial stability in bread matrices (Alpers et al., 2021; D'Alessandro et al., 2024).

The pH stability observed during storage (5.34 ± 0.02), is particularly important, as fluctuations in acidity can significantly alter not only the sensory profile, including taste and aroma, but also impact probiotic cell viability. An excessively low pH may result in increased stress on probiotic cells, while a rise in pH could promote the growth of spoilage microorganisms. The consistent pH values throughout the shelf-life suggested a stable internal environment, which supports the integrity of both the sensory and microbiological characteristics of the product.

The product also demonstrated relatively high, but stable, a_w (0.948 ± 0.003), which indicates the retention of moisture within the bread matrix. This is beneficial for maintaining the desired softness and chewiness over time, key attributes for consumer acceptability. High a_w typically increases the risk of microbial spoilage; however, in this case, no such spoilage was observed during the study period, likely due to the low pH. Importantly, the high moisture content did not induce germination of *B. coagulans* spores, indicating that the environmental conditions (e.g., nutrient availability, absence of triggering stressors) remained favourable for maintaining the probiotic in its dormant, spore form (Setlow, 2003). This balance contributes to both the shelf-life stability and functional integrity of the product. Bread hardness exhibited a gradual increase from 6.56 ± 0.20 N, after baking, to 23.49 ± 1.49 N (T28). The texture evolution exhibited a pattern consistent with the inherent staling processes observed in conventional bakery products. The progressive increase in hardness over the storage period is primarily attributed to well-documented phenomena such as starch retrogradation and moisture redistribution within the bread matrix, which are characteristic processes influencing textural firming in bakery science (Kulp & Ponte, 1981; Y. Zhang et al., 2024).

The colour stability was well-maintained throughout the storage period, and only minor fluctuations in total colour difference ($\Delta E^* < 3$) were observed. This consistent colour profile strongly suggests that the incorporation of *B. coagulans* Unique IS-2 spores does not significantly promote undesirable Maillard reactions or oxidative discoloration during either processing or subsequent storage, and align with previous studies on probiotic-enriched bread, where the presence of *B. coagulans* GBI-30 6086 did not significantly alter colour parameters (L^* , a^* , and b^*) relative to untreated controls (Almada-Érix et al., 2022). This finding is particularly relevant for consumer acceptance, as perceptible changes in colour are often perceived as indicators of undesirable chemical transformations or potential microbial spoilage.

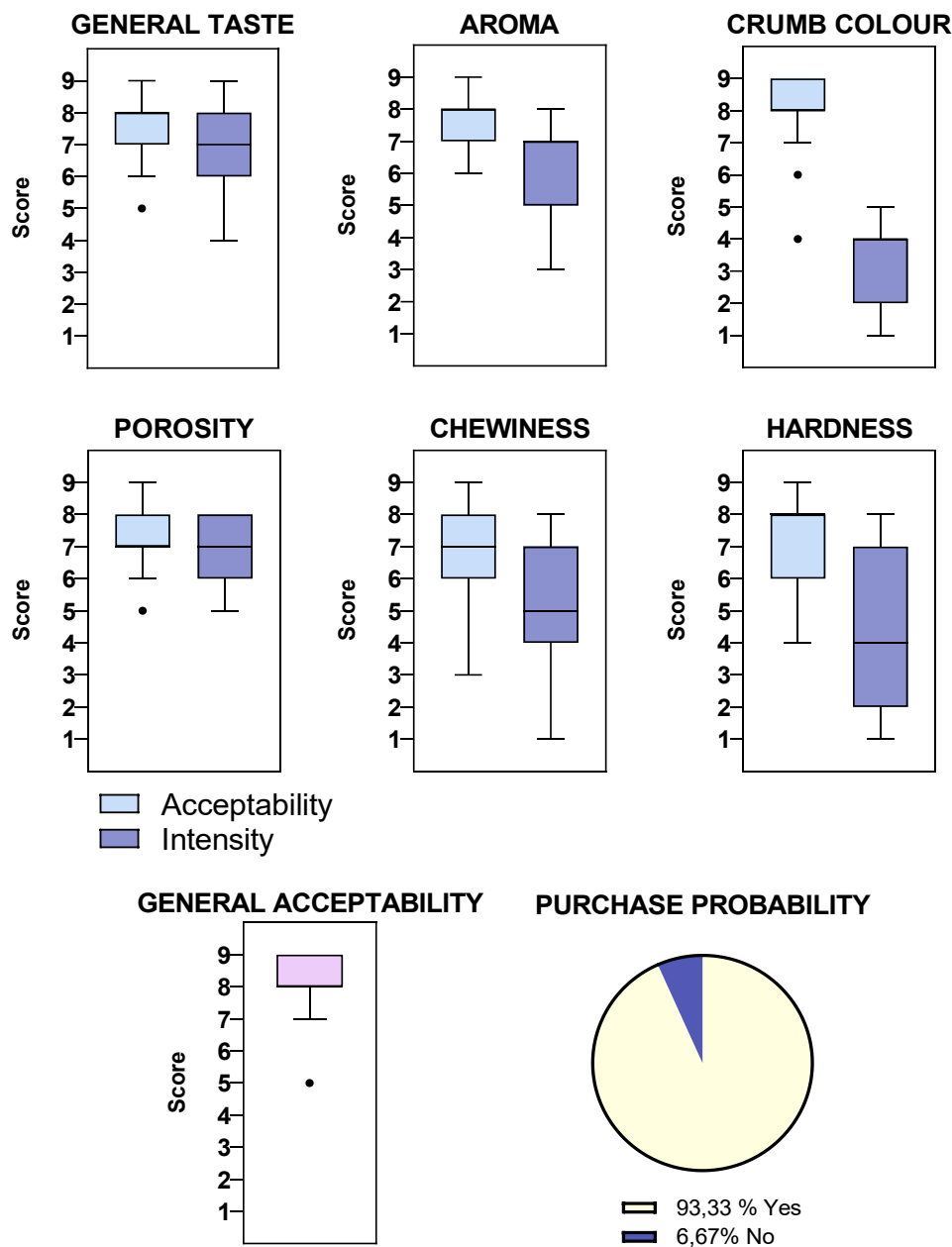


Figure 54. Sensory analysis results of sandwich bread containing the probiotic strain *B. coagulans* Unique IS-2. The boxplots illustrate the scores for sensory attributes, categorized by acceptability (light blue) and intensity (dark blue), while the pie chart illustrates the scores about the purchase probability, as evaluated by 15 untrained panellists.

To assess consumer acceptance, a sensory evaluation was conducted the day after sample production with a panel of 15 untrained panellists (66.7% female, 33.3% male; 23–69 years), and the results are summarized in Figure 54.

As no control samples were included, panellists were informed that they were assessing sandwich bread enriched with probiotics. Consequently, their evaluations focused on the intrinsic product characteristics rather than comparing probiotic addition against a control. Overall, the bread received favourable evaluations across a wide range of sensory parameters, reflecting high consumer acceptability.

The attribute "General Acceptability" obtained a notably high median score of 8.07 ± 1.10 , indicating broad consumer approval. Similarly, other hedonic dimensions such as general taste (7.40 ± 1.12), aroma (7.47 ± 0.99), crumb colour (7.93 ± 1.39), and porosity (7.33 ± 0.98) were rated highly, suggesting that the probiotic incorporation did not negatively affect organoleptic quality. Textural parameters also received satisfactory scores, with chewiness (6.93 ± 1.71) and hardness (7.00 ± 1.73) being within desirable ranges, indicating a pleasant mouthfeel.

These findings were further reinforced by a high purchase intent, with 93.33% of the panellists reporting that they would consider buying the product, highlighting the market potential of the probiotic formulation.

Sensory acceptability remains one of the main challenges in the development of probiotic foods, particularly in fermented matrices (Gámbaro & McSweeney, 2020).

In this context, the present findings are especially significant, as they demonstrate that the incorporation of *B. coagulans* spores into bread can be optimized without major formulation changes, ensuring commercial feasibility. The presence of probiotics did not alter the product's natural flavour or aroma, a critical factor for maintaining consumer familiarity and facilitating successful product adoption.

In this context, the findings are particularly relevant, as they demonstrate that *B. coagulans* spores can be incorporated into bread without substantial formulation adjustments while maintaining the product natural flavour and aroma, thus supporting commercial feasibility. These findings collectively demonstrate the potential of sandwich bread as a viable food matrix for delivering *B. coagulans* Unique IS-2, particularly regarding its post-baking stability and consumer acceptance. However, the ultimate efficacy of such a product is contingent upon the optimization of the probiotic dosage.

4.2.3 Beverage Products

4.2.3.1 Alcohol-free Beer

The stability and properties of alcohol-free beer enriched with the probiotic strain *B. coagulans* Unique IS-2, were assessed according to the experimental design outlined in Figure 55.

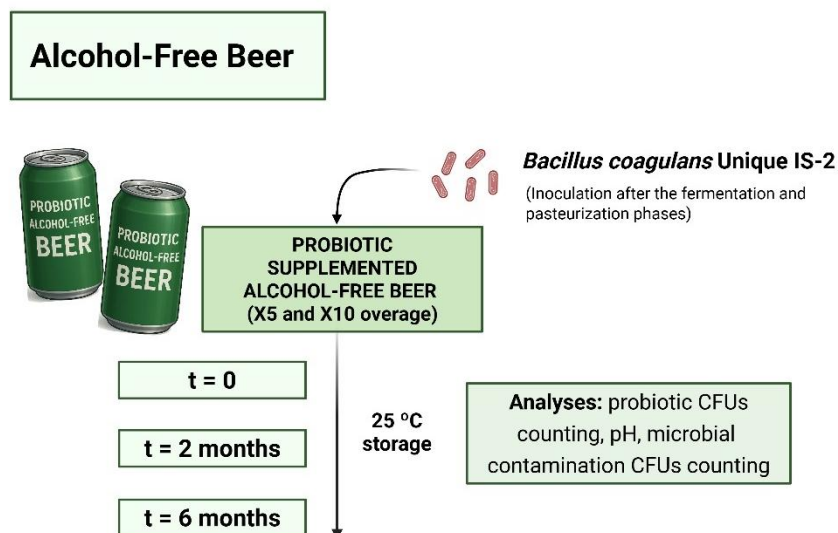


Figure 55. Schematic representation of the experimental design. Alcohol-free beer was supplemented with *B. coagulans* Unique IS-2 following completion of the fermentation and pasteurization steps. Two formulations were prepared, corresponding to 5-fold and 10-fold overages of the clinical dose (2×10^9 CFU), and stored at 25 ± 5 °C. Evaluated parameters included viable probiotic spore count (CFU) and pH and were assessed at 0, 2, and 6 months of storage, while microbiological contamination analyses were conducted at the beginning and end of the storage period. The figure was created in <https://BioRender.com>.

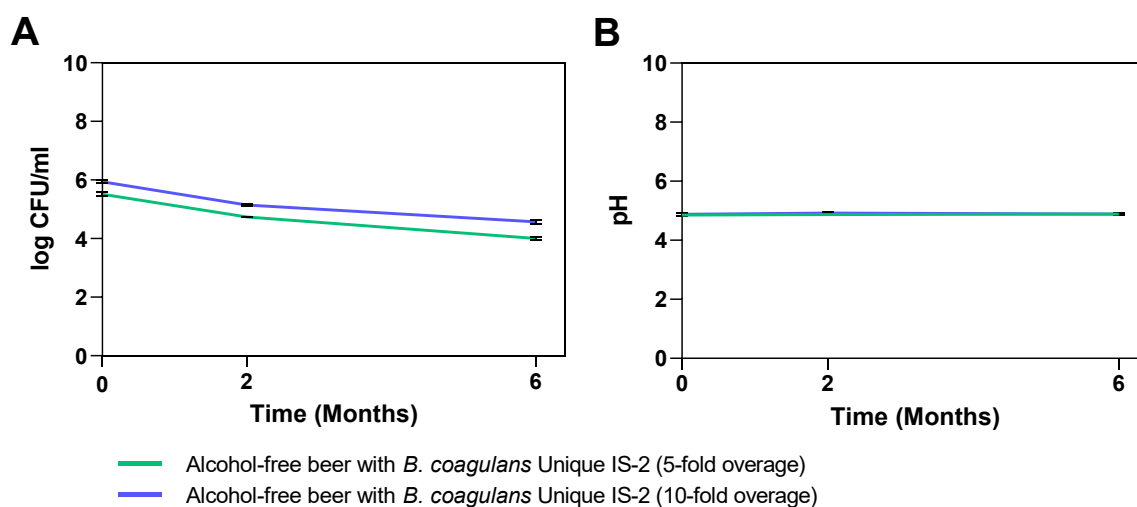


Figure 56. Probiotic viability and pH of alcohol-free beer fortified with two different overage, 5-fold and 10-fold of probiotic strain *B. coagulans* Unique IS-2, over 6 months storage at room temperature (25 ± 5 °C). A) Viability of *B. coagulans* Unique IS-2 spores expressed as log CFU/ml. B) pH.

The incorporation strategy for *B. coagulans* Unique IS-2 spores into the alcohol-free beer was developed, as described in Section 3.2.18 (Materials and Methods) to ensure the delivery of a functional probiotic dose in the final product. The target probiotic concentration for the beer was set at 6.66 log CFU/ml per 440 ml serving, which corresponds to the clinically validated daily dosage of the strain. To effectively counteract the anticipated viability reduction due to the harsh environment of beer, a 5-fold and 10-fold overages were applied, resulting in an initial inoculum of 7.36 log CFU/g and 7.66 CFU/g, respectively.

As shown in Figure 56, the initial probiotic concentration in the 5-fold overage version was 5.52 ± 0.07 log CFU/g while the 10-fold overage version was 5.94 ± 0.05 log CFU/g. This suggests potential initial losses during the production or bottling process.

Over the 6-month storage period, both formulations demonstrated a decline in viable *B. coagulans* Unique IS-2 spores. The 10-fold overage formulation consistently maintained a higher CFU count compared to the 5-fold overage formulation throughout the storage period. At 6 months, the 10-fold overage beer retained approximately 4.57 ± 0.06 log CFU/g, while the 5-fold overage beer showed a concentration of about 4.00 ± 0.06 log CFU/g. Neither formulation was able to maintain the targeted 6.66 log CFU/ml per 440 ml serving, from the beginning of the stability study. This substantial reduction highlights the challenges associated with maintaining high probiotic viability in the alcohol-free beer matrix over an extended period. The complex composition of beer, including its low pH, CO₂ levels, hop compounds, and potential residual oxygen, can exert significant stress on probiotic spores, even those as robust as *B. coagulans* (Hinojosa-Avila et al., 2024). Among the various inhibitory factors present in beer that affect microorganisms, hop resistance appears to be a key determinant of microbial viability. The primary antimicrobial compounds in hops are the iso- α -acids (IAAs), which inhibit microbial growth by acidifying the cytoplasm and chelating essential ions such as manganese. Nevertheless, certain microorganisms have evolved specific resistance mechanisms that enable their survival in hop-rich environments. These include multidrug efflux transporters encoded by the *horA* and *horC* genes, which actively expel IAAs from the cell; HitA, a membrane protein that enhances manganese uptake; and ATP-dependent transporters that assist in the removal of hydronium ions. Such adaptations are essential for microbial survival in beer, where hop resistance plays a key role in determining viability (Borges Martins da Silva et al., 2024; Munford et al., 2017b; Schurr et al., 2015).

The pH analysis revealed excellent stability for both alcohol-free beer formulations throughout the 6-month storage period. The pH for both the 5-fold and 10-fold overage beers remained remarkably consistent, hovering around pH 4.87 ± 0.01 and 4.90 ± 0.02 , respectively with no significant fluctuations observed. This constancy in pH indicates that the incorporation of *B. coagulans* Unique IS-2 spores did not induce substantial metabolic activity or acid production that would alter the beer's acidity. Furthermore, it confirms the inherent chemical stability of the alcohol-free beer matrix itself under the storage conditions. Maintaining a stable pH is crucial for preserving the sensory attributes (taste, flavour) and physicochemical integrity of the product, as well as for ensuring microbial safety by limiting the growth of spoilage microorganisms.

These findings are consistent with previous studies indicating that spore-forming probiotics, such as *B. coagulans*, typically do not cause noticeable changes in sensory characteristics when incorporated into beers. For example, Majeed et al. (2022) reports stability studies on alcoholic beer, demonstrating that the inclusion of the probiotic strain *B. coagulans* MTCC 5856 did not alter specific gravity, pH, or alcohol content, indicating its compatibility and maintenance of beverage quality during both ambient and accelerated storage conditions.

The results demonstrate the potential of incorporating *B. coagulans* Unique IS-2 spores into alcohol-free beer; however, achieving and maintaining target probiotic levels throughout a typical shelf life presents a considerable challenge. While the spores showed persistence for 6 months, their concentrations fell below the desired functional threshold for a single serving very early in the storage period. The slightly superior retention in the 10-fold overage group suggests that strategies involving higher initial inoculation might extend the period of functionality, but it may not be sufficient to meet stringent targets.

The observed probiotic decline could be attributed to a combination of factors inherent to the beer matrix. For this kind of matrix improving results solution should be considered like a product reformulation, microencapsulation of spores or optimization of the brewing and bottling process to minimize oxygen ingress (Santos et al., 2024).

4.2.4 Plant-based Beverage Products

4.2.4.1 Fermented Tiger Nut Beverage

The stability and properties of tiger nut beverage enriched and fermented with different probiotic strains were assessed according to the experimental design outlined in Figure 57.

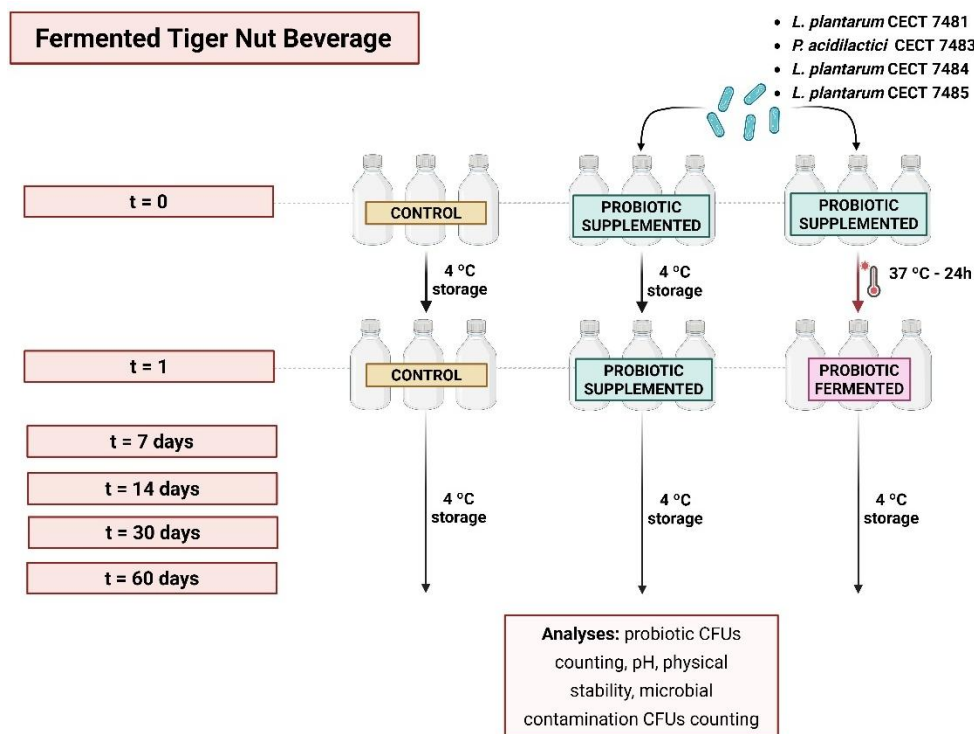


Figure 57. Schematic illustration of the experimental design. Three tiger nut beverage variants were prepared: a control (without probiotics), a probiotic-supplemented beverage, and a probiotic-fermented beverage. Each variant was produced for each one of the following probiotic strains: *L. plantarum* CECT 7484, CECT 7485, CECT 7481 and *P. acidilactici* CECT 7483. Probiotic inoculation was carried out prior to fermentation at 37°C for 24 h, followed by storage at $4 \pm 2^{\circ}\text{C}$. Analyses were performed before and after fermentation, and at 7, 14, 30, and 60 days of storage. Evaluated parameters included viable probiotic counts (CFU), pH, physical stability, and microbiological contamination (post-fermentation and at day 60). The figure was created in <https://BioRender.com>.

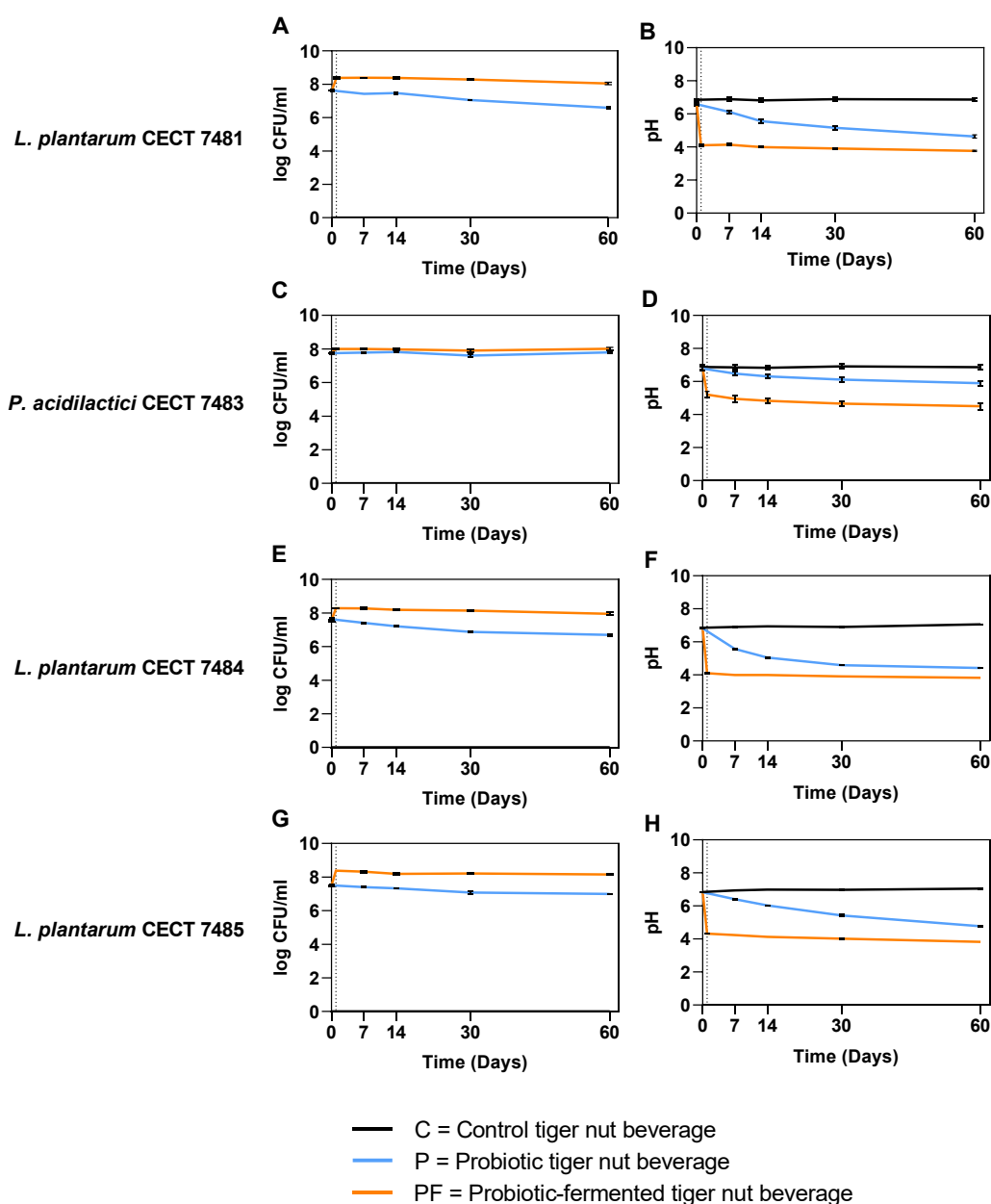


Figure 58. Probiotic viability and pH evolution in tiger nut without probiotic (Control, C), probiotic-supplemented (P), probiotic-fermented (PF) with four different strains, monitored over 60 days of storage at 4 °C. A-C-E-G) Probiotic viability expressed as log CFU/ml. B-D-F-H) pH.

The inoculation of the different probiotic strains in tiger nut beverage was designed, as detailed in Section 3.2.4 (Materials and Methods), to ensure a target probiotic concentration in the final products. To achieve a target of 6.96 log CFU/ml per 330 ml serving, the inoculation dose was standardized at 7.96 log CFU/ml, applying a 10-fold overage to account for potential viability losses.

Figure 58 (panels A, C, E, G) illustrate the evolution of viable counts for each strain in P and PF tiger nut beverages. Immediately after inoculation (T0), all strains exhibited similar concentrations (7.48–7.75 log CFU/ml), confirming consistency in the inoculation process. Under fermentation conditions (24 h at 37 °C), all strains demonstrated the ability to grow in the tiger nut matrix; however, the three *L. plantarum* strains exhibited significantly higher fermentative potential, with an average increase of 0.79 ± 0.09 log CFU/ml, compared to *Pediococcus acidilactici* CECT 7483, which increased by only 0.25 ± 0.11 log CFU/ml. This observation aligns with the findings of Banwo & Taiwo (2025), who reported that *L. plantarum* LB113 exhibited strong fermentative performance and high survival rates under GI conditions when used to ferment a plant-based yogurt made from tiger nut and date palm milk, highlighting its adaptability and probiotic potential in non-dairy substrates.

During 60 days of refrigerated storage, most probiotic-supplemented and probiotic-fermented samples maintained viable counts above the target threshold (6.96 log CFU/ml), with two exceptions: *L. plantarum* CECT 7484 and CECT 7485 in the supplemented (P) versions, which declined slightly below the threshold (6.65 and 6.59 log CFU/ml, respectively). In contrast, *P. acidilactici* CECT 7483 deviated from this general pattern, maintaining stable CFU counts in both P and PF. This behaviour suggests that its robustness in the fermented matrix may be partly due to its comparatively mild acidifying activity, which reduces self-induced acid stress during fermentation. In contrast, the other strains appear to experience autoinhibition, as their more intense acid production likely accelerates cellular injury and viability loss in the fermented version.

Overall, the maintenance of high viable counts confirms tiger nut beverage as a suitable carrier for probiotic delivery, in line with findings for other non-dairy matrices such as soy and oat-based beverages (Granato et al., 2010).

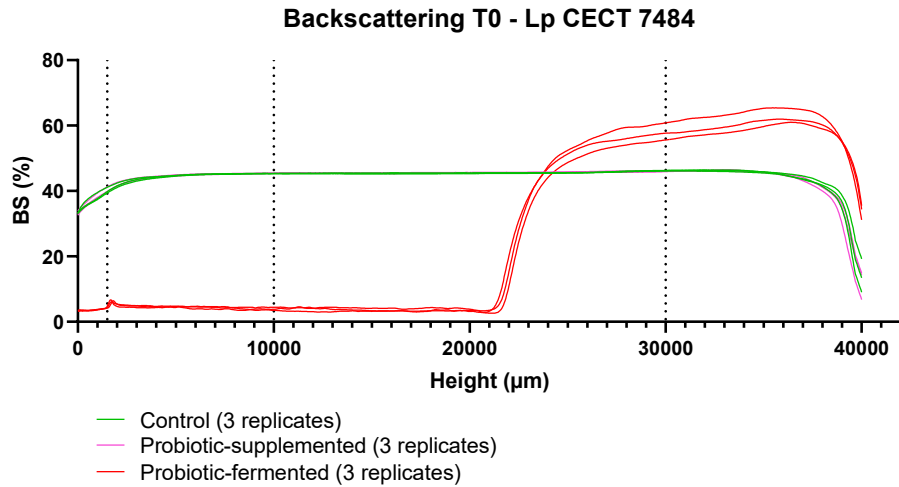
Figure 58 (panels B, D, F, and H) depict pH changes during storage. All fermented samples (PF) exhibited a marked pH reduction after fermentation, dropping from an initial average of 6.79 ± 0.09 to 4.10–4.33 for *L. plantarum* strains and to 5.20 ± 0.20 for *P. acidilactici* CECT 7483. Following the initial 24 h drop, a further pH decrease was observed along 60 days-storage, with values ranging from 0.28 to 0.69 units. *P. acidilactici* CECT 7483 exhibited the lowest acidification, while *L. plantarum* CECT 7484 showed the highest.

Control samples (C) maintained original pH, while probiotic-supplemented but non-fermented samples (P) showed gradual acidification during storage, likely due to residual metabolic activity at low temperature. This effect was more pronounced in *L. plantarum*

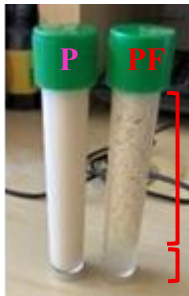
strains, where pH decreased an average of -2.14 ± 0.01 , compared to -0.89 ± 0.05 for *P. acidilactici*.

While moderate pH reduction improves microbial stability, excessive acidification may compromise palatability, a trade-off well documented in plant-based probiotic beverages (Hidalgo-Fuentes et al., 2024). Conversely, *P. acidilactici* milder acidification supports its use in formulations where maintaining neutral sensory attributes is desirable.

A1



A2



B

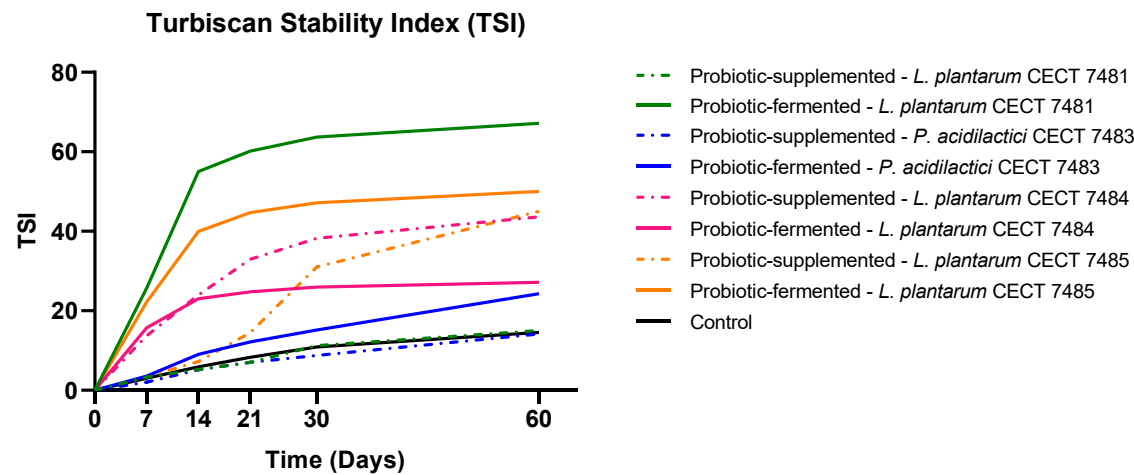


Figure 59. Physical stability of tiger nut beverage without probiotics (C, Control), probiotic-supplemented (P) and probiotic-fermented (PF) with different strains: *L. plantarum* CECT 7481, *P. acidilactici* CECT 7483, *L. plantarum* CECT 7484, *L. plantarum* CECT 7485), during 60 days of storage at 4 ± 2 °C. A1) Representative image of the first physical stability measurement (T0) for C, P, and PF versions of *L. plantarum* CECT 7484. A2) T0 images of P and PF tiger nut beverage samples with *L. plantarum* CECT 7484. B) Evolution of the Turbiscan Stability Index (TSI) for C, P, and PF beverages.

Table 33. Variation in backscattering (Δ BS) between initial (Day 0) and final (Day 60) measurements across sedimentation (1.5–10 mm), flocculation (10–30 mm), and creaming (30–40 mm) zones.

Strain	Treatment	Sedimentation Zone* (1.5-10 mm)	Flocculation Zone* (10-30 mm)	Creaming Zone* (30-40 mm)
<i>L. plantarum</i> CECT 7481	P	-13.65 $\pm$ 2.22 ^{bA}	-8.15 $\pm$ 0.77 ^{bA}	5.13 $\pm$ 0.91 ^{aA}
	PF	10.66 $\pm$ 0.79 ^{aA}	2.37 $\pm$ 3.94 ^{aA}	0.69 $\pm$ 3.23 ^{bA}
	C	-21.51 $\pm$ 2.82 ^{cA}	-10.11 $\pm$ 2.05 ^{bA}	10.05 $\pm$ 6.23 ^{aA}
<i>P. acidilactici</i> CECT 7483	P	-23.81 $\pm$ 0.48 ^{bB}	-10.07 $\pm$ 0.42 ^{aA}	5.23 $\pm$ 1.47 ^{bA}
	PF	-29.64 $\pm$ 0.63 ^{cB}	-24.12 $\pm$ 0.19 ^{bB}	11.10 $\pm$ 1.01 ^{aB}
	C	-21.51 $\pm$ 2.82 ^{aA}	-10.11 $\pm$ 2.05 ^{aA}	10.05 $\pm$ 6.23 ^{aB}
<i>L. plantarum</i> CECT 7484	P	-36.34 $\pm$ 1.05 ^{cC}	-23.81 $\pm$ 1.00 ^{cC}	11.39 $\pm$ 3.34 ^{bA}
	PF	8.15 $\pm$ 0.90 ^{aA}	-0.39 $\pm$ 1.54 ^{aB}	3.20 $\pm$ 2.88 ^{aA}
	C	-21.51 $\pm$ 2.82 ^{bA}	-10.11 $\pm$ 2.05 ^{bA}	10.05 $\pm$ 6.23 ^{aB}
<i>L. plantarum</i> CECT 7485	P	-40.32 $\pm$ 1.26 ^{cD}	-16.62 $\pm$ 2.21 ^{cB}	16.15 $\pm$ 3.47 ^{aB}
	PF	8.08 $\pm$ 0.47 ^{aA}	-0.96 $\pm$ 1.36 ^{aB}	7.22 $\pm$ 1.48 ^{bAB}
	C	-21.51 $\pm$ 2.82 ^{bA}	-10.11 $\pm$ 2.05 ^{bA}	10.05 $\pm$ 6.23 ^{aA}

* Δ BS $\pm$ SD

C = Control

P = probiotic-supplemented

PF = probiotic-fermented

Two-way ANOVA followed by Tukey's post hoc test was applied.

Different lowercase letters within a column indicate significant differences between treatments on the same microorganism ($p < 0.05$).

Different uppercase letters within a column indicate significant differences between microorganisms within the same treatment ($p < 0.05$).

The evolution of physical stability of tiger nut beverages subjected to probiotic supplementation (P) or fermentation (PF) with different strains was monitored during 60 days of refrigerated storage using the TSI and Δ BS analysis (Figure 59-B; Table 33, respectively).

Initial measurements were performed immediately after fermentation (Day 0). At this time point, all PF samples already exhibited marked instability, as evidenced in the example of *L. plantarum* CECT 7484 shown in Figure 59 (panels A and B), with the exception of samples fermented with *P. acidilactici*. This behaviour indicates that significant structural rearrangements occurred during the fermentation process. In contrast, the control (C) and most probiotic-supplemented (P) formulations displayed a more homogeneous dispersed system at Day 0.

The early destabilization observed in PF systems is consistent with fermentation-induced modifications of the colloidal matrix, a phenomenon frequently reported for lactic-fermented plant-based beverages. Acidification and microbial metabolic activity likely altered protein–

polysaccharide interactions, reducing electrostatic repulsion and favouring particle association (Erem & Kilic-Akyilmaz, 2024; Kizzie-Hayford et al., 2015). Macroscopically, phase separation was observed, consisting of a translucent aqueous lower phase and a grainy, opaque upper fraction (Figure 59, A2), which is technologically undesirable from a consumer acceptance standpoint (Kizzie-Hayford et al., 2016).

The evolution of TSI during storage revealed distinct destabilization patterns depending on both processing strategy and strain. PF samples fermented with *L. plantarum* strains (CECT 7481, CECT 7484, and CECT 7485) exhibited a rapid increase in TSI during the first 15 days of storage, indicating intense structural reorganization shortly after fermentation. Thereafter, TSI curves approached a plateau, suggesting that the multiphase systems formed during fermentation reached a relatively stable configuration. Among these strains, *L. plantarum* CECT 7484 showed the lowest destabilization kinetics, compared to the other *L. plantarum* strains.

In contrast, PF samples fermented with *Pediococcus acidilactici* CECT 7483 displayed consistently lower and more gradual TSI increases (TSI < 27 throughout storage). This reduced destabilization coincides with the more moderate acidification achieved by this strain (final pH $\approx$ 5.2), which limits protein charge neutralization and aggregation phenomena. Conversely, fermentation with *L. plantarum* strains resulted in lower final pH values (4.1–4.3), conditions that are known to promote protein denaturation, reduced electrostatic stabilization, and enhanced particle association in plant-based dispersions, thereby compromising colloidal stability.

Unlike PF samples, which exhibited initially higher destabilization, P samples showed a gradual development of destabilization during storage. This trend was particularly pronounced for *L. plantarum* CECT 7485 and CECT 7481, which reached TSI values exceeding 40 and 55, respectively, by Day 14. Unlike PF systems, where instability was largely induced during processing, P formulations underwent a slow, storage-dependent reorganization of the dispersed phase.

Importantly, Δ BS analysis (Table 33) provided mechanistic insight into the nature of the observed instability. While TSI quantifies the overall extent of destabilization, Δ BS spatial profiles allowed discrimination among particle migration phenomena, including sedimentation and creaming. The combined evaluation of TSI, Δ BS zoning, and visual inspection therefore represents a robust framework for the assessment of physical stability in complex plant-based matrices.

Marked differences in Δ BS between Day 0 and Day 60 were observed among treatments (C, P, PF) and strains across the three optically defined zones (sedimentation, flocculation, and creaming), confirming that both the magnitude and the dominant mechanism of destabilization were strongly strain- and process-dependent.

In the sedimentation zone, control samples consistently exhibited strongly negative Δ BS values, indicative of clarification near the bottom of the tube due to upward migration of dispersed particles. Probiotic supplementation intensified this effect for *L. plantarum* CECT 7484 and CECT 7485, suggesting enhanced density-driven separation during storage. In contrast, PF samples fermented with *L. plantarum* strains showed positive Δ BS values in this zone, reflecting particle accumulation and sedimentation over time. Notably, PF samples fermented with *P. acidilactici* CECT 7483 deviated from this pattern, exhibiting strongly negative Δ BS values comparable to or lower than those of the control, indicating limited sediment formation despite fermentation.

In the flocculation zone, Δ BS values were predominantly negative across treatments and strains, reflecting depletion of scattering material from the central region. P samples generally exhibited lower negative Δ BS values than PF formulations (values closer to zero), indicating a higher depletion of particles from this zone. An exception was again observed for *P. acidilactici* CECT 7483.

Overall, these results indicate that the central region of the sample functions as a transient migration zone, supplying particles to either the sedimentation or creaming regions. The absence of sustained positive Δ BS values in this zone confirms that destabilization was dominated by particle migration processes rather than by the formation of stable flocculated structures.

In the creaming zone, Δ BS values were positive for all treatments, confirming particle accumulation at the top of the sample during storage. P samples exhibited higher Δ BS values than PF formulations, indicating a more pronounced creaming tendency. This difference can be attributed to the fact that creaming in PF systems was already evident in the first timepoint and reached a plateau rapidly, whereas in P samples creaming developed progressively during storage.

Significant differences among microorganisms within the same treatment were observed under several experimental conditions, confirming the presence of statistically significant strain-specific effects on the extent of creaming and sedimentation.

Collectively, these findings demonstrate that fermentation induces immediate and pronounced physical destabilization of tiger nut beverages, whereas probiotic supplementation leads to slower, storage-driven instability. Both the extent and the dominant mechanism of destabilization are strongly strain-dependent. Among the tested microorganisms, *P. acidilactici* CECT 7483 consistently exhibited the most favourable behaviour, characterized by moderate acidification, reduced particle aggregation, and limited phase separation during storage.

From a product development perspective, these results are highly relevant, as phase separation negatively affects visual homogeneity and mouthfeel, key sensory attributes influencing consumer acceptance of plant-based beverages (García-Barón et al., 2025). Although PF formulations may offer advantages in terms of microbial stability (Abbaspour, 2024), their early onset of physical destabilization highlights the need for targeted formulation strategies. These may include the incorporation of hydrocolloids to enhance steric stabilization, optimization of fermentation parameters to limit excessive protein aggregation, and the selection of exopolysaccharide-producing strains to increase viscosity and mitigate phase separation (Boukid, 2024; Kizzie-Hayford et al., 2016).

Overall, physical stability remains a critical challenge in the development of plant-based probiotic beverages. Strain selection emerges as a pivotal factor governing not only microbial performance but also physicochemical compatibility with the food matrix. In this context, *P. acidilactici* CECT 7483 appears as the most promising for the formulation of stable probiotic tiger nut beverages, whereas *L. plantarum* strains may be more suitable for applications where a more pronounced acidic profile is technologically or sensorially desirable.

4.2.4.2 Fermented Soy and Oat Beverages

The stability and properties of soy and oat beverages fermented with the probiotic strain *L. paracasei* AB0918 in combination with a commercial starter, were assessed according to the experimental design outlined in Figure 60.

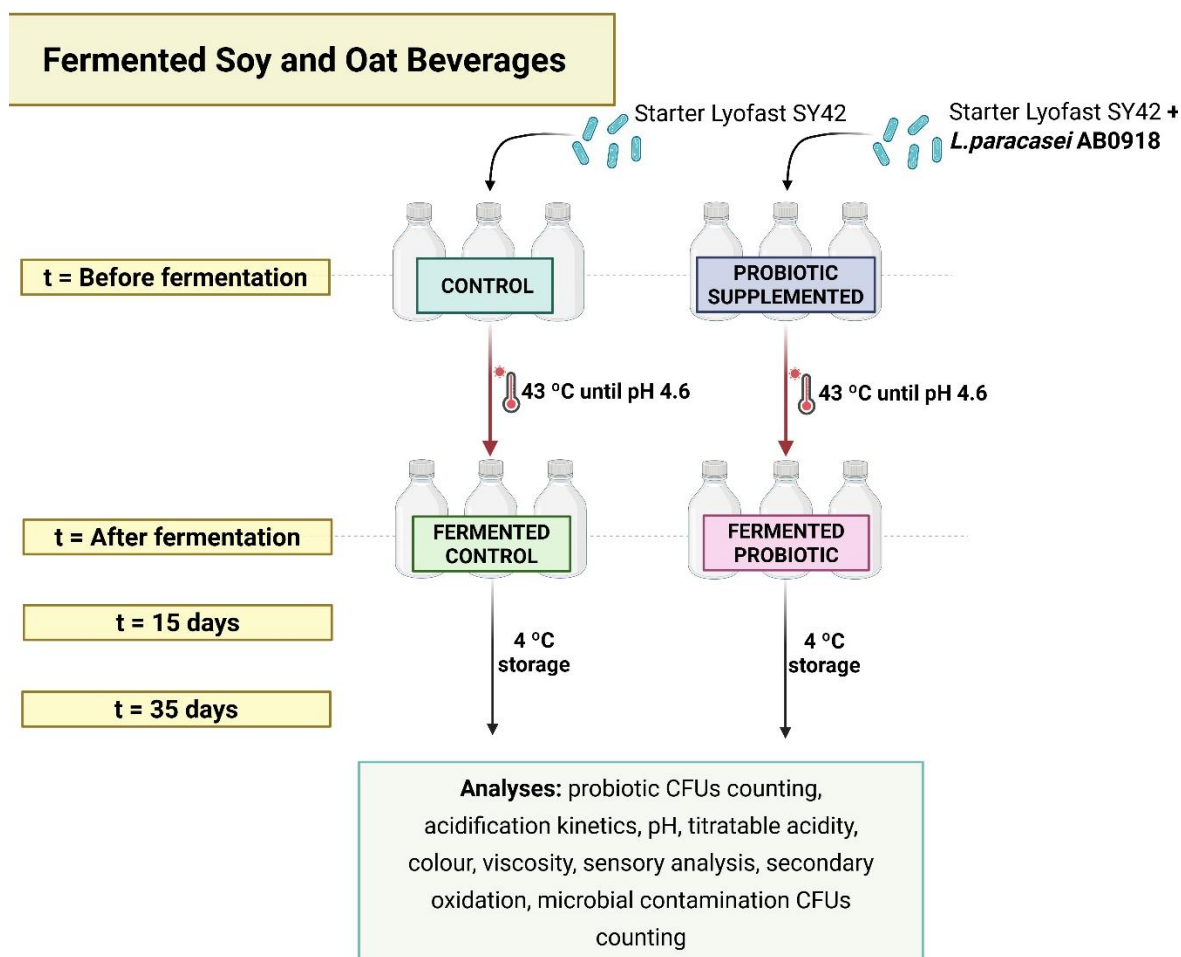


Figure 60. Schematic illustration of the experimental design. Two variants were produced for each plant-based beverage (oat- and soy-based): a fermented control (starter culture only) and a probiotic-fermented beverage. Both formulations were inoculated before fermentation using the starter culture Lyofast SY42; the probiotic variant additionally contained *L. paracasei* AB0918. Fermentation was carried out at 43 °C until the product reached pH 4.6, after which samples were stored at 4 ± 2 °C. Analyses were performed immediately after fermentation and at 15 and 35 days of refrigerated storage. Evaluated parameters included viable probiotic counts (CFU), pH TA, and microbiological contamination (post-fermentation and at day 35). Colour and viscosity were assessed after fermentation and at 15 and 35 days. Acidification kinetics was continuously monitored during fermentation and additional analyses on freshly produced samples included secondary lipid oxidation (MDA–TBARS) and sensory acceptability. The figure was created in <https://BioRender.com>.

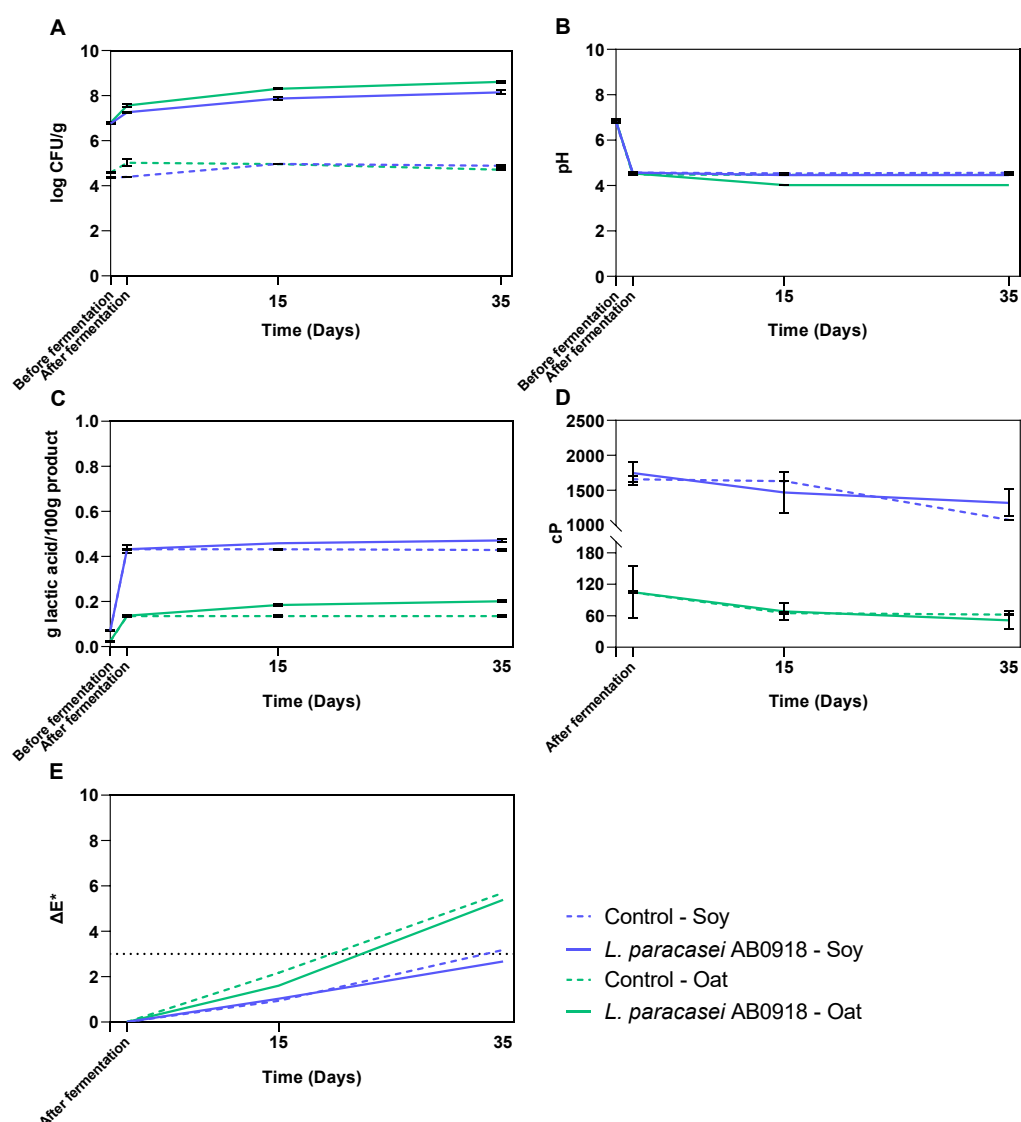


Figure 61. Probiotic viability and physicochemical characteristics of fermented soy and oat beverage during 35 days of storage at 4 ± 2 °C. The beverages were fermented with the starter Lyofast SY42 either supplemented with *L. paracasei* AB0918 (PF) or without the probiotic strain (Control, C). A) Probiotic viability expressed as log CFU/g. B) pH. C) TA expressed as g of lactic acid / 100g of product. D) Viscosity expressed as centipoises. E) Colour variation expressed as ΔE^* .

The inoculation of *L. paracasei* AB0918 into the plant-based beverage matrices was performed according to the protocol described in Section 3.2.5 (Materials and Methods). The procedure aimed to achieve a final concentration of 7 log CFU/mL per 200 mL serving. No initial overage was applied.

As shown in Figure 61, viable cell counts (log CFU/mL) slightly increased throughout refrigerated storage in all fermented samples, with counts remaining above 7 log CFU/mL for both soy and oat PF matrices. The CFU counts increased during fermentation at 43 °C

with the greater increase observed in oat (soy: $+0.50 \Delta \log \text{CFU/g}$, oat: $+0.76 \Delta \log \text{CFU/g}$). After fermentation the probiotic samples reached respectively $7.57 \pm 0.05 \log \text{CFU/g}$ for oat and $7.26 \pm 0.02 \log \text{CFU/g}$ for soy. The incorporation and maintenance of the probiotic strain during storage was successful. During storage the probiotic strain maintained a viability of $8.16 \pm 0.54 \log \text{CFU/g}$ in oat and $7.76 \pm 0.45 \log \text{CFU/g}$ in soy.

These results demonstrate that both soy and oat matrices can sustain probiotic viability under refrigerated conditions, although the oat matrix appeared to provide a more favourable environment for fermentation (average probiotic increase: $+0.50 \log \text{CFU/mL}$ in soy and $+0.76 \log \text{CFU/mL}$ in oat; $p=0.001$), possibly due to its higher content of fermentable carbohydrates.

These findings are consistent with previous studies reporting high survival of *Lactocaseibacillus paracasei* in soy- and oat-based beverages during cold storage. Ruiz De La Bastida et al. (2023) demonstrated that soy beverages fermented with *L. paracasei* INIA P272 maintained viability above $9.56 \log \text{CFU/mL}$ during 28-days refrigerated storage, while Iraporda et al. (2022) reported good *L. paracasei* BGP1 stability in soy beverage with values around $2 \times 10^7 \text{CFU/mL}$ after 30 days of storage at 4°C , and even higher counts in the inulin-supplemented versions. Similarly, Gokavi et al. (2005) confirmed that oat-based beverages fermented with *L. paracasei* ssp. casei sustained high viability for up to 10 weeks at 4°C , with populations ranging from 7.39×10^7 to $4.49 \times 10^8 \text{CFU/mL}$.

All formulations exhibited a sharp decrease in pH during fermentation that was blocked once pH reached ~ 4.6 . During storage, all samples showed significant post-acidification, with oat PF displaying the most pronounced pH reduction (oat: -0.52 ± 0.01 ; soy: -0.11 ± 0.03). These results indicate that bacterial metabolic activity slowed markedly in the increasingly acidified and refrigerated environment, particularly in the soy matrix, yet did not cease entirely.

Titrateable acidity increased significantly during fermentation in both matrices, with a more substantial rise observed in soy samples (PF oat beverage: $+0.113 \pm 0.005$; PF soy beverage: $+0.362 \pm 0.016$). Furthermore, titrateable acidity in oat PF samples continued to increase significantly during storage, reaching $0.201 \pm 0.005 \text{g lactic acid/100 g product}$, compared with $0.135 \pm 0.002 \text{g lactic acid/100 g}$ in the corresponding control.

The concurrent evolution of pH and titrateable acidity during storage indicates that the fermented products maintained a relatively stable acid–base balance over time, although the

probiotic formulations (PF) consistently exhibited a higher tendency toward post-acidification than their respective controls, particularly in the oat-based matrix.

These observations are consistent with previous studies on plant-based probiotic beverages. Maffei et al. (2023) demonstrated that soy drinks fermented with *L. paracasei* ssp. *paracasei* exhibit rapid acidification during fermentation, followed by minimal changes during cold storage. In contrast with our results, Ziarno et al. (2025) demonstrated that oat-based fermented beverages fermented with yogurt starter and/or *L. plantarum* 299v/*L. acidophilus* La-5 exhibit pH stability during storage, highlighting species-dependent differences in metabolic activity within plant-based fermentation systems.

Viscosity results showed clear matrix-dependent differences. The soy beverage exhibited substantially higher viscosity ($C = 1454,87 \pm 328.39$ cP and $PF = 1512,52 \pm 271.92$ cP) than the oat beverage ($C = 77.23 \pm 23.84$ and $PF = 75.02 \pm 27.48$ cP), which can be attributed to the greater protein content and the formation of a more stable colloidal network during soy beverage fermentation. In contrast, the oat beverage, mainly structured by soluble β -glucans and starch components (Anttila et al., 2004), displayed lower viscosity values. A reduction in viscosity measured after shaking was observed during storage in both matrices, particularly in the oat formulations, indicating a greater susceptibility of these systems to structural changes over time.

Colour variation, expressed as ΔE^* , increased progressively in all formulations during the 35-day storage period, indicating colour evolution over time. The magnitude of ΔE^* was notably higher in oat-based beverages ($C = 5.69$; $PF = 5.39$) compared to soy-based samples ($C = 3.18$; $PF = 2.67$), suggesting that oat formulation was more prone to colour shifts during storage. Taken together, these results show that fermentation with a yogurt starter supplemented with *L. paracasei* AB0918 successfully acidified both soy and oat beverages, resulting in stable products with moderate acidification, during 35 days of refrigerated storage. The probiotic strain maintained high viability throughout storage, confirming its adaptability to plant-based substrates. The soy beverage generally exhibited higher acidity, viscosity, and microbial stability, indicating a more favourable matrix for probiotic survival and texture retention, while the oat beverage showed lower viscosity and more pronounced colour changes, confirming that the physicochemical properties and stability of plant-based fermented beverages depend strongly on the substrate composition and fermenting bacteria.

Table 34. Acidification kinetics of soy and oat beverages during fermentation with yogurt starter with or without *L. paracasei* AB0918.

		Starter Lyofast SY42 (Control)	Starter Lyofast SY42 + <i>L. paracasei</i> AB0918
Soy samples	Te ± SD (min)	283.33 ± 15.53 ^b	284.00 ± 17.09 ^b
	Vm ± SD (pHu/min)	−0.020 ± 0.001 ^b	−0.021 ± 0.002
	Tm ± SD (min)	136.00 ± 3.46 ^b	132.67 ± 4.16 ^b
	Ta ± SD (min)	32.67 ± 4.16	25.33 ± 6.43
Oat samples	Te ± SD (min)	200.67 ± 2.31 ^a	200.67 ± 4.16 ^a
	Vm ± SD (pHu/min)	−0.025 ± 0.002 ^a	−0.023 ± 0.004
	Tm ± SD (min)	102.00 ± 3.46 ^a	106.00 ± 6.93 ^a
	Ta ± SD (min)	17.33 ± 6.43	20.00 ± 3.46
Acidification kinetics parameters:			
Te = Time when the sample reaches pH 4.6			
Vm = the maximum acidification rate			
Tm = Time required to reach the maximum acidification rate			
Ta = Time for the pH to drop by 0.08 units after the start of fermentation (fermentation start speed)			
One-way ANOVA with multiple comparisons was applied across treatment and matrices for each acidification parameter. Different superscript letters indicate statistically significant differences ($p < 0.05$).			

Table 34 summarizes the acidification kinetics of soy and oat beverages fermented with the yogurt starter (Lyofast SY42), either alone (control) or supplemented with *L. paracasei* AB0918. No statistically significant differences were detected between the control and the supplemented formulations in either matrix.

Overall, fermentation in soy yielded higher values of Te and Tm, compared with oat. These differences indicate a slower acidification rate in soy relative to oat. This behaviour is consistent with the compositional characteristics of the two substrates: oat beverages generally contain higher levels of readily fermentable carbohydrates, including maltose and glucose released from enzymatic starch hydrolysis, whereas soy beverages have a higher protein content, a stronger buffering capacity, and lower concentrations of simple sugars. These properties can delay early bacterial growth and acidification, as documented in previous studies on plant-based fermentation systems (Klajn et al., 2021).

Taken together, these results demonstrate that both soy and oat matrices can be effectively fermented by the yogurt starter culture, reaching the technological target pH of 4.6 within approximately 3–5 hours, with oat fermentations progressing more rapidly.

The inclusion of *L. paracasei* AB0918 produced only minor modifications in acidification kinetics in either substrate, suggesting that this strain did not substantially alter the metabolic performance of the starter culture under the tested conditions. Nevertheless, its presence may

still contribute to the probiotic potential of the final product. Overall, the findings underscore the key role of substrate composition in determining fermentation dynamics in plant-based dairy alternatives. A limitation of this study is the absence of a fermentation trial with *L. paracasei* AB0918 as a single inoculum, which prevents direct attribution of the observed effects to the probiotic strain. Previous work indicates that *L. paracasei* can independently ferment soy-based substrates, showing adequate acidification and metabolic activity (Ruiz De La Bastida et al., 2023). Future investigations should therefore assess the performance of *L. paracasei* alone in oat and soy matrices to determine whether co-inoculation with yogurt starters is necessary. Demonstrating effective single-strain fermentation could streamline production, reduce ingredient inputs, and lower processing costs.

Table 35. Comparative analysis of secondary lipid oxidation (MDA-TBARS) in plant-based beverages: impact of yogurt starter and *L. paracasei* AB0918 fermentation.

Sample	Average $\pm$ SD (MDA $\mu\text{mol/L}$)	Oxidation Reduction (%) vs. Control
Soy beverage (Control)	29.99 ± 7.81^a	
Starter Lyofast SY42	0.34 ± 0.05^b	98.87
Starter + <i>L. paracasei</i> AB0918	1.10 ± 1.14^b	96.33
Oat beverage (Control)	8.77 ± 3.76^a	
Starter Lyofast SY42	4.35 ± 1.69^b	50.38
Starter + <i>L. paracasei</i> AB0918	4.80 ± 1.60^b	45.28
Both pairwise t-tests and one-way ANOVA with multiple comparisons were applied across the three formulations for each matrix, yielding consistent results. Different superscript letters indicate statistically significant differences ($p < 0.05$).		

Table 35 presents a comparative analysis of secondary lipid oxidation in fermented oat and soy beverages following forced oxidation, in which samples were held at 50 °C for 48 hours prior to analysis.

The soy beverage control (unfermented matrix) exhibited the highest lipid oxidation level (29.99 ± 7.81 MDA $\mu\text{mol/L}$). Fermentation drastically reduced lipid oxidation ($p < 0.05$), reaching values of 0.34 ± 0.05 MDA $\mu\text{mol/L}$ for the yogurt starter and 1.10 ± 1.14 MDA $\mu\text{mol/L}$ for the probiotic formulation. These reductions correspond to 98.87% and 96.33%, respectively, indicating that fermentation almost completely inhibited secondary lipid oxidation in the soy beverage. No significant difference was observed between the starter

alone and the formulation containing *L. paracasei* AB0918, suggesting that both fermentation treatments were similarly effective in mitigating oxidative degradation.

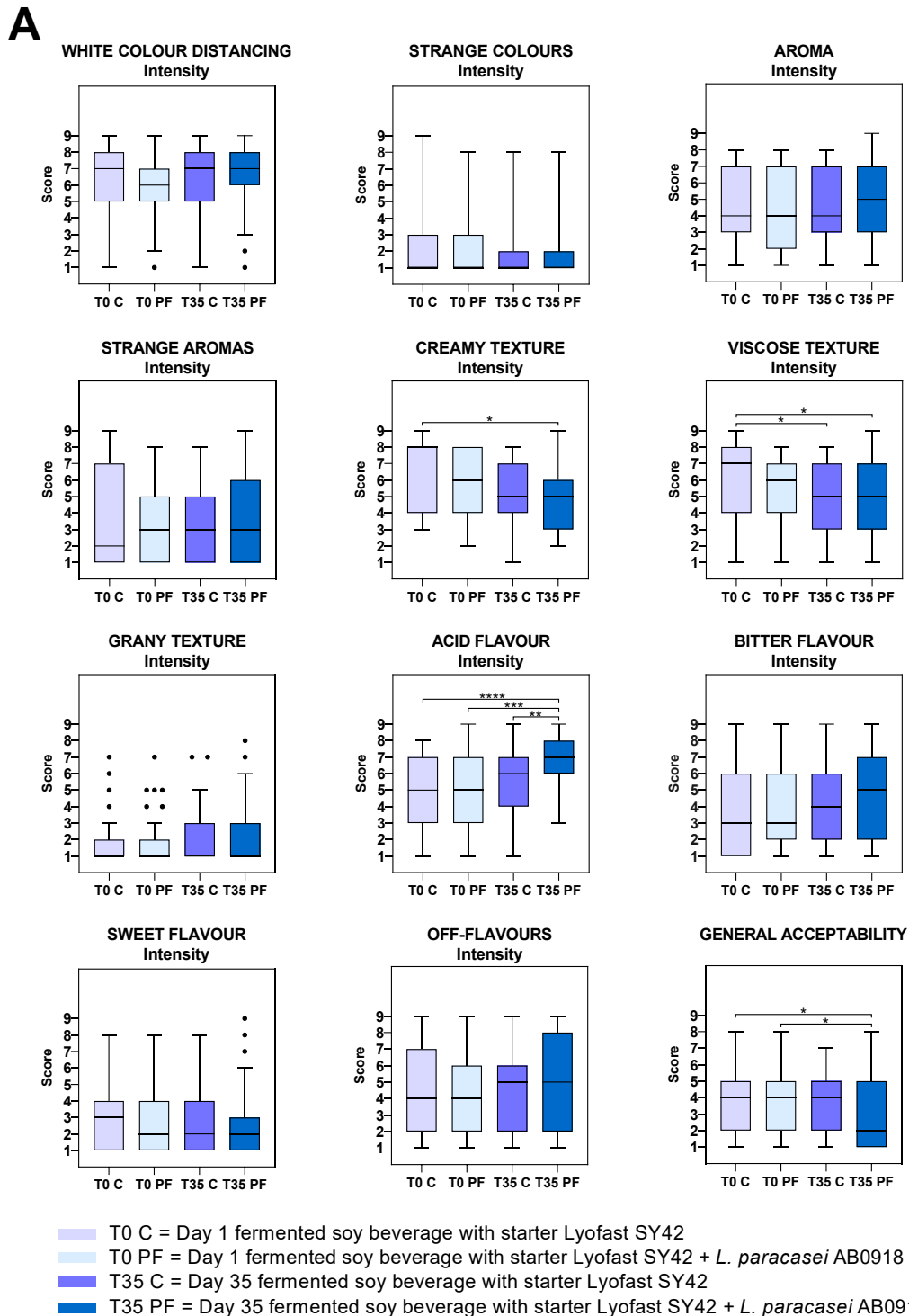
This near-complete inhibition of secondary lipid oxidation aligns with previous findings that lactic acid fermentation of soy matrices significantly enhances antioxidant capacity and reduces oxidative stress markers through acidification and conversion of isoflavone glycosides into aglycones (Sirilun et al., 2017). Isoflavone aglycones such as genistein and daidzein exhibit higher antioxidant activity compared to their glycoside forms, contributing to radical scavenging and lipid peroxidation inhibition (Wójciak et al., 2024). Moreover, LAB fermentation can increase total phenolic content and release bioactive peptides with antioxidant properties, further strengthening oxidative stability (H. Li et al., 2025). These mechanisms are complemented by enzymatic systems in LAB, including NADH peroxidase, thioredoxin reductase, and glutathione peroxidase, which neutralize reactive oxygen species (Ampemohotti et al., 2025).

In the oat beverage, the unfermented control presented a significantly higher ($p < 0.05$) MDA value compared with oat beverages fermented with the yogurt starter and with *L. paracasei* AB0918. The corresponding oxidation reduction percentages were 50.38% and 45.28%, respectively, compared with the unfermented oat beverage. These results indicate that fermentation substantially decreased secondary lipid oxidation in the oat matrix, and that both the starter culture and the probiotic formulation exerted comparable antioxidant effects ($p > 0.05$).

The less pronounced reduction in oat compared to soy may be attributed to differences in lipid composition and phenolic profiles (Moretto et al., 2021). Oats contain β -glucans, phenolic acids and avenanthramides, which provide inherent antioxidant protection, resulting in lower baseline oxidation (L. Chen et al., 2020; Soycan et al., 2019). Fermentation further enhances antioxidant capacity by increasing phenolic acids such as ferulic and caffeic acid and improving their bioavailability (Yu et al., 2023).

Overall, the findings demonstrate that fermentation markedly mitigates lipid oxidation in both soy and oat beverages, improving oxidative stability, with soy showing a more pronounced effect, probably due to its higher initial oxidation level. The yogurt starter alone was highly effective, and the addition of *L. paracasei* did not confer a significant additional benefit in terms of TBARS reduction. This agrees with studies indicating comparable antioxidant effects among LAB strains, mediated by radical scavenging and enzymatic systems. However, it is worth noting that strain-specific differences in antioxidant

mechanisms have been reported, with some *L. paracasei* strains exhibiting strong DPPH and ABTS radical scavenging activity and upregulation of genes encoding glutathione peroxidase and thioredoxin reductase under oxidative stress (Ampemhotti et al., 2025). These properties suggest potential synergistic effects when combined with plant phenolics, even if not evident in TBARS measurements.



To evaluate consumer acceptance of fermented soy and oat beverages, a sensory analysis was conducted on four formulations for each base matrix: a freshly prepared control (C) and a probiotic-fermented sample (PF) at production (T0), and the corresponding samples after 35 days of refrigerated storage (T35). The evaluation involved 39 untrained panellists (69.2% female, 30.85% male; 19–59 years), and the results are summarized in Figure 62-A (soy) and Figure 62-B (oat).

For soy beverages, no significant differences were detected at T0 between C and PF for appearance, aroma, texture, flavour, or general acceptability. These results show that incorporation of *L. paracasei* AB0918 did not alter the initial sensory profile of fermented soy beverages. Over storage, however, significant time-dependent changes emerged. Creamy and viscous texture decreased markedly in PF at T35 (creamy: 5.05 ± 1.98 ; viscosity: 4.74 ± 2.27), compared with T0 C (6.41 ± 2.11 ; 6.15 ± 2.24), and viscosity T0 C also decreased significantly in T35 C (5.25 ± 1.98). These shifts likely reflect progressive destabilization of the protein–polysaccharide network and redistribution of water during refrigerated storage, a phenomenon widely described in lactic-fermented plant beverages (Wu et al., 2022).

Acid flavour increased significantly during storage in both formulations, but more intensely in PF, rising from 5.00 ± 2.20 to 7.08 ± 1.68 . Such post-acidification may be attributed to residual metabolic activity and organic acid accumulation during cold storage. General acceptability declined significantly only in PF at T35 (2.71 ± 2.08) relative to both its T0 value (3.95 ± 2.21) and T0 C (4.08 ± 2.11), whereas no significant C–PF differences were observed at T35.

Oat beverages exhibited similar tendencies. At T0, C and PF did not differ significantly in aroma, flavour, texture, or general acceptability, and white colour was the only attribute showing a baseline difference, with lower scores in PF (4.49 ± 1.99) than in C (5.82 ± 1.82). During storage, PF samples showed increases in aroma intensity and bitterness at T35 (aroma: 5.33 ± 2.52 ; bitterness: 4.21 ± 2.98) compared with T0 C values (3.85 ± 2.19 ; 2.54 ± 1.96). Acid flavour increased significantly in both formulations but was most pronounced in PF (T35: 7.03 ± 1.82), consistent with post-acidification patterns observed in oat-based fermentations with *Lacticaseibacillus* spp. (Yang et al., 2024). Sweetness declined significantly only in T35 PF (2.72 ± 1.62), while off-flavours increased substantially in T35 PF (5.67 ± 2.94). General acceptability decreased significantly during storage in both formulations, though PF T35 (2.74 ± 2.21) scored significantly lower than all other samples.

Collectively, these findings show that co-fermentation with *L. paracasei* AB0918 yields soy- and oat-based beverages with sensory characteristics comparable to controls at production. However, refrigerated storage induces substantial sensory modifications, especially increased acidity and, in oat beverages, the development of bitterness and off-flavours. These changes reflect established mechanisms of post-acidification, carbohydrate depletion, and metabolite accumulation in fermented plant beverages during shelf life (Yang et al., 2024). Overall, the results confirm that *L. paracasei* AB0918 is suitable for inclusion in plant-based fermented beverages but underscore the need to mitigate post-acidification and preserve organoleptic stability over storage.

4.2.4.3 Fermented Fruit Juices

Within the scope of applying probiotics in fermented fruit juices, two trials were conducted:

4.2.4.3.1 TEST 1: Screening and Functional Evaluation of Probiotic Strains for Flavonoid Biotransformation in Orange Juice

Flavonoids are abundant bioactive compounds in citrus fruits, primarily present as glycosylated derivatives such as hesperidin and narirutin. These forms exhibit limited bioavailability compared to their aglycone counterparts (Zhang et al., 2021a). Microbial fermentation, particularly by LAB, offers a promising strategy to enhance flavonoid functionality through enzymatic hydrolysis, releasing aglycones with improved absorption and biological activity (Guo et al., 2021b). This section describes the screening and functional evaluation of AB-Biotics strains for their potential to produce aglycones and phenolic acids in orange juice fermentation, aiming to develop functional probiotic beverages.

The stability and properties of orange juice fermented with different probiotic strains were assessed according to the experimental design outlined in Figure 63. Notably, this study followed a distinct experimental pathway compared with previous approaches, as a preliminary strain selection was first conducted using an *in silico* screening strategy, enabling the identification of candidate strains prior to their technological and functional evaluation in the food matrix.

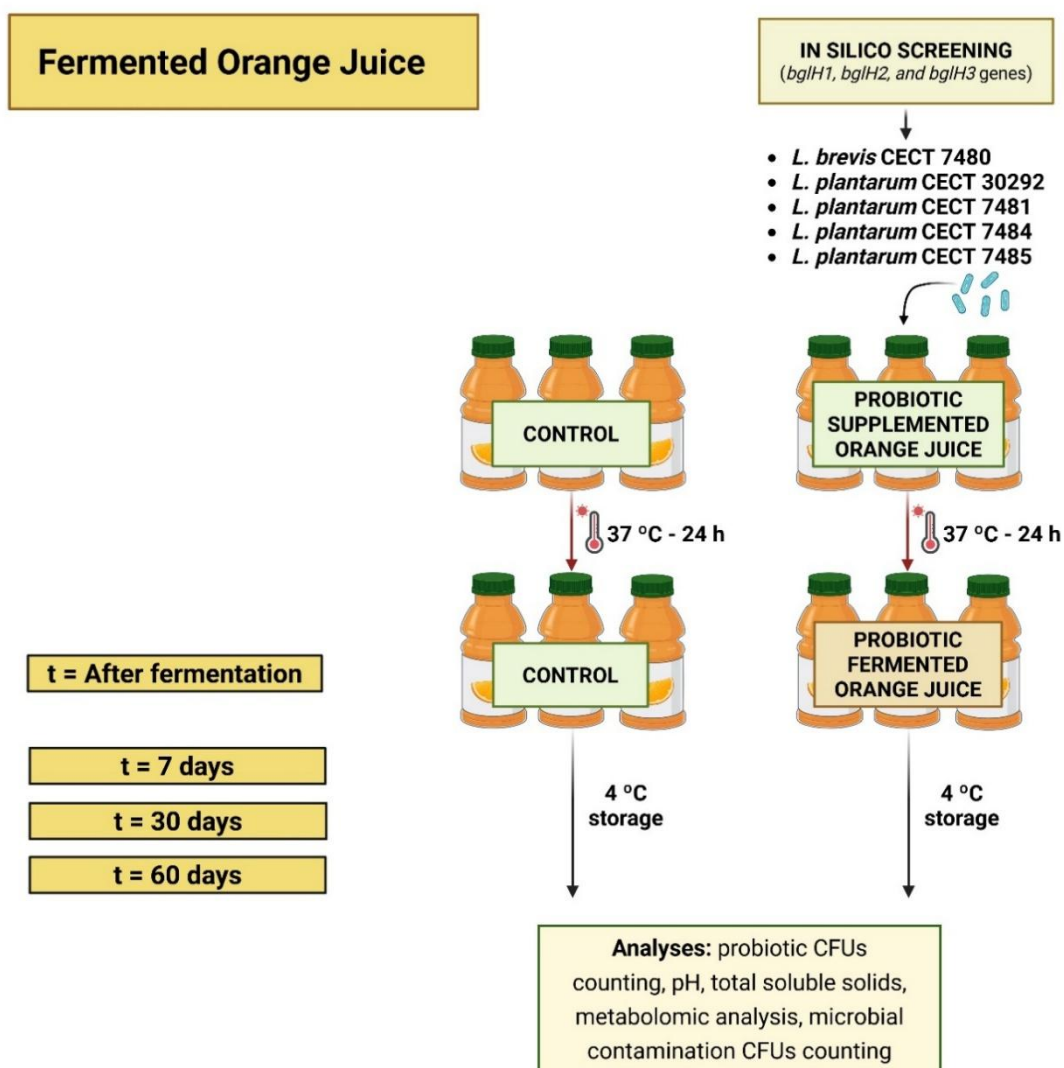


Figure 63. Schematic illustration of the experimental design. Orange juice was supplemented with probiotic strains selected through in silico screening for the presence of β -glucosidase-encoding genes (*L. brevis* CECT 7480, *L. plantarum* CECT 30292, CECT 7481, CECT 7484, and CECT 7485). Following inoculation, samples were incubated at 37 °C for 24 h and subsequently stored at 4 ± 2 °C for 60 days. Analyses were conducted immediately after fermentation and at 7, 30, and 60 days of storage. Evaluated parameters included viable probiotic counts (CFU), pH, TSS, and microbiological contamination (post-fermentation and at day 60). A metabolomic analysis was also performed. The figure was created in <https://BioRender.com>.

Table 36. In silico screening of the beta-glucosidase genes *bglH1*, *bglH2* and *bglH3* in different strains.

Strain	<i>bglH1</i>	<i>bglH2</i>	<i>bglH3</i>	Selected
<i>Lactiplantibacillus plantarum</i> CECT 30292	+	–	–	YES
<i>Lactiplantibacillus plantarum</i> CECT 7485	–	+	+	YES
<i>Lactiplantibacillus plantarum</i> CECT 7528	–	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 7529	–	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 8675	–	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 8677	–	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 8678	–	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 7481	–	+	+	YES
<i>Lactiplantibacillus plantarum</i> CECT 7484	+	+	+	YES
<i>Lactiplantibacillus plantarum</i> CECT 7315	+	+	+	
<i>Lactiplantibacillus plantarum</i> CECT 7527	+	+	+	
<i>Lactiplantibacillus plantarum</i> WCFS1	+	+	+	
<i>Lactiplantibacillus plantarum</i> 299v	+	+	+	
<i>Levilactobacillus brevis</i> CECT 7480	–	–	–	YES

A plus sign (+) indicates gene presence, whereas a minus sign (–) indicates absence

To identify candidate strains with the capacity to generate aglycones, the genomes of selected AB-Biotics strains, together with additional reference strains, were screened for the presence of three β -glucosidase genes *bglH1*, *bglH2*, and *bglH3* previously decriimplicated in *Lactiplantibacillus plantarum*-driven olive fermentation (Vaccalluzzo et al., 2022).

The comparative genomic analysis revealed a heterogeneous distribution of these loci across the evaluated panel (Table 36). As expected, most *L. plantarum* strains encoded at least one of the target β -glucosidase genes. Among them, *bglH2* and *bglH3* were the most frequently detected, suggesting that these variants may represent conserved functional determinants within the species. In contrast, *Levilactobacillus brevis* CECT 7480 lacked all three gene variants, reinforcing the species-specific nature of these enzymatic functions.

Based on the diversity of their *bglH* gene complements and their relevance to strategic market applications, five strains were selected for further functional characterization.

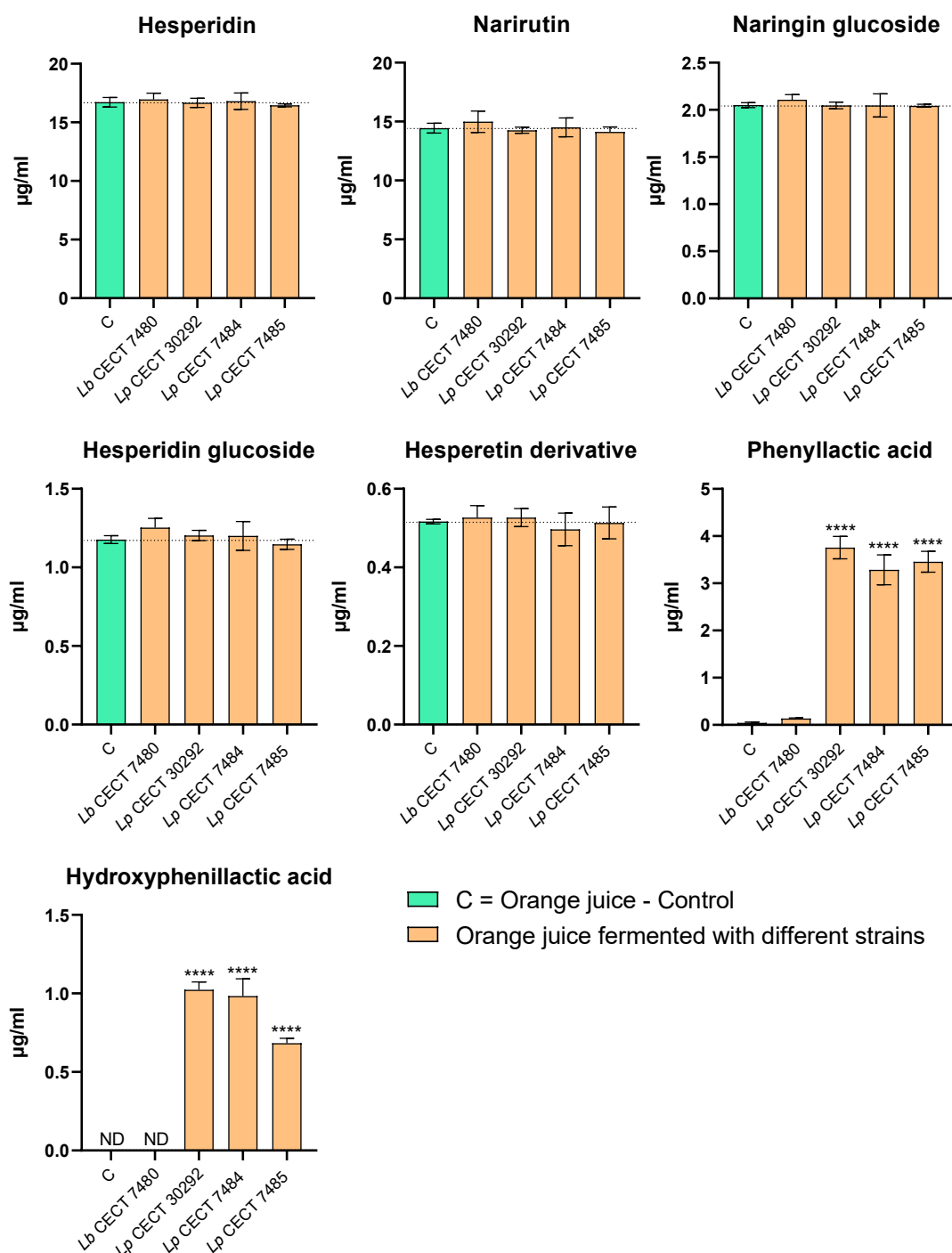


Figure 64. Quantitative metabolomic analysis of citrus flavonoids and phenolic metabolites in orange juice (Control, C) and samples fermented for 24 h at 37 °C with different probiotic strains: *L. brevis* CECT 7480, *L. plantarum* CECT 30292, CECT 7484, CECT 7485. Statistical significance was assessed using one-way ANOVA. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

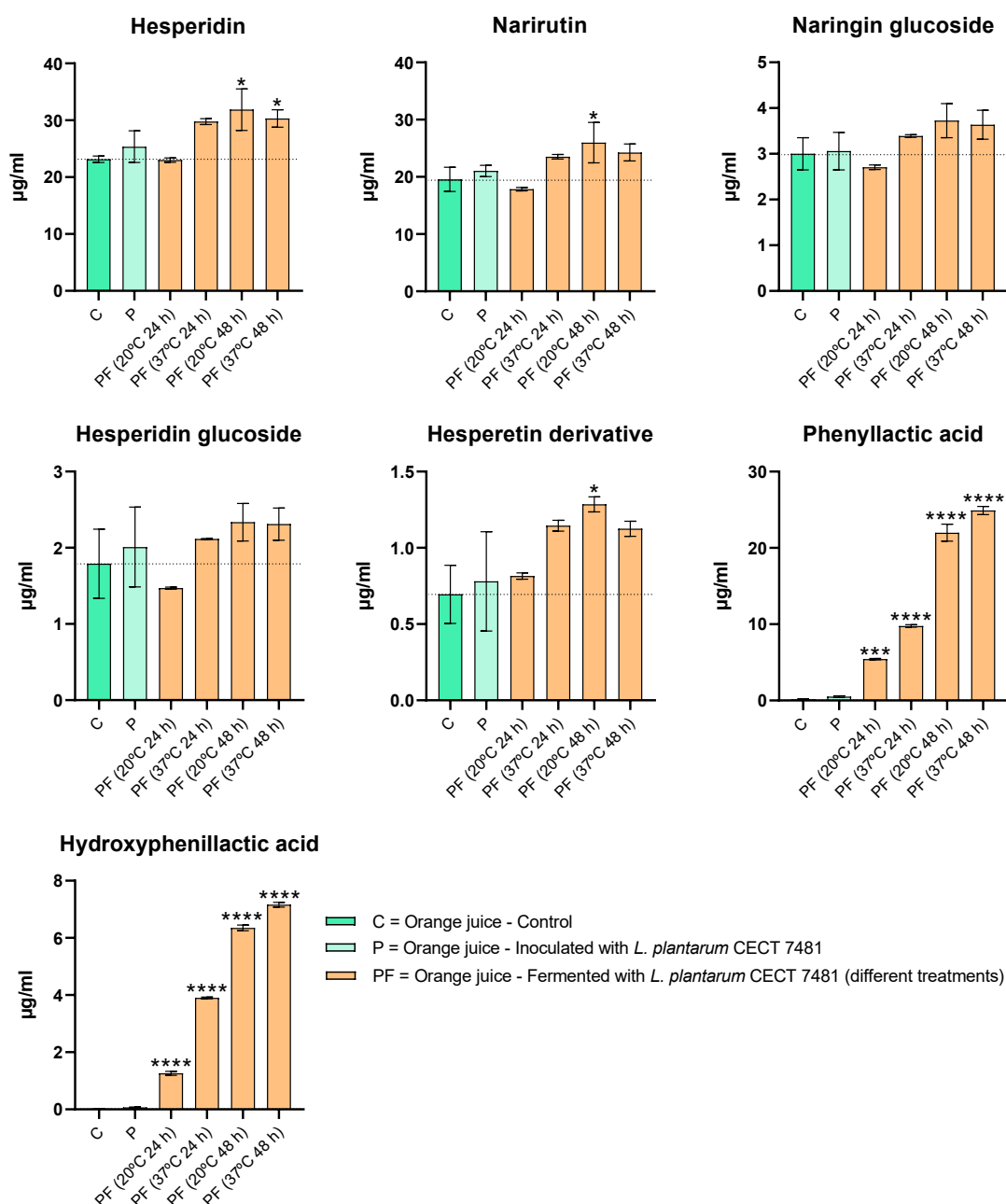


Figure 65. Quantitative metabolomic analysis of citrus flavonoids and phenolic metabolites in orange juice (Control, C), probiotic-supplemented orange juice non-fermented (P) and samples fermented for 24 h at 20/37 °C or 48h at 20/37 °C, with probiotic strain *L. plantarum* CECT 7481. Statistical significance was assessed using one-way ANOVA. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To assess the functional impact of selected strains on flavonoid biotransformation and phenolic metabolite production, targeted metabolomic analysis was performed on orange juices fermented for 24 h at 37 °C with four out of the five strains (*L. brevis* CECT 7480, *L. plantarum* CECT 30292, *L. plantarum* CECT 7484, *L. plantarum* CECT 7485) previously identified as promising candidates in the in silico screening (Figure 64). In addition, orange

juice fermented with *L. plantarum* CECT 7481 was evaluated under four different fermentation conditions (Figure 65), as this strain showed higher commercial relevance and was therefore further investigated to determine whether variations in fermentation parameters could influence fermentation performance and metabolite outcomes.

Hesperidin remained stable at 16.72 ± 0.40 $\mu\text{g/mL}$ in the control versus 16.67 ± 0.39 , 16.45 ± 0.13 , and 16.81 ± 0.69 $\mu\text{g/mL}$ for *L. plantarum* CECT 30292, *L. plantarum* CECT 7485, and *L. plantarum* CECT 7484, respectively; *L. brevis* CECT 7480 showed 16.96 ± 0.54 $\mu\text{g/mL}$. Similarly, narirutin concentrations were 14.44 ± 0.42 $\mu\text{g/mL}$ in the control and ranged from 14.12 ± 0.43 to 14.99 ± 0.90 $\mu\text{g/mL}$ across strains. Naringin glucoside remained unchanged (2.05 ± 0.03 $\mu\text{g/mL}$ in control vs. $2.04\text{--}2.10 \pm 0.05$ $\mu\text{g/mL}$ in fermented samples). Minor derivatives such as hesperidin glucoside (1.18 ± 0.02 $\mu\text{g/mL}$ in control vs. $1.15\text{--}1.25 \pm 0.06$ $\mu\text{g/mL}$) and hesperetin derivative (0.52 ± 0.006 $\mu\text{g/mL}$ in control vs. $0.50\text{--}0.53 \pm 0.04$ $\mu\text{g/mL}$) also showed no significant variation. These findings indicate that short-term fermentation does not promote rutinoside hydrolysis under these conditions, corroborating previous reports that deglycosylation by LAB is strain-dependent and often requires extended fermentation or specific enzymatic activity (Guo et al., 2021b; König et al., 2023). Conversely, phenolic acids exhibited pronounced changes. Phenyllactic acid (PLA) increased from 0.044 ± 0.011 $\mu\text{g/mL}$ in the control to 3.76 ± 0.24 , 3.46 ± 0.22 , and 3.29 ± 0.31 $\mu\text{g/mL}$ in juices fermented with Lp 30292, Lp 7485, and Lp 7484, respectively ($p < 0.0001$), while *L. brevis* 7480 produced only 0.13 ± 0.014 $\mu\text{g/mL}$. Hydroxyphenyllactic acid (HPLA) was absent in the control but reached 1.02 ± 0.05 $\mu\text{g/mL}$ (Lp 30292), 0.68 ± 0.03 $\mu\text{g/mL}$ (Lp 7485), and 0.98 ± 0.11 $\mu\text{g/mL}$ (Lp 7484) ($p < 0.0001$). These results confirm strain-dependent phenolic metabolism, consistent with previous observations for *Lactobacillus* spp. (Maione et al., 2025; Mu et al., 2012; Valerio et al., 2004). Both metabolites are recognized antimicrobial compounds derived from phenylalanine and tyrosine catabolism, supporting the role of *L. plantarum* in bioprotective activity during juice fermentation.

The analysis of *L. plantarum* CECT 7481 under different fermentation conditions demonstrated that process parameters strongly influenced metabolite profiles (Figure 65). Hesperidin increased significantly under 48 h treatments, reaching 31.87 ± 3.66 $\mu\text{g/mL}$ at 20°C and 30.32 ± 1.53 $\mu\text{g/mL}$ at 37°C , compared to the control (23.14 ± 0.56 $\mu\text{g/mL}$) ($p < 0.05$). Narirutin followed a similar trend, increasing from 19.56 ± 2.12 $\mu\text{g/mL}$ in the control to 25.99 ± 3.53 $\mu\text{g/mL}$ at 20°C for 48 h and 24.27 ± 1.49 $\mu\text{g/mL}$ at 37°C for 48 h. The

higher narirutin levels observed after prolonged fermentation suggest a time-dependent effect associated with increased flavonoid availability rather than de novo synthesis. Previous studies on lactic acid fermentation of orange matrices have demonstrated that extended fermentation promotes matrix disintegration and modification of pulp and fibre components, facilitating the release of flavanone glycosides such as narirutin from bound fractions (Razola-Díaz et al., 2024). In addition, *Lactiplantibacillus plantarum* is known to possess a broad repertoire of carbohydrate-active enzymes, which may indirectly enhance flavonoid accessibility through polysaccharide and glycoside turnover during prolonged fermentation (Plessas, 2022). Minor flavonoid derivatives exhibited modest but significant changes. Hesperidin glucoside increased from $1.79 \pm 0.45 \mu\text{g/mL}$ in the control to $2.34 \pm 0.25 \mu\text{g/mL}$ (20 °C/48 h), while hesperetin derivative rose from $0.69 \pm 0.19 \mu\text{g/mL}$ to $1.28 \pm 0.05 \mu\text{g/mL}$ under the same condition ($p < 0.05$), supporting the hypothesis of biotransformation toward more bioavailable aglycones, as previously demonstrated in citrus matrices fermented by LAB (Razola-Díaz et al., 2024).

Phenolic acids showed the most pronounced changes. PLA increased from $0.16 \pm 0.07 \mu\text{g/mL}$ in the control to $5.43 \pm 0.08 \mu\text{g/mL}$ (20 °C/24 h), $9.77 \pm 0.16 \mu\text{g/mL}$ (37 °C/24 h), and reached $22.00 \pm 1.11 \mu\text{g/mL}$ (20 °C/48 h) and $24.91 \pm 0.54 \mu\text{g/mL}$ (37 °C/48 h) ($p < 0.0001$). HPLA, absent in the control ($0.03 \pm 0.00 \mu\text{g/mL}$), was detected exclusively in fermented juices, increasing to $1.26 \pm 0.07 \mu\text{g/mL}$ (20 °C/24 h) and up to $7.16 \pm 0.09 \mu\text{g/mL}$ under 37 °C/48 h ($p < 0.0001$). These results confirm that amino acid-derived phenolic metabolism is strongly modulated by fermentation time and temperature, consistent with previous findings for *Lactobacillus* spp. (Kasperek et al., 2024).

The absence of significant reductions in hesperidin and narirutin suggests limited β -rutosidase activity under the tested conditions. However, the increase in hesperetin derivatives points to β -glucosidase-mediated hydrolysis, in agreement with genomic predictions of bglH1 gene presence, which encodes a phosphor- β -glucosidase involved in glycoside breakdown (Gänzle, 2015).

The simultaneous production of PLA and HPLA highlights the metabolic versatility of lactic acid bacteria, as these compounds originate from phenylalanine catabolism via the phenylpyruvate pathway (Valerio et al., 2004; Wenger et al., 2023). Beyond their role as metabolic intermediates, PLA and HPLA are recognized for their antioxidant and antimicrobial properties, which may enhance the functional profile and shelf-life of fermented juices (Maione et al., 2025; Parappilly et al., 2024).

Overall, the findings indicate that short fermentation primarily influences phenolic acids, whereas prolonged fermentation with *L. plantarum* can modulate flavonoid profiles and promotes the synthesis of bioactive metabolites, albeit with modest flavonoid deglycosylation. Optimization of fermentation parameters or strain selection may be required to maximize aglycone release, which is critical for improving bioavailability and health benefits. This strain- and condition-dependent modulation of phenolic composition highlights the importance of controlled fermentation as a strategy to improve the nutritional and preservative properties of citrus-based beverages. Such an approach aligns with current trends in developing non-dairy probiotic drinks enriched with phenolic compounds and antimicrobial metabolites to deliver added health benefits.

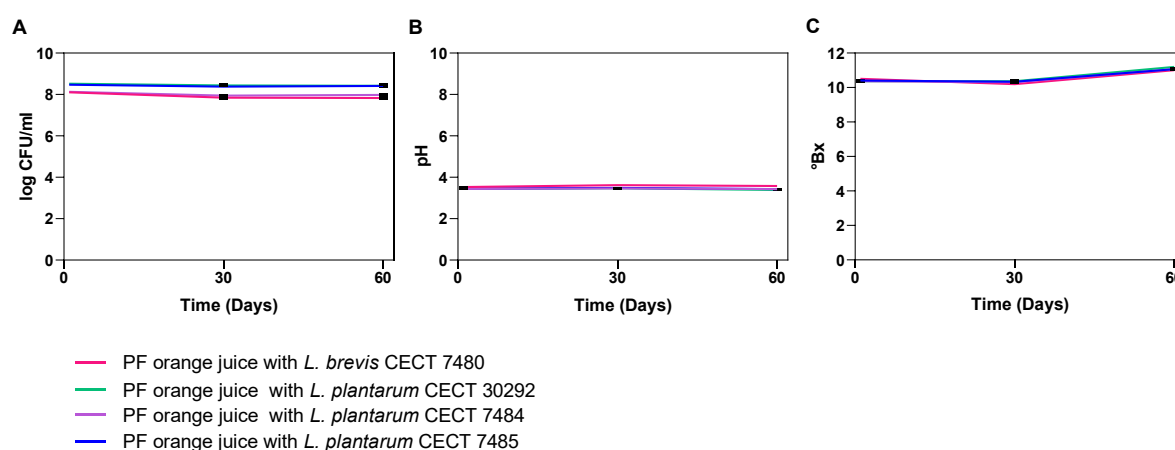


Figure 66. Probiotic viability and physicochemical characteristics of orange juice fermented (PF) with different strains (*L. brevis* CECT 7480, *L. plantarum* CECT 30292, CECT 7484, CECT 7485), during 60 days of storage at 4 ± 2 °C. A) Probiotic viability expressed as log CFU/ml. B) pH. C) TSS expressed as °Brix.

The inoculation of the different probiotic strains was designed, as detailed in Section 3.2.6 (Materials and Methods), to ensure a target probiotic concentration in the final product of 6.96 log CFU/ml per 330 ml serving, and a 10-fold overage was applied, resulting in an initial inoculum of 7.96 log CFU/ml to compensate for potential viability loss during extended storage.

Figure 66-A presents the evolution of probiotic viability during storage. In this study, T0 corresponds to the cell count measured immediately after the fermentation step. As observed, *L. plantarum* CECT 7485 and CECT 30292 exhibited the greatest increases in CFU during the 24 h fermentation at 37 °C. Following this initial growth phase, all fermented orange juice formulations maintained probiotic populations above 7.83 log CFU/g throughout 60

days at 4° C, with viability losses remaining below 0.27 log units. This high level of stability is consistent with previous reports demonstrating that *L. plantarum* strains are capable of sustaining populations exceeding 10⁷ CFU/mL for extended periods (≥60 days) in citrus-based and other fruit juice matrices. Such persistence is attributed to the species' robust ecological fitness in acidic, sugar-rich environments, which restrict competitor proliferation while supporting the long-term maintenance of lactic acid bacteria following fermentation (Rizzi et al., 2024b). Mechanistically, the orange juice matrix likely provides multiple microniches and protective solids (pectin/soluble fibres), moderating acid stress and osmotic shocks and thereby limiting death rates; similar matrix mediated protection and high survival during 30–60 days have been reported for *L. plantarum* in carrot–orange systems and other vegetable/fruit blends, especially when prebiotics (e.g., inulin) are present (Valero-Cases & Frutos, 2017).

The pH (Figure 66-B) remained stable throughout storage across all treatments, with slight strain-dependent differences: *L. brevis* 7480 showed the highest pH (3.58 ± 0.05), indicating less acidification during fermentation, whereas *L. plantarum* strains maintained lower values (3.43–3.45), consistent with their stronger acidifying capacity. This stability is desirable for sensory quality and microbial safety, as low pH inhibits spoilage organisms while maintaining product palatability (Di Cagno et al., 2013).

TSS values (Figure 66-C) showed a slight but significant increase ($p < 0.05$) from ~10.5 to ~11 during 60 days of refrigerated storage, consistent with previous findings in fermented citrus juices (Sharaf et al., 2014). This minor rise can be attributed to residual enzymatic hydrolysis and is considered technologically negligible for product quality.

The results demonstrate that probiotic-fermented orange juice is a stable product during 60 days of refrigerated storage, retaining both high probiotic viability and consistent physicochemical properties. This stability supports its potential as a functional beverage with extended shelf life. Among tested strains, no major differences were observed in viability or physicochemical parameters, suggesting that all candidates are suitable for industrial application. These findings align with previous studies on fruit-based probiotic beverages, confirming that lactic acid bacteria can maintain viability in acidic matrices without compromising sensory quality (Di Cagno et al., 2013; Szutowska, 2020). Future work could explore the impact of packaging, oxygen exposure, and additional functional ingredients on long-term stability.

4.2.4.3.2 TEST 2: Functional Evaluation of Probiotic Strain *L. plantarum* CECT 7481 in Orange, Apple and Peach Juices

The stability and properties of different fruit juices enriched and fermented with *L. plantarum* CECT 7481, were assessed according to the experimental design outlined in Figure 67.

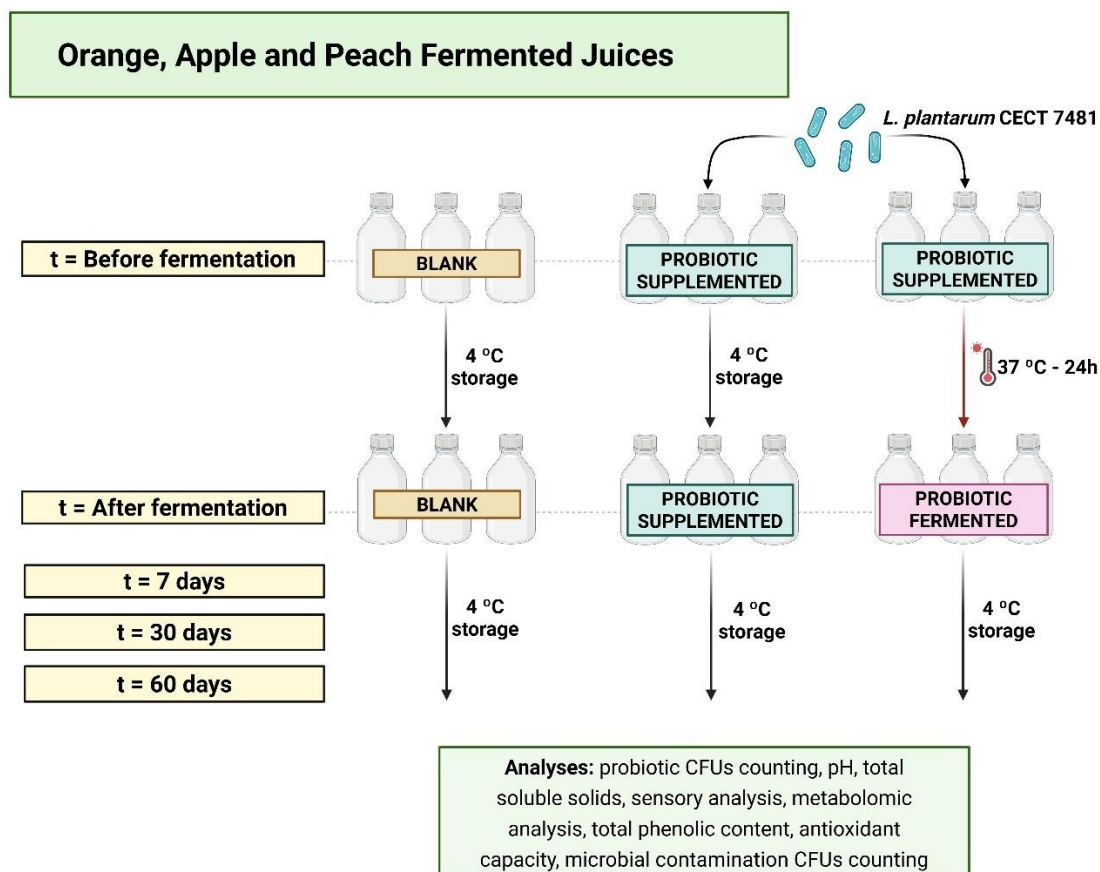


Figure 67. Schematic illustration of the experimental design. For each fruit juice type (orange, apple, and peach), three variants were prepared: a blank (without probiotics), a probiotic-supplemented juice, and a probiotic-fermented juice. All probiotic treatments contained *L. plantarum* CECT 7481. In the fermented variants, inoculation was followed by incubation at 37 °C for 24 h prior to refrigerated storage at 4 ± 2 °C. Samples were collected before fermentation, immediately after fermentation, and at 7, 30, and 60 days of storage. Evaluated parameters included viable probiotic counts (CFU), pH, TSS, and microbiological contamination (post-fermentation and at day 60). Additionally, sensory evaluation, metabolomic profiling, and antioxidant capacity analyses (TPPC, DPPH-RSA) were performed on freshly produced samples. The figure was created in <https://BioRender.com>.

Fruit and vegetable juices represent promising carriers for probiotics, offering a non-dairy alternative to deliver health-promoting microorganisms and bioactive compounds. However, the stability of probiotics and the functional impact of fermentation in these matrices remain relatively underexplored compared to traditional dairy systems. In this context, our study focused on the fermentation of orange, apple, and peach juices using *L. plantarum* KABP051

(CECT 7481), a well-characterized probiotic strain. The work aimed to evaluate microbial viability, physicochemical changes, and sensory acceptance, while also investigating metabolomic shifts associated with fermentation. Attention was given to the formation of bioactive molecules such as phenyllactic acid, N-acetyl glutamine, nicotinic acid, and acetylcholine, which may enhance the nutritional and functional profile of fermented juices. This research provides evidence that fruit juices can serve as effective probiotic carriers and highlights the potential of controlled lactic fermentation to produce safe, palatable, and functionally enriched beverages.

An article derived from this part of the research was published in *Foods* in November 2024 (DOI: 10.3390/foods13233851), and the following pages reproduce the full disclosed version of that publication (Rizzi et al., 2024b).

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***Lactiplantibacillus plantarum* KABP051: Stability in Fruit Juices and Production of Bioactive Compounds During Their Fermentation**

by Francesca Rizzi ^{1,2}, Bibiana Juan ¹, Jordi Espadaler–Mazo ², Marta Capellas ¹ and Pol Huedo ^{2,3,*}

¹ Centre d'Innovació, Recerca i Transferència en Tecnologia dels Aliments (CIRTTA), XIA, TECNIO, Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona (Cerdanyola del Vallès), 08193 Barcelona, Spain

² R&D Department, AB-Biotics S.A. (Part of Kaneka Corporation), 08174 Barcelona, Spain

³ Basic Sciences Department, Universitat Internacional de Catalunya, 08195 Barcelona, Spain

* Author to whom correspondence should be addressed.

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Abstract

The lactic fermentation of fruit and vegetable juices by well-characterised probiotics remains relatively underexplored. We have investigated the stability and impact of *Lactiplantibacillus plantarum* KABP051 fermentation on orange, apple, and peach juices by microbiological, physicochemical, and sensory evaluation means. For each fruit juice, three

different samples were analysed: original fruit juice without probiotic as blank (B), fruit juice inoculated with 10^7 CFU/mL of probiotic without fermentation (P), and fruit juice inoculated with 10^7 CFU/mL of probiotic and fermented at 37 °C for 24 h (PF). P samples displayed good stability throughout the study, and PF samples showed an initial increase in CFUs accompanied by a change in pH, confirming the ability of the probiotic to ferment these juices. After 60 days of refrigeration, PF samples contained $>10^7$ CFU/mL. Total phenolic content and antioxidant capacity were equivalent in F, P, and PF. Remarkably, deep metabolomic analyses confirmed malolactic fermentation and revealed the production of several bioactive compounds including the antimicrobial substance phenyllactic acid, the immunomodulatory and anti-fatigue amino acid N-acetyl glutamine, the vitamin B3 form nicotinic acid, the monoterpene (–)- β -pinene, and the neurotransmitter acetylcholine, among others, during probiotic fermentation. Finally, a hedonic analysis involving 51 participants showed that probiotic fermented orange juice is well accepted by panellists, with scores comparable to those of the control juice. Overall, we here show that fruit juices are excellent carriers for the delivery of the probiotic *L. plantarum* KABP051 and its non-alcoholic fermentation can result in tasty functional fruit juices enriched with health-promoting compounds.

Keywords: probiotics; fermentation; functional foods; bioactive compounds; fruit juices

1. Introduction

In recent years, and especially after the pandemic period, consumers have gained awareness about the importance of preventive health. In this context, gut microbiota is gaining momentum, with increasing evidence on the importance of maintaining its balance for human health [1,2]. It is known that gut microbiota composition not only influences the digestive system but also has an impact on distant organs and systems by the different gut–organ axes, including the so-called gut-brain, gut-liver, gut-lung, gut-muscle and gut-skin axes [3,4,5,6,7,8]. Different probiotics have shown efficacy in the modulation of these axes, exerting beneficial effects beyond the digestive tract [9].

The term “functional food”, apparently coined in Japan in the 1980s–90s, defines a food that, beyond basic dietary benefits, exerts additional health effects in the body [10]. Probiotics, in line with the latter term, are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [11]. Recently, the International Scientific Association for Probiotics and Prebiotics (ISAPP) has defined the terms “fermented food” and “probiotic fermented food” [12]. According to ISAPP, “fermented foods” are “foods made through desired microbial growth and enzymatic conversions of food components”, and “probiotic fermented food” are “food fermented by or containing probiotic(s) with strain-specific evidence”. As per definition, the number of live probiotic cells must be sufficient to exert the beneficial effects validated in preclinical and clinical studies throughout the shelf life of the product [12,13].

Fermented foods are the oldest and most traditional version of functional foods and have been part of the human diet for millennia [14,15,16]. Several bioactive metabolites have been identified as a result of bacterial protein, lipid, and carbohydrate metabolism during the fermentation process. The production of vitamins and antioxidants as well as an increase in

the bioavailability of minerals has been reported for many lactic acid bacteria species [17]. A wide assortment of biologically active peptides of bacterial origin has also been described in fermented products displaying antioxidant, anti-microbial, anti-fungal, anti-inflammatory, anti-diabetic, and anti-atherosclerotic activity, among others [18]. Thus, fermented probiotic products, which contain active bacteria and biomolecules, exert health-promoting effects by modulating the intricate network that involves probiotic-, biomolecules- gut microbiota-, and host interactions.

Food and beverage matrices are a combination of countless sets of physicochemical parameters and conditions (pH, water activity, temperature, etc.) that may affect probiotic viability. Moreover, each probiotic strain has different traits and growth and survival needs [11]. Therefore, every probiotic strain should be individually investigated and selected according to the characteristics of the food and the microbe.

Historically, most marketed probiotic fermented foods have been of dairy origin, limiting their utilisation by consumers following strict vegetarian or vegan diets, as well as those with food intolerances. Therefore, the addition of probiotics to fruit juices, a plant-based matrix, could not only increase the nutritional value of the product but also benefit a wider population [19].

Fruits are naturally rich in minerals, vitamins, dietary fibres, antioxidants, and many other beneficial nutrients that make them essential components of a healthy and balanced diet. Due to new healthy trends, sales of fruit and vegetable juices have increased in recent years [20].

Lactiplantibacillus plantarum is frequently used as a starter or adjunct cultures in fermentation of raw materials from plant and animal origin because of its environmental resilience and metabolic versatility [21]. *L. plantarum* strains display a high level of genome diversity, yet their shared core genome reveals a remarkably nomadic lifestyle, ranging from fruits and vegetables to the intestinal tract of herbivores and omnivores, stools, urine, or milk [22]. *L. plantarum* KABP051 is one such nomadic strain, independently isolated both from human faeces and milk, which has shown a beneficial effect in various clinical trials [23,24,25,26] and been subjected to extensive characterisation. For these reasons, we selected this strain to investigate its behaviour in natural fruit juices.

The aim of the present study was to assess the stability (viability) of the probiotic *L. plantarum* KABP051 in orange, apple, and peach juices during two months of refrigerated storage, and to investigate the impact of its presence and fermentation on the physicochemical, nutritional, and organoleptic characteristics of the juices by microbiological, metabolomic, and sensory evaluation approaches.

2. Materials and Methods

2.1. Materials

2.1.1. Bacterial Strains

A freeze-dried culture of the probiotic lactic acid bacteria *Lactiplantibacillus plantarum* KABP051 (CECT7481, DR7TM) was supplied by AB-Biotics SA (KANEKA Corp., Barcelona, Spain) and stored at -20 °C in a sealed aluminium bag until use.

2.1.2. Fruit Juices

Three different commercial UHT 100% fruit juices were purchased in local supermarkets in Barcelona, Spain, from local brands: orange (“Orange juice without pulp”, Hacendado, Barcelona, Spain), apple (“Organic apple juice”, Bonpreu, Barcelona, Spain), and peach (“Organic peach juice”, Bonpreu, Barcelona, Spain).

2.2. Methods

2.2.1. Inoculation and Fermentation of Fruit Juices

To obtain an initial probiotic concentration of 10⁷ CFU/mL (corresponding to 10⁹ CFU/serving in 240 mL), all juices were inoculated with the freeze-dried *L. plantarum* strain KABP051 under aseptic conditions. For each fruit juice, three different samples were prepared: original fruit juice without probiotic as blank (B), fruit juice with 10⁷ CFU/mL of probiotic without fermentation (P), and fruit juice inoculated with 10⁷ CFU/mL and fermented at 37 °C for 24 h (PF). Samples were stored at 4 °C for 60 days (**Figure S1**).

2.2.2. Stability Analyses

Microbiological and physicochemical changes in the fruit juices were determined at days 0 (before and after inoculation), 1, 7, 30, and 60 to assess product quality and probiotic stability as follows:

Physicochemical Analyses

B, P, and PF juices were tempered to 20 °C before physicochemical analyses. pH was measured using a pH-Meter (Basic 20 Crison Instruments, S.A., Barcelona, Spain), and total soluble solids (TSS) were analysed using an optical refractometer (Analogue hand-held refractometer PREF-B32-001, Labbox Labware, S.L., Barcelona, Spain), and the results were reported as °Brix [27].

Bacterial Viable Count

CFU enumeration was determined by the standard plate count method in triplicate. Serial dilutions of probiotic juices were prepared using a peptone buffer, and 100 µL of the appropriate dilutions were seeded onto de Man, Rogosa, and Sharpe (MRS) agar and incubated anaerobically at 37 °C for 48 h. To discard bacterial contamination, samples were analysed before probiotic inoculation and at the end of the study. The presence of coliforms, mesophilic aerobic bacteria, psychrotrophic bacteria, and yeasts/moulds was investigated by seeding samples onto Chromogenic Coliform Agar, Plate Count Agar, and Rose-Bengal Chloramphenicol Agar. These plates were incubated 24 h at 37 °C, 72 h at 30 °C, 7 days at 7 °C, and 7 days at 30 °C, respectively.

2.2.3. Determination of Total Phenolic Content

Total phenolic content in B, P, and PF samples was determined by using an adaptation of the Folin-Ciocalteu (F-C) assay [28]. In brief, 1.97 mL of Milli-Q water and 175 µL of Folin

& Ciocalteu's phenol reagent were added to 100 μL of the appropriate filtered and diluted sample, standard or blank. Milli-Q water, instead of juice, was used for the control. After 8 min of incubation at room temperature, 375 μL of 20% Na_2CO_3 solution were added, and the mixture was incubated for 30 min in complete darkness at 40 $^\circ\text{C}$. The final mixture absorbance was measured using a spectrophotometer at 765 nm.

A gallic acid standard calibration curve (0–500 mg/L) was used for the quantification of total phenolic compounds, and the results were expressed as gallic acid equivalent (GAE) using units of mg/L.

2.2.4. Determination of DPPH Radical Scavenging Activity *In Vitro*

To infer the potential antioxidant activity (AC) of B, P, and PF juices, an adaptation of the 2,2-difenil-1-picrilhidrazil (DPPH) radical scavenging method [29,30] was followed. Fifty μL of each juice sample was filtered and diluted until absorbance values within the calibration curve were obtained. Juice samples, standards, and blank were mixed with 1.95 mL of methanolic DPPH 0.1 mM solution and kept at complete darkness for one hour at room temperature. Methanol instead of juice was used for the blank. The final mixture absorbance was measured using a spectrophotometer at 515 nm.

A Trolox five-point standard calibration curve (0.1–0.7 mM) was used for the quantification of antioxidant capacities, and the results were expressed as Trolox equivalent (TE) using units of $\mu\text{mol/L}$.

2.2.5. Metabolomic Analyses

Metabolomic analyses of B, P, and PF juices were carried out to investigate the production of potential bioactive compounds by *L. plantarum* KABP051 during fermentation. Juice samples were centrifuged (14,000 $\times$ g), filtered (0.2 μm), and stored at -20 $^\circ\text{C}$ until needed. Metabolomic analyses were performed by MS-Omics (Copenhagen, Denmark) as follows.

For the SCFA analyses, samples were acidified using hydrochloride acid, and deuterium labelled internal standards were added. Samples were analysed in a randomised order without extraction. Analysis was performed using a high polarity column (ZebronTM ZB-FFAP, GC Cap. Column 30 m $\times$ 0.25 mm $\times$ 0.25 μm) installed in a GC (7890B, Agilent, Madrid, Spain) coupled with a time-of-flight MS (Pegasus[®] BT, LECO, Madrid, Spain). The system was controlled by ChromaTOF[®] (LECO Instrumentos S.L., Madrid, Spain). Raw data were converted to netCDF format using Chemstation (Agilent), before the data were imported and processed in Matlab R2014b (Mathworks Inc., Natick, MA, USA) using the PARADISE software version 6 described by Johnsen et al. [31].

The semi-polar metabolite analysis was carried out using a Thermo Scientific Vanquish LC coupled to an Orbitrap Exploris 240 MS, Thermo Fisher Scientific (Madrid, Spain). Samples were extracted as follows: 75 μL samples were mixed with 25 μL aqueous solution of zink nitrate hexahydrate (5% w/v) and ammonium thiocyanate (20% w/v). After centrifugation at 15,000 $\times$ g for 15 min at 4 $^\circ\text{C}$, supernatant was filtered through a 0.22 μm filter. An electrospray ionisation interface was used as ionisation source. Analysis was performed in polarity switching ionisation mode.

The UPLC was performed using a slightly modified version of the protocol described by Doneanu et al. [32]. Peak areas were extracted using Compound Discoverer 3.2 (Thermo Scientific). The identification of compounds was performed at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; Level 2a: identification by retention times (compared against in-house authentic standards) and accurate mass (with an accepted deviation of 3 ppm); Level 2b: identification by accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra; and Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm).

Finally, a targeted metabolomic analysis following the same semi-polar metabolite method was conducted to verify the identity and the concentration of the following compounds: Nicotinic acid (CAS: 59-67-6), Phenyllactic acid (CAS: 828-01-3), Acetylcholine (CAS: 51-84-3) and N-Acetyl-L-glutamine (CAS: 2490-97-3).

2.2.6. Consumer Acceptability

A sensory evaluation with 51 untrained panellists recruited amongst faculty staff and students (27 females and 24 males; aged 23–63 years old) was conducted to assess whether the average consumer could detect differences between the products. Prior to the tasting, the panellists were provided with detailed instructions regarding the product, the evaluation parameters, and the methodology to be followed during the assessment. Consumers were asked to evaluate the intensity and acceptability of different parameters as well as the purchase probability of fermented probiotic orange juice. Two different samples were tested: original orange juice without probiotic and orange juice with not less than 10^7 probiotic CFU/mL fermented at 37 °C for 24 h.

The evaluation sessions took place 52 h after the inoculation and the juices were served at 5 ± 3 °C, poured in portions of 25 mL into white cups, identified with random three-digit codes and offered simultaneously to panellists. Bottled water was available to rinse the mouth between samples. A nine-point scale was used for the evaluation of acceptability and intensity of aroma, colour, bitterness, sweetness, acidity, and strange flavours, with 1 = Dislike extremely/Extremely low intensity and 9 = Like extremely/Extremely intense. The parameter “Purchase probability” was evaluated with a five-point scale, 1 = Definitely will not buy and 5 = Definitely will buy. The parameter “Overall preference” was evaluated by placing the three samples in order of preference: 1 = Worst; 2 = Intermediate; 3 = Best.

2.2.7. Statistical Analysis

All experiments were carried out in triplicate, and values are reported as mean $\pm$ standard deviation (SD). Physicochemical, microbiological, and sensory evaluation tests were analysed using *t*-test. Metabolomic results were analysed using *t*-test, and the Benjamini–Hochberg correction was applied to control the false discovery rate (FDR = 0.1). The principal components analysis (PCA) models and their loading plots were calculated on the relative concentrations of the variables annotated on levels 1, and 2a.

Differences with a two-sided *p* value < 0.05 were considered statistically significant. Statistical analyses were performed with software PAST v4 (PCA and loading plots) and GraphPad PRISM v8 (all other analyses).

3. Results

3.1. *Lactiplantibacillus plantarum* KABP051 Stability in Supplemented and Fermented Orange, Apple and Peach Juices During 60 Days at 4 °C

The initial inoculum was calculated to reach the clinical dose (not less than 10^9 CFU) per serving, defined as 240 mL/day of 100% fruit juices [33]. As shown in **Figure 1A**, CFU counts of orange and apple supplemented (non-fermented) juices maintained good stability throughout the study, while peach juice showed a remarkable reduction in bacterial viability after 4 weeks. Initial counts were 7.12 ± 0.00 log CFU/mL in orange juice, 7.02 ± 0.03 log CFU/mL in apple juice, and 7.08 ± 0.05 log CFU/mL in peach juice. After 60 days of cold storage, the bacterial load was 6.65 ± 0.06 log CFU/mL in orange juice, 6.41 ± 0.01 log CFU/mL in apple juice, and 5.69 ± 0.05 log CFU/mL in peach juice. No bacterial/fungal contamination was detected throughout the study.

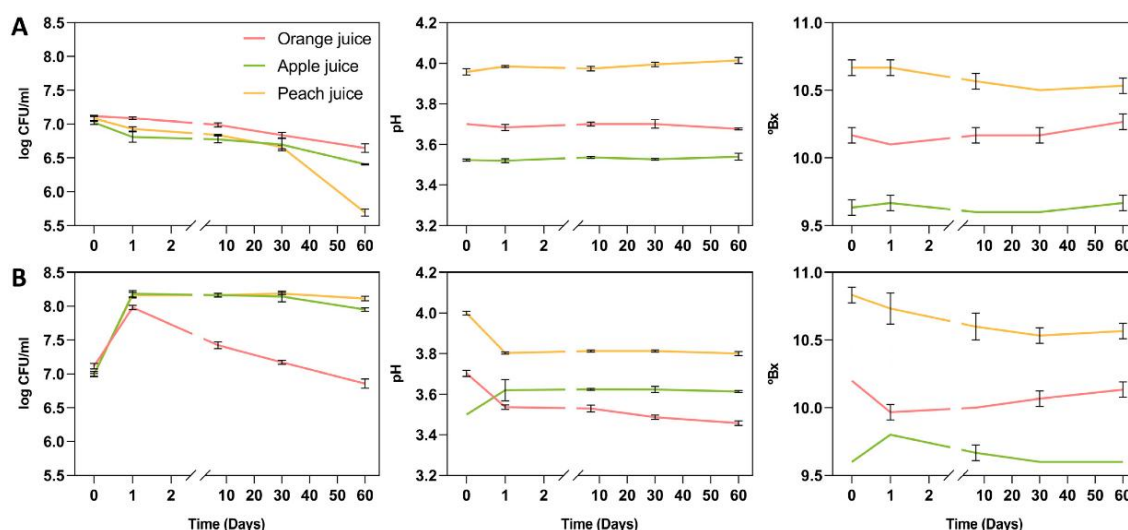


Figure 1. Results of stability analysis (log CFU/mL, pH and total soluble solids (°Bx) in orange, apple, and peach juices inoculated with *Lactiplantibacillus plantarum* KABPTM 051 and stored 60 days at 4 °C without fermentation (A) and after a fermentation of 24 h at 37 °C (B).

The initial pH values of orange, apple, and peach juices were 3.69 ± 0.01 , 3.51 ± 0.01 and 3.95 ± 0.01 , respectively. pH remained stable in all juices throughout the two months of storage (**Figure 1A**). The average of total soluble solids that initially measured 10.1 ± 0.1 °Bx in orange juice, 9.6 °Bx in apple and 10.7 ± 0.1 °Bx in peach juices showed a similar trend to pH, without significant changes throughout the study (**Figure 1A**).

In the 24 h-fermented samples (**Figure 1B**), the number of CFU/mL in all three juices was significantly higher than the initial inoculum. The initial average count was 7.11 ± 0.04 log CFU/mL in orange juice, 7.00 ± 0.04 log CFU/mL in apple juice, and 6.99 ± 0.02 log CFU/mL in peach juice. After fermentation, *L. plantarum* KABP051 concentration increased up to 7.98 ± 0.04 log CFU/mL in orange juice, 8.18 ± 0.05 log CFU/mL in apple juice, and 8.17 ± 0.04 log CFU/mL in peach juice. After 60 days of cold storage, CFU counts were 6.86 ± 0.07 log CFU/mL in orange juice, 7.95 ± 0.02 log CFU/mL in apple juice, and 8.11 ± 0.03 log CFU/mL in peach juice.

The pH of orange and peach juices significantly decreased after fermentation from 3.70 ± 0.02 to 3.54 ± 0.01 and from 4.00 ± 0.01 to 3.80 ± 0.01 , respectively (both $p \leq 0.0001$). In contrast, the pH of apple juice showed a slight but significant increase after fermentation from 3.50 to 3.62 ± 0.05 ($p = 0.017$). The pH of the three juices remained stable during the two months of cold storage. No major changes were noted for total soluble solids (TSS) in the three juices after fermentation, although a slight decrease was observed for orange and apple juices and a small increase was noted for the apple juice. These results indicate that *L. plantarum* KABP051 is stable and can ferment fruit juices, increasing CFU counts and modifying the pH of the matrix.

3.2. Total Phenolic Content and DPPH Radical Scavenging Activity

Table 1 shows the results of the total phenolic content (TPC) and DPPH radical scavenging activity of day 1 samples. Among blank samples, apple juice showed the lowest TPC, followed by peach juice and orange juice. After fermentation, only orange juice showed a TPC increment (from 709.9 ± 22.5 to 746.1 ± 27.6 mg GAE/L), although it did not reach statistical significance. The DPPH radical scavenging activity of blank juices correlated with their TPC, as the apple sample showed the lowest potential antioxidant capacity, followed by peach and orange juice. As for TPC results, no statistically significant differences in antioxidant potential were noted in fermented juices ($p > 0.05$).

Table 1. Total phenolic compounds (TPC) and DPPH radical scavenging activity (DPPH-RSA) of orange, apple, and peach juices.

	Orange Juice		Apple Juice		Peach Juice	
	TPC	DPPH-RSA	TPC	DPPH-RSA	TPC	DPPH-RSA
	(Gallic Acid mg/L)	(Trolox μ mol/L)	(Gallic Acid mg/L)	(Trolox μ mol/L)	(Gallic Acid mg/L)	(Trolox μ mol/L)
B	709.9 ± 22.5	2309 ± 127.5	262.8 ± 21.3	137.7 ± 16.1	314.4 ± 2.3	783.1 ± 4.9
P	682.3 ± 2.9	2722.9 ± 131.4	264.8 ± 1.1	136.4 ± 31.5	320.5 ± 23.6	782.2 ± 18.5
PF	746.1 ± 27.6	2731.3 ± 190.3	269.7 ± 14.9	139.5 ± 59.5	312.4 ± 8.6	790.8 ± 23.6

DPPH: 2,2-difenil-1-picrilhidrazil. B: Blank; P: probiotic (non-fermented); PF: probiotic fermented.

3.3. Metabolome Profiling of Fruit Juices

To further characterise the functionality of fruit juices, metabolomic analyses were conducted in day 1 samples. First, the presence of short-chain fatty acids (SCFA) was investigated by targeted approaches (**Figure 2**). Formic acid was the most abundant SCFA in blank juices at around 10, 7, and 12 mM concentration in orange, apple, and peach juices, respectively (**Table S1**). Acetic acid was also detected in the three juices at lower concentration: around 1, 2, and 2 mM in orange, apple, and peach juices, respectively. The other SCFAs analysed were found at very low concentrations. The presence of probiotic did not modify the concentration of any of the SCFAs, but probiotic-assisted fermentation

significantly increased levels of acetic acid in orange (from 0.98 ± 0.1 mM to 1.96 ± 0.11 mM) and peach (from 2.12 ± 0.19 mM to 3.51 ± 0.32 mM) juices (**Figure 2C**).

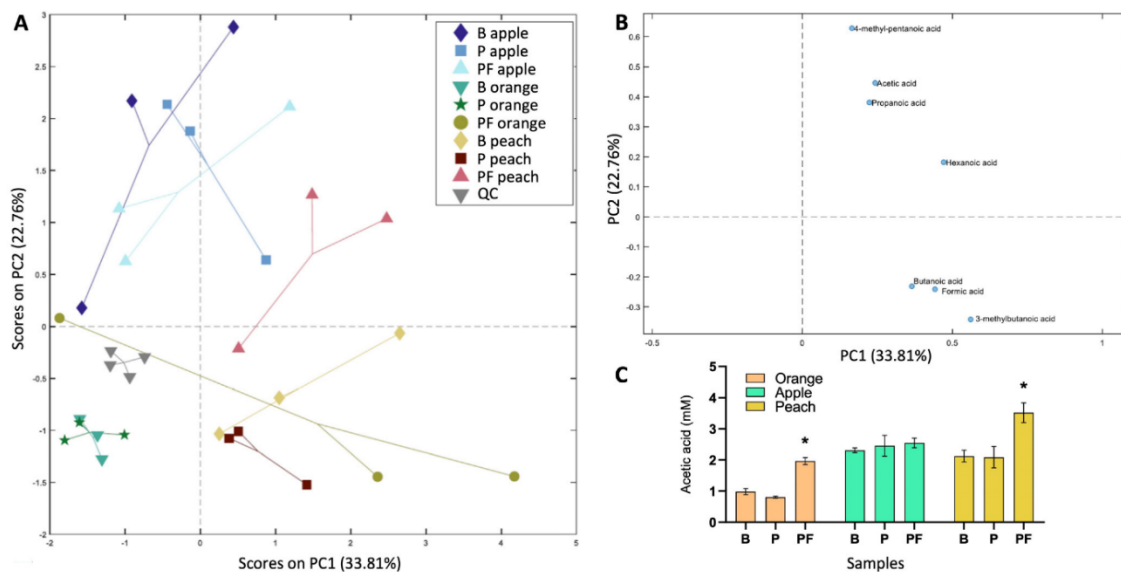


Figure 2. Metabolomic analysis of SCFAs in fruit juices. (A) Score plot of PC2 over PC1 in the model calculated on the relative concentrations of SCFAs in the reduced dataset. Data have been autoscaled. (B) Loading plot from PCA model calculated on the relative concentrations of SCFAs in the reduced dataset. Data have been autoscaled, and overlapping labels were removed to improve readability. (C) Acetic acid concentration (mM) in blank (B), probiotic-supplemented (P), and probiotic-fermented (PF) fruit juices. * $p < 0.05$.

An untargeted semi-polar metabolite analysis was also conducted to identify additional beneficial compounds produced by *L. plantarum* KABP051 during the fermentation of fruit juices. A total of 672 compounds were detected, of which 32 metabolites could be identified as level 1; 56 compounds were annotated as level 2a; 21 were annotated as level 2b, and, with less certainty, 128 compounds were detected in level 3. Finally, 439 compounds were distinguished from the background but remained unknown. The first two principal components explained 51.69% (PC1) and 26.51% (PC2) of the total variation (**Figure 3A,B**).

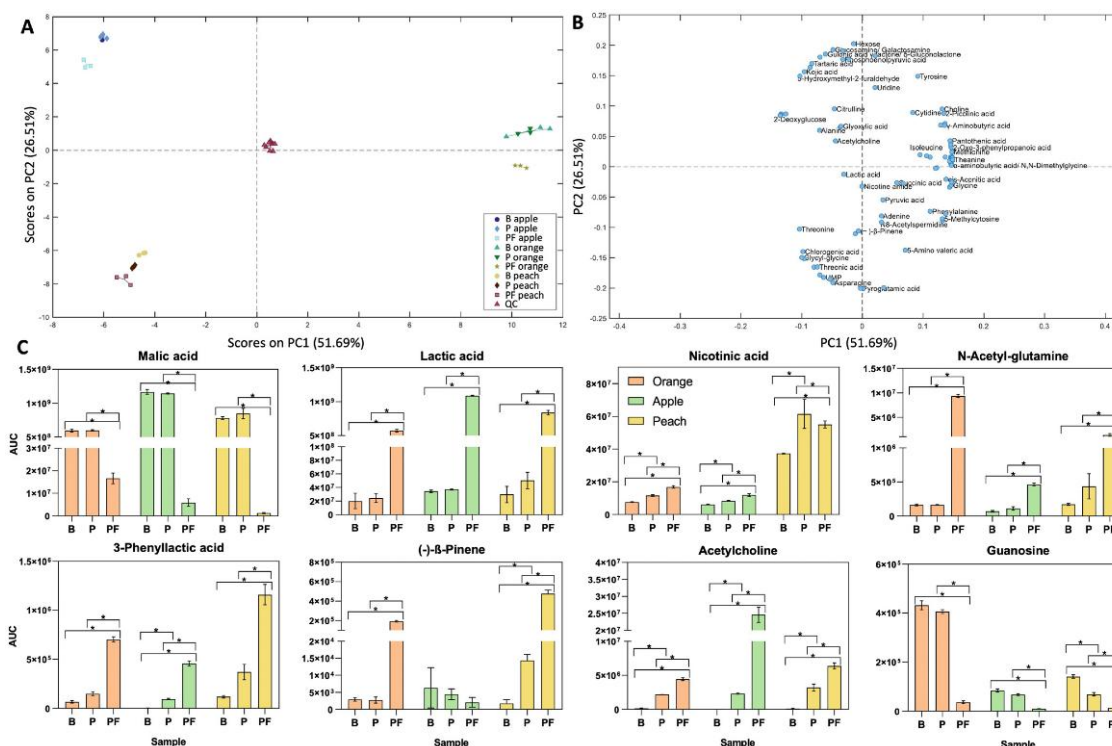


Figure 3. Semi-polar metabolite profile. (A) Score plot of PC2 over PC1 in the model calculated on the relative concentrations of the variables annotated on levels 1 and 2a in the reduced dataset. (B) Loading plot from PCA model calculated on the relative concentrations of the variables annotated on levels 1 and 2a in the reduced dataset. Data have been autoscaled, and overlapping labels were removed to improve readability. (C) Relative abundances (Area Under the Curve) of compounds of interest detected in level 1 and 2a in blank (B), probiotic-supplemented (P), and probiotic-fermented (PF) fruit juices. * $p < 0.05$.

The presence of *L. plantarum* KABP051 in fruit juices, with or without fermentation, modified the concentration of 22 compounds (Table S2). Eleven compounds significantly increased in some of the P or PF samples, while the concentration of the rest of the compounds decreased, evidencing some metabolic pathways. For instance, in PF samples, the concentration of malic acid drastically decreased in favour of lactic acid (Figure 3C), confirming that malic acid is a major carbon source for *L. plantarum* KABP051 growth. Likewise, nicotinamide decreased while nicotinic acid increased in PF samples, indicating that *L. plantarum* KABP051 can modify vitamin B forms, changing the vitamin profile of the juices. Moreover, probiotic fermentation raised the concentration of additional bioactive compounds including n-acetyl glutamine (NAG), phenyllactic acid (PLA), (–)-β-pinene, and acetylcholine (ACh) (Figure 3C), among others (Table S2). In addition to malic acid, the nucleosides adenosine, guanosine, and cytidine were amongst the top reduced compounds in juices supplemented with *L. plantarum* KABP051 (Table S2).

The precise concentration of nicotinic acid, ACh, PLA, and NAG was determined by targeted metabolomic analysis (Table 2). These results validated those observed for the untargeted analyses (Figure 3C) and confirmed that *L. plantarum* KABP051 can significantly synthesise these biomolecules at μM range. While nicotinic acid, ACh, and PLA were already present at detectable concentrations in the blank juices, the amino acid NAG was not detected in any blank sample but was produced during fermentation in the three juices, up to a maximum of 11.26 ± 0.39 μM in orange juice.

Table 2. Concentration (average $\pm$ SD) in μ M of Nicotinic acid, Acetylcholine, Phenyllactic acid, and N-Acetyl-L-Glutamine detected in fruit samples by targeted metabolomics.

	Apple			Orange			Peach		
	B	P	PF	B	P	PF	B	P	PF
Nicotinic acid	1.56 $\pm$ 0.10	2.36 $\pm$ 0.03 *	3.42 $\pm$ 0.09 *	2.42 $\pm$ 0.06	3.9 $\pm$ 0.22 *	5.85 $\pm$ 0.09 *	13.82 $\pm$ 0.17	20.21 $\pm$ 0.37 *	19.24 $\pm$ 0.51 *
Acetylcholine	0.09 $\pm$ 0.00	0.29 $\pm$ 0.00 *	2.41 $\pm$ 0.11 *	0.09 $\pm$ 0.00	0.29 $\pm$ 0.01 *	0.51 $\pm$ 0.01 *	0.09 $\pm$ 0.00	0.32 $\pm$ 0.00 *	0.64 $\pm$ 0.02 *
Phenyllactic acid	0.23 $\pm$ 0.04	1.32 $\pm$ 0.01 *	5.16 $\pm$ 0.15 *	0.5 $\pm$ 0.05	1.34 $\pm$ 0.05 *	7.57 $\pm$ 0.29 *	1.56 $\pm$ 0.07	3.68 $\pm$ 0.24 *	12.04 $\pm$ 0.40 *
N-Acetyl-L-Glutamine	<LOD	<LOD	0.3 $\pm$ 0.01 *	<LOD	<LOD	11.26 $\pm$ 0.39 *	<LOD	0.16 $\pm$ 0.02	1.28 $\pm$ 0.19 *

B: Blank; P: probiotic (non-fermented); PF: probiotic fermented. LOD: Limit Of Detection. * $p < 0.05$; t-test PF vs. B.

3.4. Sensory Evaluation

A total of 51 independent panellists were enrolled to evaluate the organoleptic properties (intensity and acceptability) of the original orange juice (Blank; B) and the fermented version (PF).

As shown in **Figure 4**, probiotic fermentation did not alter colour perception by panellists. The intensity of the aroma was not significantly altered in probiotic-fermented juice, but its acceptability was significantly reduced. Strange flavour perception significantly increased in fermented samples without affecting its acceptability. Although a significant increase in bitter and acid intensity was noted for the fermented version, it did not translate into a reduced acceptability. The intensity of sweet taste significantly decreased in fermented juices, but it did not affect acceptance scores. Finally, and in line with sensory results, similar purchase probability was declared by participants between original and 24 h-fermented orange juices (**Figure 4B**). These results indicate that, although fermentation for 24 h affected different organoleptic parameters, overall acceptance is comparable to the original orange juice.

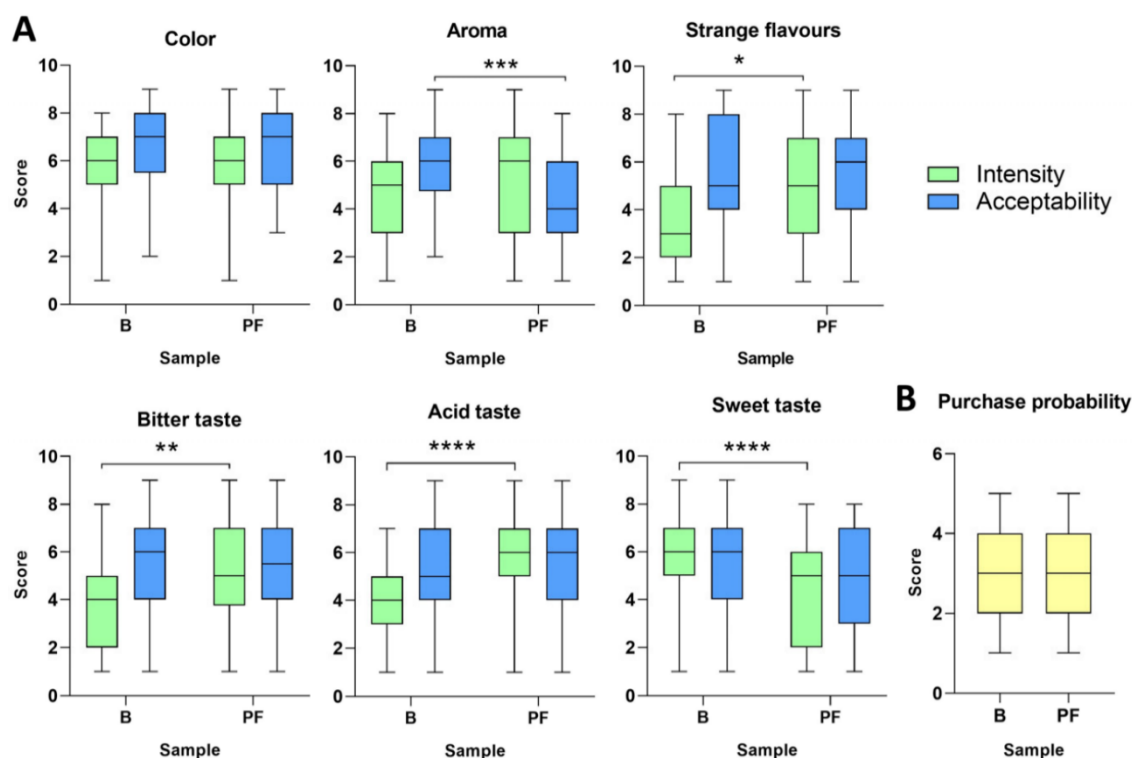


Figure 4. Results of the sensory evaluation involving 51 untrained panellists. **(A)** Results of organoleptic parameters and **(B)** score of the purchase probability of blank juices (**B**) and probiotic-fermented (PF) orange juices. A nine-point scale was used for the evaluation of acceptability and intensity of aroma, colour, bitterness, sweetness, acidity, and strange flavours, with 1 = Dislike extremely/Extremely low intensity and 9 = Like extremely/Extremely intense. The parameter “Purchase probability” was evaluated with a five-point scale, with 1 = Definitely will not buy and 5 = Definitely will buy. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. Discussion

The fermentation of fruit and vegetable juices by well-documented probiotics represents a promising nutritional approach for the development of novel non-dairy functional beverages. It is paramount to select the best probiotic strains that combine excellent *in vitro*, *in vivo*, and clinical evidence with an adequate stability. *Lactiplantibacillus plantarum* is one of the most used species for vegetable fermentation [34], likely because of its metabolic versatility and environmental resilience. Our results demonstrate that probiotic strain *L. plantarum* KABP051 displays an excellent viability in orange, apple, and peach juices, both in probiotic-fermented and non-fermented forms, throughout 60 days of cold storage. Only the non-fermented peach sample displayed probiotic CFU counts below the recommended 10^6 – 10^7 CFU/mL dose [19,35] at day 60, indicating this is the most unfavourable condition for probiotic stability tested in our study. Nonetheless, this problem could be solved by increasing the initial inoculum. Interestingly, probiotic fermentation increased CFU counts of *L. plantarum* KABP051 in all juices, reaching CFUs per serving of 240 mL [33] higher than the therapeutic dose ($>10^9$ CFUs) [23,24] in the three fermented juices for up to 60 days of cold storage (Figure 1B), thus qualifying as “probiotic fermented foods” [12]. These results suggest that the *L. plantarum* KABP051 fermentation of fruit juices might reduce probiotic production costs while maximising its viability and health benefits.

Different studies have investigated the stability of some probiotic strains in non-fermented fruit juices. For instance, the popular probiotics *L. rhamnosus* GG (ATCC53103), *L. casei* DN-114 001, and *B. lactis* BB-12 as well as *L. paracasei* NFBC43338 showed good survival in orange and pineapple juices, with CFUs above the recommended dose for up to 12 weeks under refrigeration [36]. On the contrary, *L. salivarius* strains UCC118 and UCC500 showed very poor viability ($<10^6$ CFU/mL) in orange and pineapple juices after only two weeks [36].

Regarding probiotic viability in fermented fruit juices, it was found that *L. casei* NRRL B442 was able to survive ($>10^6$ CFU/mL) in fermented pineapple juice for 42 days in refrigerated conditions, while the addition of sucrose reduced *L. casei* NRRL B442 viability to 28 days [37]. Interestingly, when *L. casei* NRRL B442 was used to ferment cashew apple juice, high CFU counts ($>10^8$ CFU/mL) were recorded throughout the storage period of 42 days at 4 °C [38]. Likewise, Dimitrovs.ki et al. [39] also found apple juice to be a suitable matrix for probiotic fermentation and viability using *L. plantarum* strain PCS 26. Our results are in accordance with previous studies, confirming that apple juice is an excellent medium for the growth and stability of *L. plantarum* KABP051, as fermented apple samples maintained $\sim 10^8$ CFU/mL after 60 days of refrigerated storage. While similar counts were noted in the fermented peach sample, a more marked CFU reduction was observed in the fermented orange samples at the end of the study. Taken together, probiotic viability in fermented juices seems to be significantly influenced by many aspects including the composition of the matrix and the strain specificity. Therefore, probiotic stability in fruit juices cannot be taken for granted and should be determined on a case-by-case basis.

While the pH of fermented orange and peach juices decreased compared to unfermented and control juices, the pH of fermented apple juice, where malic acid is the most abundant organic acid [40], showed a slight increment. This can be explained by the malolactic fermentation (transformation of malic acid into lactic acid), as lactic acid ($pK_a = 3.86$) is a weaker acid than malic acid ($pK_{a1} = 3.40$) and therefore, reduction of malic acid in favor of lactic acid translates into a slight increase of apple juice pH [41].

In a previous study, Yuasa et al. [42] reported that *L. plantarum* SI-1 and *L. pentosus* MU-1 exclusively utilise L-malic acid to produce lactic acid, without affecting total sugar concentration of citric juices. Our results confirm this notion and show that malolactic fermentation is the main energy metabolic pathway of strain KABP051 not only in orange and apple but also in peach juices.

TPC and antioxidant (DPPH-RSA) capacity remained unaltered in all fermented and unfermented juices (Table 1). Although an increase in TPC and DPPH-RSA was noted for the probiotic-fermented orange juice, it did not reach statistical significance. In this line, only the flavanones naringin and hesperidin, but not their aglycone forms, were detected in orange juice. Probiotic introduction and fermentation did not modify flavanone's profile. In a previous work, de la Fuente et al. [43] showed that *L. brevis* strain POM and *L. plantarum* strains TR-71 and TR-14 were able to ferment orange juice-milk beverages resulting in increased antioxidant capacity, which was in part attributed to the production of phenyllactic acid (PLA) by lactobacilli. Although we also detected a great increase in the phenolic compound PLA after probiotic fermentation, it did not translate into a significant increase in TPC and antioxidant capacity. Of note, de la Fuente et al. [43] conducted a 72 h fermentation

in milk-based beverages with different conditions from those assessed in our work, which may explain discrepancies in antioxidant and TPC results.

In order to identify metabolic products of interest, different metabolomic approaches were performed. The results showed that the fermentation not only increased the probiotic biomass but also allowed for the production of molecules of medical, nutraceutical, and biotechnological interest. The short-chain fatty acid (SCFA) profile was enhanced with a significant increment in the acetic acid content in all fermented beverages. SCFAs play an important role in human health; therefore, introducing a fortified food, rich in these compounds, could contribute to maintaining a correct endogenous SCFA balance [44]. Acetic acid has antioxidant, antibacterial, anti-diabetic, and anti-tumour properties and prevents cardiovascular disorders [45,46]. Moreover, acetic acid in the gut is the preferred substrate for the butyrate-producing bacteria *Roseburia* spp. and *Faecalibacterium prausnitzii* to produce butyric acid, the major carbon source of enterocytes [47].

Semi-polar metabolite profiling confirmed malolactic fermentation, typically used in wine production [48], and revealed the production of several additional bioactive compounds. Among the common compounds produced by the probiotic fermentation of all juices, nicotinic acid, acetylcholine (ACh), phenyllactic acid (PLA), and n-acetyl glutamine (NAG) were verified and quantified by targeted approaches (Table 2).

Nicotinic acid is a form of vitamin B3 and is an essential human nutrient. According to EFSA [49], dietary reference values for niacin range between 13 to 19 mg/day for adults. Among the juices investigated, nicotinic acid was particularly high in peach (1.7 mg/L), and the presence of the probiotic significantly increased its concentration up to ~2.4 mg/L. Some probiotic lactobacilli have been reported to produce a number of vitamins belonging to the B-complex, such as folate, vitamin B12, and thiamine [50,51]. However, to our knowledge, this is the first time that the synthesis of niacin during fruit juice fermentation by *L. plantarum* has been described.

Acetylcholine is a neurotransmitter that has a wide variety of functions in the brain and other organs. ACh production has been described in many gram-negative and gram-positive bacteria including lactobacilli [52]. It is well known that some lactobacilli can produce ACh [53], but to our knowledge this is the first time that a *L. plantarum* strain has shown the ability to synthesise this neurotransmitter in orange, peach, and apple juices. Intestinal ACh exerts a localised immunomodulatory effect [54] and can improve intestinal barrier function [53,55]. The highest levels of ACh were found in the probiotic-fermented apple juice.

PLA is typically produced during fermentation of dairy products [56] but has been also detected in supernatants of different *Lactobacillus* spp. strains grown in the presence of pomegranate extract [57] and in fermented orange juices containing milk [43]. It has demonstrated broad spectrum antimicrobial activity against spoilage organisms and pathogens, both bacteria and fungi, which prolongs fermented food shelf life [56,58]. PLA content drastically increased in all probiotic-fermented juices, peach juice being the most concentrated sample.

Glutamine is very abundant in the human body, but it is unstable in liquid media, and N-acetyl-l-glutamine (NAG) has been used as supplement to avoid instability and intolerance

issues [59]. Different clinical studies have reported that enteral or parenteral nutrition formulas containing this amino acid help maintain the structural and functional integrity of the gut [60,61]. Supplements containing glutamine forms are widely used in sports nutrition, especially because of its immunomodulatory role, but recent studies have also evidenced some improvement in fatigue markers including increased glycogen synthesis and reduced ammonia accumulation [62]. Interestingly, NAG was only detected in probiotic-fermented juices, while its concentration in probiotic-supplemented and blank samples was below the limit of detection. These results suggest that fermentation assisted by *L. plantarum* KABP051 de novo produces this interesting amino acid, which was absent from original juices.

Besides the production of interesting bioactive compounds, the fermentation of fruit juices also evidenced the ability of *L. plantarum* KABP051 to consume some nutrients (Figure 3 and Table S2). Of note, the probiotic consumed the purines adenosine, guanosine, and cytidine in all fermented juices. Purines are the major substrate for uric acid formation and crystallisation, which could lead to gout [63]. Therefore, the consumption of a probiotic that has the ability to reduce dietary purines in the gut could be of interest for patients prone to hyperuricemia [64], as reported for *Lactobacillus gasseri* strain PA-3 [65].

For the sensory evaluation, orange juice was selected because it is the most popular and consumed juice worldwide [66] and Spain is the leading orange producer in the EU, according to USDA (2020). A sensory evaluation study was conducted to gain insights into consumers' perception and acceptability. The fermentation of orange juice by the probiotic KABP051 only reduced aroma acceptability and increased the intensity of strange flavours and bitter and acid taste, whereas that of sweet taste was reduced. Similarly, Yuasa et al. [42] reported that the fermentation of mandarin and orange juices by *L. plantarum* SI-1 increased sourness and acidic bitterness due to an increase in lactic acid and total organic acids, which is also observed in our analyses.

The present study has certain limitations. First, as an attempt to evaluate the functionality of probiotic juices, DPPH-RSA capacity and TPC experiments were performed, and results showed no differences compared to commercial beverages. Therefore, although *L. plantarum* KABP051 has shown probiotic effects in several trials [23,24,25,26], future *in vitro*, *in vivo*, or clinical studies are required to confirm that fermented fruit juices retain and improve probiotic properties. Second, as proof of concept, only probiotic fermented orange juices were assessed in a sensory evaluation study. Thus, additional sensory experiments are required for peach and apple fermented beverages to confirm that their organoleptic parameters and overall acceptance fit consumer tastes. Finally, the metabolomic analyses performed in our study were not suitable for the detection of medium- and long-chain fatty acids (LCFA), and therefore it is unknown whether *L. plantarum* KABP051 metabolism could influence the composition of these relevant compounds. Future targeted approaches should be conducted to elucidate if probiotic fermentation may improve the profile of LCFA, such as conjugated linolenic acid, which displays remarkable beneficial effects [67].

In conclusion, we here show that fermentation of fruit juices by *L. plantarum* KABP051 may result in tasty functional probiotic juices that are enriched with a number of health-promoting compounds. Thus, the development of these functional juices may have a dual function to

the host: the delivery of a well-characterised probiotic combined with fruit juices with increased nutritional value.

Supplementary Materials

The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods13233851/s1>, Figure S1: Schematic representation of the experimental design; Table S1: Concentration of SCFA (mM) in fruit juices; Table S2: Additional compounds detected, consumed or produced by *L. plantarum* KABP051 in fruit juices.

Author Contributions

Conceptualization, F.R., B.J., J.E.-M., M.C. and P.H.; Data curation, J.E.-M.; Formal analysis, J.E.-M.; Investigation, F.R. and P.H.; Methodology, F.R.; Resources, B.J. and M.C.; Supervision, B.J., M.C. and P.H.; Validation, B.J. and M.C.; Visualization, F.R.; Writing—original draft, F.R. and P.H.; Writing—review & editing, B.J., J.E.-M., M.C. and P.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

The original contributions presented in the study are included in the article/[supplementary material](#), further inquiries can be directed to the corresponding author.

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Conflicts of Interest

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Abbreviations

The following abbreviations are used in this manuscript:

CFU	colony forming units	probiotic
PF	probiotic-fermented	
B	blank	
ISAPP	International Scientific Association for Probiotics and Prebiotics	
MRS	de Man, Rogosa, and Sharpe	
TSS	total soluble solids	
TPC	total phenolic content	
GAE	gallic acid equivalent	
TE	trolox equivalent	
AC	antioxidant activity	
DPPH	2,2-difenil-1-picrilhidrazil	
NAG	n-acetyl glutamine	
PLA	phenyllactic acid	
ACh	acetylcholine	
FDR	false discovery rate	
PCA	principal components analysis	
SCFA	short-chain fatty acids	

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4.3 MICROBIOLOGICAL QUALITY ASSESSMENT RESULTS

Across all evaluated food matrices, microbiological quality remained within acceptable limits throughout storage.

Yeasts and moulds were consistently below 100 CFU/g, with the majority of matrices showing no detectable counts at either sampling point. Detectable levels were observed only in cereal bars, chocolate, chocolate creams, and soy- and oat-based beverages; however, counts in these products always remained <100 CFU/g, indicating minimal fungal proliferation during storage.

Total coliforms remained below 20 CFU/g (solid matrices) and <2 CFU (liquid matrices), in all finished products, supporting good hygienic conditions during processing and handling. Psychrotrophic microorganisms were generally absent across matrices, with the exception of oat-based beverage and popping candy samples, where low counts (<20 CFU/mL or g) were detected at the end of shelf life, and peanut butter, which showed <20 CFU/g both at the beginning and end of storage.

For liquid products subjected to heat treatment, microbiological analyses were performed before and after thermal processing. In nonfermented orange juice inoculated with *Bacillus coagulans* Unique IS-2 spores, pretreatment samples contained <100 CFU/mL yeasts and moulds and <10 CFU/mL coliforms. After HTST treatment, both groups were completely undetectable, demonstrating that the applied time–temperature combination was effective in eliminating these populations. A similar pattern was observed for the isotonic beverage, where coliforms detected before HTST (<100 CFU/mL) were fully inactivated posttreatment.

Total aerobic mesophilic counts were strongly influenced by the presence of probiotic strains capable of aerobic growth on PCA. All strains except the obligate anaerobe *Bifidobacterium longum* CECT 7894 showed varying degrees of aerobic proliferation, with *Bacillus coagulans* Unique IS-2 spores producing the highest increases. Consequently, mesophilic counts in matrices containing these strains were markedly elevated, reflecting probiotic viability rather than contamination.

In addition, *B. coagulans* Unique IS-2 spores were able to germinate and grow on PCA following heat shock treatment, thereby impacting the aerobic spore forming bacteria counts in matrices containing this strain.

Aerobic spore-formers were also detected in the ice cream matrices containing the I3.1 blend, with counts remaining relatively stable across storage. Mean values were 1.22×10^3

CFU/g for yogurt ice cream and 1.30×10^3 CFU/g for chocolate ice cream at both the beginning and end of shelf life, reflecting the presence of background spores.

Finally, in fermented soy and oat beverage formulations exhibited measurable levels of aerobic spore forming bacteria. In oat beverages, counts decreased from 1.02×10^3 CFU/g at the beginning to 2.40×10^2 CFU/g at the end of shelf life, suggesting reduced spore germination or growth inhibition during storage. Conversely, in soy beverages, counts increased from 1.33×10^2 CFU/g initially to 1.15×10^3 CFU/g by end of shelf life.

5 GENERAL DISCUSSION

5.1 COMPREHENSIVE INTERPRETATION OF RESULTS

The research presented in this doctoral thesis provides compelling experimental evidence that probiotic performance in food and beverage systems cannot be regarded as a fixed, strain intrinsic property, but rather as an emergent phenotype arising from complex, multilevel interactions between microbial genomic architecture, stress response capacity, ecological lifestyle, and the physicochemical constraints imposed by the food matrix (Flach et al., 2018; Kaur et al., 2025; Sendra et al., 2016; You et al., 2023). This system level perspective is consistent with recent advances in microbial ecology and applied probiotic science, which increasingly recognize that microbial robustness, functionality, and technological suitability cannot be reliably inferred from taxonomic identity alone (George et al., 2018; Kathiriya et al., 2023).

Across more than twenty matrices: dairy, plant-based beverages, fruit juices, isotonic drinks, confectionery, bakery goods, chocolate, bars, gummies, popping candy, and fermented systems, strain performance followed certain reproducible patterns that aligned with predictable ecological strategies and genomic signatures.

Rather than behaving as passive or static biological additives, probiotics interacted dynamically with the constraints and opportunities defined by each matrix, resulting in marked variability in survival, metabolic activity, and sensory impact.

By integrating these findings, this General Discussion situates the results of the thesis within a broader conceptual framework encompassing microbial ecology, food science, consumer perception, and emerging sustainability imperatives, thereby highlighting both the potential and the current limitations of probiotic incorporation into complex food and beverage systems and outlining key directions for future research and innovation.

Beyond these mechanistic insights, the findings presented here intersect with a broader historical and conceptual shift occurring within contemporary food science. Since the advent of Pasteurian principles and the industrialization of food processing, the dominant trajectory of the food industry has been toward increasingly sterile, microbially depleted products—an approach that has undoubtedly been essential for controlling pathogens and spoilage microorganisms, but which has also inadvertently reduced the ecological and functional

complexity of the human diet. This trajectory stands in contrast to the traditional global dietary landscape, in which fermented foods rich in metabolically active and ecologically diverse microbial communities played a central role in nutrition, preservation, and health. Over the last decade, however, a renewed scientific and societal interest in fermented foods has emerged, driven by advances in microbiome research and growing recognition that the absence of a beneficial microbiota in modern processed foods may represent a missed opportunity for enhancing health-relevant functionality. From this perspective, the work conducted in this thesis can be viewed as part of a broader effort to reintroduce controlled, well-characterized, safe microbial biodiversity into food systems, thereby restoring certain dimensions of microbial functionality that were progressively diminished through ultra-processing and sterilization. While maintaining stringent food safety standards, the intentional incorporation of specific probiotic strains into diverse matrices offers a scientifically grounded way to balance microbial safety with microbial functionality, aligning modern food production with emerging paradigms in microbial ecology, personalized nutrition, and sustainable health-promoting food design.

5.2 GENOME ARCHITECTURE, ECOLOGICAL LIFESTYLE, AND LIFE-HISTORY STRATEGY AS PREDICTORS OF MATRIX-DEPENDENT PROBIOTIC PERFORMANCE

Across all tested systems, strains characterised by broad ecological competence consistently outperformed ecological specialists for example in hostile, non-dairy matrices, such as acidic and oxygen-rich fruit juices, underscoring the relevance of ecological theory as a powerful conceptual framework for interpreting microbial behaviour in food systems.

The consistently superior performance of *Lactiplantibacillus plantarum* in non-dairy matrices is fully coherent with its genomic organisation and nomadic ecological lifestyle. Comparative genomics has shown that *L. plantarum* possesses one of the largest and most plastic genomes among lactic acid bacteria (LAB), characterised by an open pangenome, extensive repertoires of carbohydrate transporters, redox and detoxification systems, membrane fatty-acid remodelling enzymes, and diversified stress-response regulators (Carpi et al., 2022; Siezen & van Hylckama Vlieg, 2011). Together, these features encode a broad and redundant “stressome” that enables active tolerance to acid stress, oxidative stress, osmotic fluctuations, and plant-derived antimicrobial compounds. This genomic

configuration explains the robust viability of *L. plantarum* under the multiple, simultaneous stresses encountered during processing, storage, and digestion (Papadimitriou et al., 2016). In contrast, the markedly poorer survival of *Bifidobacterium* in matrices such as orange juice reflects fundamental genomic and ecological constraints. *Bifidobacterium* species typically harbour smaller, more specialised genomes optimised for the anaerobic, carbohydrate-rich, and highly buffered environment of the human colon, with limited oxidative stress defences and reduced metabolic flexibility (Deb, 2022). As a consequence, these taxa are highly sensitive to acidic pH, dissolved oxygen, and polyphenolic compounds characteristic of fruit juices, highlighting the cost of ecological specialisation outside the host niche. Genome specialization and ecological niche breadth are closely linked across bacteria: generalist taxa typically exhibit broader niche ranges and greater functional diversity, whereas specialists are constrained to narrower ecological contexts with correspondingly reduced adaptive capacity. This pattern is well documented in trait-based and comparative ecological studies showing that generalists occupy diverse environments while specialists remain limited to specific niches adaptability (Beier et al., 2022; Bell & Bell, 2021; von Meijenfeldt et al., 2023).

Crucially, however, the results of this thesis also demonstrate that adaptability is not dictated by genome size alone. *Pediococcus acidilactici* represents an instructive counterexample: despite a comparatively compact genome (~1.9–2.0 Mb), this species displayed notable robustness across diverse food environments. Comparative genomic analyses indicate a highly dynamic and open pangenome enriched in accessory genes, mobile elements, carbohydrate utilisation pathways, and defensive functions, which plausibly compensate for a streamlined core genome and enable resilience across heterogeneous ecological contexts (Z. Li et al., 2021). These findings reinforce the concept that functional density, regulatory architecture, and pangenome dynamics are more predictive of performance than absolute genome length.

A central outcome of this work is the recognition that high technological robustness can arise through fundamentally different biological routes. In the present study, both *L. plantarum* and *Bacillus* (*Heyndrickxia*) *coagulans* emerged as high-potential candidates across challenging matrices, yet their resilience is underpinned by contrasting life-history strategies. *L. plantarum* is non-sporulating and remains metabolically active within the food matrix; its performance reflects active stress tolerance and metabolic engagement enabled by genomic breadth and regulatory flexibility (typical genome size ~3.0–3.3 Mb). By

contrast, *B. coagulans* achieves robustness primarily through endospore formation, a dormant but extremely stress-resistant state that confers exceptional tolerance to heat, desiccation, low pH, and bile, consistent with closed genomes in the ~3.5–3.7 Mb range for probiotic strains. This distinction is not merely descriptive: it implies that *L. plantarum* contributes robustness through continuous physiological activity, whereas *B. coagulans* contributes robustness through passive persistence.

The predictive value of ecological lifestyle classification is further reinforced by strain-level evidence. Nomadic LAB consistently showed high viability across matrices, corroborating previous findings that *L. plantarum* strains do not cluster phylogenetically by isolation source and lack niche-specific genomic signatures (Fidanza et al., 2021; Martino et al., 2016). In this context, the comparative case of *Lactiplantibacillus plantarum* CECT 7481 and DR7, presented in our conference poster (Rizzi, et al., 2024a), offers a striking illustration of how ecological lifestyle traits can manifest at a global scale. CECT 7481 was originally isolated in 2004 from saliva and stool samples of healthy children living in remote Peruvian jungle communities with no exposure to ultra-processed foods or antibiotic pressures, whereas DR7 was isolated years later in Malaysia from a traditionally fermented cow's-milk product (Chong et al., 2019b). Remarkably, deep genomic and phenotypic characterisation confirmed that these two isolates are equivalent, despite originating from geographically distant, culturally unrelated, and nutritionally distinct ecosystems. This unexpected biogeographic convergence suggests that certain *L. plantarum* lineages may constitute exceptionally successful, environmentally versatile “global generalist” strains whose distribution reflects ecological opportunity rather than geographic proximity.

To date, continent-scale bacterial dissemination across has been examined almost exclusively within epidemiological frameworks focused on pathogens; however, this case underscores the scientific value of extending such biogeographic perspectives to beneficial microorganisms.

The broad ecological cycle of *L. plantarum*, including facultative anaerobiosis, the presence of bile salt hydrolase genes associated with gastrointestinal passage, and transient colonisation of plant niches, provides a plausible evolutionary basis for the worldwide occurrence of such strains. Importantly, independent research groups, working with unrelated strain collections and selection pipelines, consistently converged on this same isolate through multi-layered screening (genomic, *in vitro*, *in vivo*, and clinical), underscoring its exceptional technological robustness and biological fitness.

Sporulation capacity emerged as a second, orthogonal axis shaping probiotic performance. From an ecological perspective, sporulation represents a bet-hedging strategy characteristic of taxa inhabiting fluctuating or intermittently lethal environments. In food systems, this strategy translates into extraordinary technological stability: multilayered spore coats, a dehydrated core, and calcium–dipicolinic acid complexes confer resistance to thermal, oxidative, osmotic, and acidic stress. Accordingly, *B. coagulans* spores displayed outstanding stability in harsh matrices including gummies, cereal bars, peanut butter, chocolate creams, and even post-pasteurisation orange juice and isotonic beverages. Spores remained metabolically quiescent during storage, preventing sensory defects. One published study by Ahire et al. (2020) demonstrated that *Bacillus coagulans* Unique IS-2 spores exhibit exceptional gastrointestinal robustness in a dynamic SHIME® model, achieving 99–102% survival across fed and fasted gastric and small-intestinal conditions, initiating partial germination in the duodenum, and fully transitioning to metabolically active vegetative cells in the colon, where they produced substantial levels of lactate and acetate as major end-products. This apparent dichotomy, prolonged quiescence in foods versus rapid activation in the host, is fully consistent with contemporary spore biology: *Bacillus* spores remain dormant unless exposed to a specific set of germination cues, including nutrient amino acids, simple sugars, bile salts, and characteristic pH and ion-flux transitions, which are abundant in the small intestine but remain absent or below activation threshold in the food matrices investigated (Bernardeau et al., 2017; Setlow, 2003, 2014).

At a broader scale, large-scale comparative ecological genomics provides a unifying framework for these observations, demonstrating that there is no universal correlation between genome size and ecological adaptability. Meta-analyses across thousands of bacterial and archaeal genomes indicate that ecological success is shaped primarily by gene content, functional density, regulatory architecture, and lifestyle strategy, rather than genome length per se (Dewar et al., 2024; Martinez-Gutierrez & Aylward, 2022; Rodríguez-Gijón et al., 2022; C. Wang et al., 2023). This explains why compact but plastic genomes (*P. acidilactici*), large and redundancy-rich genomes (*L. plantarum*), and sporulation-enabled strategies (*B. coagulans*) can all support robust survival across food matrices.

Finally, dairy-adapted LAB such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* provide a contrasting endpoint of this ecological spectrum. These taxa exhibit reductive evolution, with loss of genes unnecessary in milk and

specialisation towards proteolysis and peptide scavenging in nutrient-rich, buffered environments (El Kafsi et al., 2014; Goh et al., 2011; Stefanovic & McAuliffe, 2019; van de Guchte et al., 2006). While this specialisation underpins excellent performance in fermented dairy matrices, it also explains their poor survival in non-dairy systems and their limited probiotic potential. As highlighted in the introduction, probiotic criteria require that strains survive transit through the gastrointestinal tract and exert demonstrable health benefits beyond basic nutrition; conditions that dairy specialists such as *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* rarely fulfil due to their limited stress tolerance and lack of functionalities associated with host interaction. Consequently, next-generation fermented foods increasingly rely on rational strain–matrix matching, combining traditional starters with clinically validated, ecologically robust probiotics to unify optimal fermentation performance with demonstrable health benefits.

Overall, the interaction between genomic adaptive traits, ecological lifestyle, and sporulation phenotype gives rise to predictable techno-functional behaviours across food environments, establishing a biologically grounded framework for precision probiotic selection and rational strain–matrix matching in product development.

5.3 FOOD MATRIX COMPOSITION AS A MODULATOR OF PROBIOTIC ROBUSTNESS

The thesis systematically demonstrates that food matrix composition profoundly modulates microbial behaviour by altering environmental pressures such as pH, water activity, redox potential, oxygen exposure, and nutrient availability.

Dairy matrices enhanced survival due to buffering effects, lipid-mediated membrane stabilization, and nutrient richness (Wasana et al., 2025).

In contrast, fruit juices and isotonic drinks imposed severe stress (low pH, phenolics, oxygen), limiting survival to strains with robust stress-response systems, membrane plasticity or in sporulated form (Guimarães et al., 2025; Maia et al., 2023).

The results further confirm that low-moisture matrices (chocolate, cereal bars, cookies, powders) offer distinct advantages for long-term stability, particularly for spore-forming probiotics such as *Bacillus coagulans*, which, in its sporulated form, can also withstand heat treatments. Low water activity, combined with fat-rich environments, effectively immobilized cells or spores, minimizing metabolic activity and degradation over extended storage periods, even under accelerated conditions (Majeed et al., 2016).

5.4 FERMENTATION AS A FUNCTIONAL AMPLIFIER RATHER THAN MERELY A PRESERVATION TOOL

Another major outcome of this thesis is the demonstration that probiotic fermentation enhances functional value beyond viability. Fermentation of milk, plant-based beverages and fruit juices by different probiotic strains produced a suite of bioactive metabolites, including phenolic derivatives, organic acids, neurotransmitter precursors and amino-acid metabolites, consistent with recent metabolomics studies showing that LAB fermentation increases the nutritional and functional profile of plant matrices (Alharbi, 2026; Cheng et al., 2026; Kumar et al., 2025).

These observations reinforce the concept that fermented probiotic foods provide a dual functional value: delivering live microorganisms and generating bioactive metabolites that may exert health effects independently or synergistically. Additionally, when probiotic strains are employed as active fermenters at industrial scale, in situ proliferation can substantially amplify cell densities during fermentation, enabling the use of markedly lower inoculum doses, thereby improving process efficiency and reducing production costs.

However, the benefits of fermentation were strain-specific and matrix-dependent, and sensory quality could be negatively impacted when metabolic activity generated off-flavours. This underscores the need for precise metabolic control and strain selection based on intended sensory and functional outcomes.

5.5 SENSORY ACCEPTANCE AS A CRITICAL BOTTLENECK FOR PROBIOTIC INNOVATION

A crucial contribution of this thesis is the systematic integration of sensory evaluation across diverse matrices. The results confirm that probiotic viability alone is insufficient for commercial success; sensory compatibility is equally decisive.

In fermented matrices, heterofermentative pathways occasionally generated off-flavours or texture changes, highlighting the need for metabolic predictability. Conversely, in non-fermented dry matrices and confectionery products, probiotic incorporation, especially of spore-forming strains, had minimal sensory impact, often yielding high consumer acceptance and purchase intent.

These findings underscore that technological feasibility must be evaluated alongside consumer perception, a decisive barrier in functional food development, particularly for innovative probiotic formats targeting emerging demographics such as children (Baker et

al., 2022). Moreover, as the present thesis did not explore formulation strategies aimed at mitigating potential organoleptic impacts associated with probiotic incorporation, targeted research will be required to optimize sensory performance in future product development.

5.6 EXPANDING CONSUMER TRENDS: FROM THE PHARMACY TO THE SUPERMARKET

A notable contextual shift influencing probiotic applications in foods is the transition of probiotics from pharmacy exclusive products to widely accessible supermarket items. Over the last decade, consumer perceptions have evolved from viewing probiotics primarily as therapeutic agents to recognizing them as everyday dietary components linked to wellness. This transition has been supported by increased scientific communication, and the familiarity gained through fermented staples such as yogurt, kefir, kimchi, and kombucha. Market reports consistently show that younger demographics, particularly millennials and Gen Z, prefer functional foods over supplements, perceiving them as more “natural,” enjoyable, and compatible with daily eating habits (Kreiter, 2025; Pione et al., 2025). The findings of this thesis strengthen the notion that technological feasibility, sensory acceptability, and stable viability across different formats are central drivers pushing probiotics into mainstream food environments. As a result, the boundary between nutritional products and health-oriented foods is becoming increasingly fluid, creating opportunities for new hybrid innovations.

5.7 CLINICAL EVIDENCE, HEALTH CONDITIONS, AND THE MICROBIOTA–HOST INTERFACE

The results also connect meaningfully with clinical evidence on probiotic efficacy. Achieving clinically effective doses in food formats requires that strains withstand processing, storage and gastrointestinal transit. The strong performance of *L. plantarum* in acidic and oxidative environments, as well as the exceptional stability of *B. coagulans* spores, suggests that these strains are highly suitable for delivering clinically relevant doses through foods. Fermented matrices further enhance functional potential by providing metabolites associated with gut, immune and metabolic health. Although this thesis did not conduct *in vivo* studies, the technological outcomes demonstrate that many food formats tested here can feasibly deliver probiotic quantities aligned with doses used in clinical research. A growing body of clinical research evaluating probiotic strains delivered through

food matrices demonstrates measurable health effects across gastrointestinal, metabolic, and neuroimmune domains. For example, a triple-blind randomized placebo-controlled trial administering 130 g/day of a probiotic-fermented dairy product to healthy adult women for 8 weeks reported significant modulation of hippocampal glutathione levels, functional brain connectivity, and gut microbiome composition compared with a placebo, highlighting neuroimmune and gut–brain axis interactions achievable through food-based delivery (Marx et al., 2026). Additional evidence from studies of fermented oat- and milk-based probiotic beverages shows enhanced strain survival and persistence in human volunteers, with *Lactiplantibacillus rhamnosus* CRL1505 detectable in a subset of subjects even one week post-consumption, underscoring the importance of matrix-dependent resilience and gastrointestinal transit in determining clinical functionality (Mangieri et al., 2025). The present thesis underscores that functional efficacy in foods cannot be dissociated from strain survival during digestion and functional metabolite production during fermentation. These mechanistic considerations help explain why only a subset of clinically validated strains maintain efficacy when delivered through non-ideal matrices. Incorporating clinical insight into food design will therefore be essential for developing next-generation functional foods that bridge mechanistic, nutritional, and technological dimensions.

5.8 EMERGENCE OF NEW DIETARY AWARENESS AND PERSONALIZED NUTRITION

Another crosscutting trend relevant to probiotic food development is the growing emphasis on personalized and preventive nutrition. Consumers increasingly seek foods tailored to digestive comfort, immune resilience, and metabolic balance; an evolution driven by heightened awareness of the microbiome role in long term health. This shift is reflected in recent microbiome based personalized nutrition research demonstrating how individualized dietary strategies, informed by microbial ecology, are progressively moving beyond traditional dietary models to support disease prevention and improved health outcomes (Nisa et al., 2025). Dietary transitions, including reduced dairy intake, increased adoption of plant-based alternatives, and rising demand for low sugar or allergen free products, create both opportunities and constraints for probiotic incorporation. The data generated in this thesis clearly show that not all strains are compatible with, for example, plant matrices, underscoring the need to align microbial physiology with emerging nutritional preferences. Looking ahead, combining matrix appropriate strains with microencapsulation technologies

and targeted metabolite producing cultures may enable the development of foods designed not only for general wellness but also for specific population subgroups such as older adults, lactose intolerant individuals, athletes, or children.

5.9 ORIGIN OF PROBIOTIC STRAINS AND ECOLOGICAL–CULTURAL DIVERSIFICATION OF FOOD CARRIERS

Classically, probiotic strains have been isolated from the human gastrointestinal tract, breast milk, and traditional dairy fermentations, sources long regarded as benchmarks for host adaptation, safety, and functional relevance. The global diversification of diets has expanded the range of candidate matrices for probiotic delivery. Traditional fermented foods from Asia, Africa, and Latin America, such as *tempeh*, *injera*, *togwa*, *pozol*, and fermented cassava products, harbour microbial communities rich in LAB and *Bacillus* species adapted to complex, fluctuating environments. These foods are increasingly studied as natural reservoirs of robust strains with high stress tolerance and desirable functional traits (Deka et al., 2021; Jung et al., 2011; Yulandi et al., 2020). The ecological origin of strains, whether from plant surfaces, cereals, fermented vegetables, or dairy environments, directly influences their techno-functional potential. The results presented in this thesis align with this ecological principle, demonstrating that strains derived from plant or nomadic habitats maintain higher stability in acidic, oxygenated matrices than gut adapted or dairy specialist strains. This ecological–cultural cross fertilization suggests that future innovation in probiotic foods may increasingly rely on biodiverse strain sources and the valorisation of traditional fermentation practices.

5.10 ECO-SUSTAINABILITY AND CIRCULAR FOOD SYSTEM INNOVATION

Probiotic food innovation is increasingly intertwined with sustainability objectives. The use of plant-based matrices, fruit and vegetable byproducts, cereal brans, and valorised side streams aligns with circular economy principles while providing nutritionally rich substrates for fermentation. Several studies have demonstrated that fermentation processes can enhance antioxidant capacity, reduce antinutritional factors, and promote the formation of novel bioactive compounds from agro-industrial residues (Di Cagno et al., 2021; Ikusika et al., 2024; Salas-Millán & Aguayo, 2024; Šelo et al., 2021; Vilas-Franquesa et al., 2024a,b).

In this context, the valorisation of plant-derived co-products has gained particular relevance. For instance, liquid co-products generated during fig processing have been shown to be rich in phenolic compounds and organic acids, exhibiting strong *in vitro* antioxidant activity and potential as a natural preservative (Viuda-Martos et al., 2015). Another study, by Sánchez-Bravo et al. (2020), demonstrated how hydroSOSustainable pistachios produced under resource-efficient agricultural practices can be efficiently transformed into technologically viable fermented foods supporting lactic acid bacteria growth and sensory quality.

Beyond plant-based substrates, emerging evidence suggests that alternative resources, such as insect-derived side streams, may also represent a promising and complementary component of circular food systems. In particular, Vilas-Franquesa et al. (2024c), demonstrated that lactic acid bacteria can effectively biotransform insect processing residues, enabling partial removal of chitin-bound proteins while simultaneously generating microbial biomass and peptides with antioxidant activity.

Together, these approaches exemplify how microbial metabolism can simultaneously drive nutritional enhancement and waste valorisation, reinforcing fermentation as a cornerstone technology for circular food system innovation. The results of this thesis further support the feasibility of integrating probiotics into such sustainable carriers, particularly nomadic lactic acid bacteria, which display an expanded metabolic repertoire and enhanced tolerance to environmental variability. Overall, the convergence of fermentation science, microbial ecology, and sustainability-driven processing strategies is likely to shape the next generation of functional foods, enabling products that deliver validated health benefits while reducing environmental impact and supporting circular bioeconomy goals.

5.11 NEXT GENERATION PROBIOTIC-RELATED SOLUTIONS

The future of next-generation probiotic foods is likely to be shaped by a progressive convergence of food science, molecular microbiology, and clinical nutrition, moving beyond the traditional paradigm of empirically selected lactic acid bacteria. Recent advances in genome-guided screening and targeted genetic approaches are already enabling the rational selection or construction of strains with defined metabolic functionalities, thereby strengthening the link between microbial genotype and functional performance in food systems.

This shift is exemplified by molecular strategies used to identify lactic acid bacteria with specific biosynthetic capacities, such as the PCR-based detection of *gadB* and *gadC* genes to efficiently select strains with high γ -aminobutyric acid (GABA)-producing potential for functional food applications, allowing a more predictable translation of genetic potential into bioactive output (Langa et al., 2024).

In parallel, synthetic biology approaches applied to food-grade microorganisms illustrate how metabolic functions that are rare or unevenly distributed within the human microbiota can be transferred into technologically relevant strains. A clear example is the heterologous expression of equol-producing pathways from *Slackia isoflavoniconvertens* into lactic acid bacteria with a history of food use, enabling the production of equol and 5-hydroxy-equol in culture media and in food matrices such as milk (Ruiz De La Bastida et al., 2021). These studies demonstrate that the functional scope of probiotic and starter cultures can be expanded through the controlled introduction of well-characterized metabolic pathways, while still relying on microorganisms commonly used in food fermentation.

Importantly, these works illustrate how both non-GMO selection approaches and recombinant strategies can be applied to obtain strains with markedly enhanced micronutrient-producing capacity, highlighting the versatility of lactic acid bacteria as platforms for next-generation functional foods. Together, these developments contribute to blurring the boundary between conventional functional foods and live biotherapeutic products.

In this context, the growing scientific interest in non-QPS commensals such as *Akkermansia muciniphila* and *Oxalobacter formigenes* reflects a broader shift towards function driven strain selection, grounded in mechanistic evidence and clinical relevance rather than historical food use. However, the translation of such microorganisms into food applications will require novel regulatory frameworks and rigorous safety-by-design strategies.

At the same time, postbiotics are emerging not merely as a technological alternative, but as a conceptual evolution of probiotic intervention, offering improved stability, reproducibility and safety while retaining biological efficacy, features that are particularly relevant for vulnerable populations and medical nutrition.

Future synbiotic formulations are also expected to become increasingly sophisticated, based on the deliberate pairing of next generation fibers with specific microbial taxa to promote predictable ecological and functional outcomes, rather than generic stimulation of bacterial growth. Moreover, the exploration of less conventional food matrices, including

insect-based products, pet foods, and animal feed such as clinically validated fermented forages, suggests that next-generation probiotics may permeate the entire food chain, from agriculture to human health. This holistic perspective is further reinforced by the expanding role of probiotics in agriculture itself, where microbial interventions may contribute to the generation of primary foods inherently enriched with beneficial microorganisms.

Collectively, these developments point towards a future in which next generation probiotic foods are no longer conceived as isolated dietary supplements, but as integral components of a continuum linking sustainable food systems, personalized nutrition, and foods for special medical purposes (FSMP), ultimately redefining the role of food in health maintenance and disease management (Abouelela & Helmy, 2024; Vijayaganapathi & Mohanasrinivasan, 2025).

6 CONCLUSIONS

The following conclusions synthesise the overarching insights derived from the multidisciplinary experimental work conducted throughout this thesis. Together, they distil how probiotic behaviour emerges within real food and beverage environments, integrating evidence across diverse microbial lineages, ecological strategies, and heterogeneous matrices. The conclusions presented below highlight the broader conceptual implications and cross-matrix patterns that inform both the scientific understanding of probiotic–matrix interactions and the practical considerations relevant to industrial product development. These consolidated statements aim to articulate the key principles that have emerged from this research and to provide a coherent foundation for future applications and directions in probiotic food design.

1. Probiotic performance in food systems arises from the interplay between strain specific genomic traits, ecological lifestyle, and the physicochemical properties of the delivery matrix.
2. Nomadic lactic acid bacteria, exemplified by *Lactiplantibacillus plantarum*, displayed broad adaptive capacity across acidic, oxygenated, and compositionally diverse environments, consistent with their genomic versatility and ecological plasticity. In contrast, host adapted and dairy specialist strains performed optimally only in matrices that approximated their native ecological niches, most notably dairy substrates, and showed markedly reduced resilience in plant based, low pH, or compositionally incompatible systems.
3. Spore forming probiotics such as *Bacillus coagulans* exhibited exceptional tolerance to low moisture, high sugar concentrations, and thermal treatments, positioning them as particularly suitable candidates for shelf stable food formats.
4. Matrix physiochemistry emerged as a central determinant of survival, with dairy products offering buffering capacity and metabolic support, plant matrices imposing substantial physicochemical stresses, and low moisture environments enabling long term stability.
5. Fermentation further enhanced functional outcomes through the generation of bioactive metabolites, though sensory consequences remained highly dependent on precise strain–matrix interactions.

6. Sensory evaluation indicated that probiotic addition had negligible impact in dry matrices, whereas fermented and dairy based products may require careful formulation to maintain consumer acceptance.
7. Development of probiotics for foods must adopt an integrative framework that jointly considers microbial ecological traits, technological feasibility, sensory compatibility, and the broader regulatory and clinical context.

7 LIMITATIONS

Although this doctoral research addressed a wide range of matrices and probiotic strains, several limitations must be acknowledged. The following are most relevant ones:

1. Matrix batches were in some cases provided by industrial partners; some formulations could not be disclosed, limiting reproducibility.
2. Repeated sampling from the same bottle (e.g., pasteurized juice) potentially enhanced oxidative exposure, influencing colour and quality evolution.
3. Storage conditions were standardized but could not simulate all real market conditions (e.g., temperature fluctuations, transport).
4. In fortified products containing probiotic blends, bacterial viability was assessed as total counts rather than at strain-specific level, reducing the depth of interpretation regarding individual strain performance and dynamics within the formulation.
5. Limited number of strains per species restricts generalizability across entire taxonomic groups.
6. Sensory panels were untrained and geographically homogeneous; broader consumer testing would strengthen insights.
7. Fermentation trials were limited to specific temperatures and inoculation ratios; optimization models were not explored.
8. No *in vivo* studies were conducted; conclusions about physiological efficacy rely on published clinical data rather than direct validation of food-delivered strains.
9. Metabolomics analyses were limited to specific substrates; wider substrates or untargeted microbial metabolite mapping could reveal additional functionalities.
10. No comparison with microencapsulation or advanced delivery technologies was included.

8 FUTURE PERSPECTIVE

A forward-looking, integrated research agenda should involve:

4.1. Predictive strain–matrix matching

- Develop computational models integrating genomic stress markers, ecological lifestyle, and matrix physiochemistry.
- Use multi-omics and AI-driven profiling to predict performance before industrial trials.

4.2. Functional metabolomics and bioactivity mapping

- Expand targeted/untargeted metabolomics to systematically map phenolic biotransformation, SCFA, neurotransmitter-related metabolites, antimicrobial and antioxidant compounds.
- Assess synergistic effects of microbe–matrix–metabolite interactions.

4.3. Sensory-driven formulation science

- Integrate sensory constraints early in strain selection.
- Use dynamic fermentation control to prevent undesirable flavour compounds.
- Explore fat, sugar, and prebiotic structuring to modulate off-flavours.

4.4. Sustainable and resilient functional foods

- Valorise agro-industrial by-products as fermentation substrates.
- Prioritize low-energy, no-refrigeration matrices.
- Develop eco-efficient packaging for oxygen-sensitive matrices.

4.5. Regulatory and clinical integration

- Align formulations with updated microbial characterization regulatory framework.
- Design clinical trials evaluating food-delivered probiotics, not only supplements.
- Explore population-specific applications (infants, elderly, athletes, stressed individuals).

4.6. Toward an integrated probiotic food ecosystem

- Together, these elements will enable the development of next-generation functional foods that are scientifically grounded, technologically feasible, sensorially acceptable, clinically aligned, and ecologically responsible.

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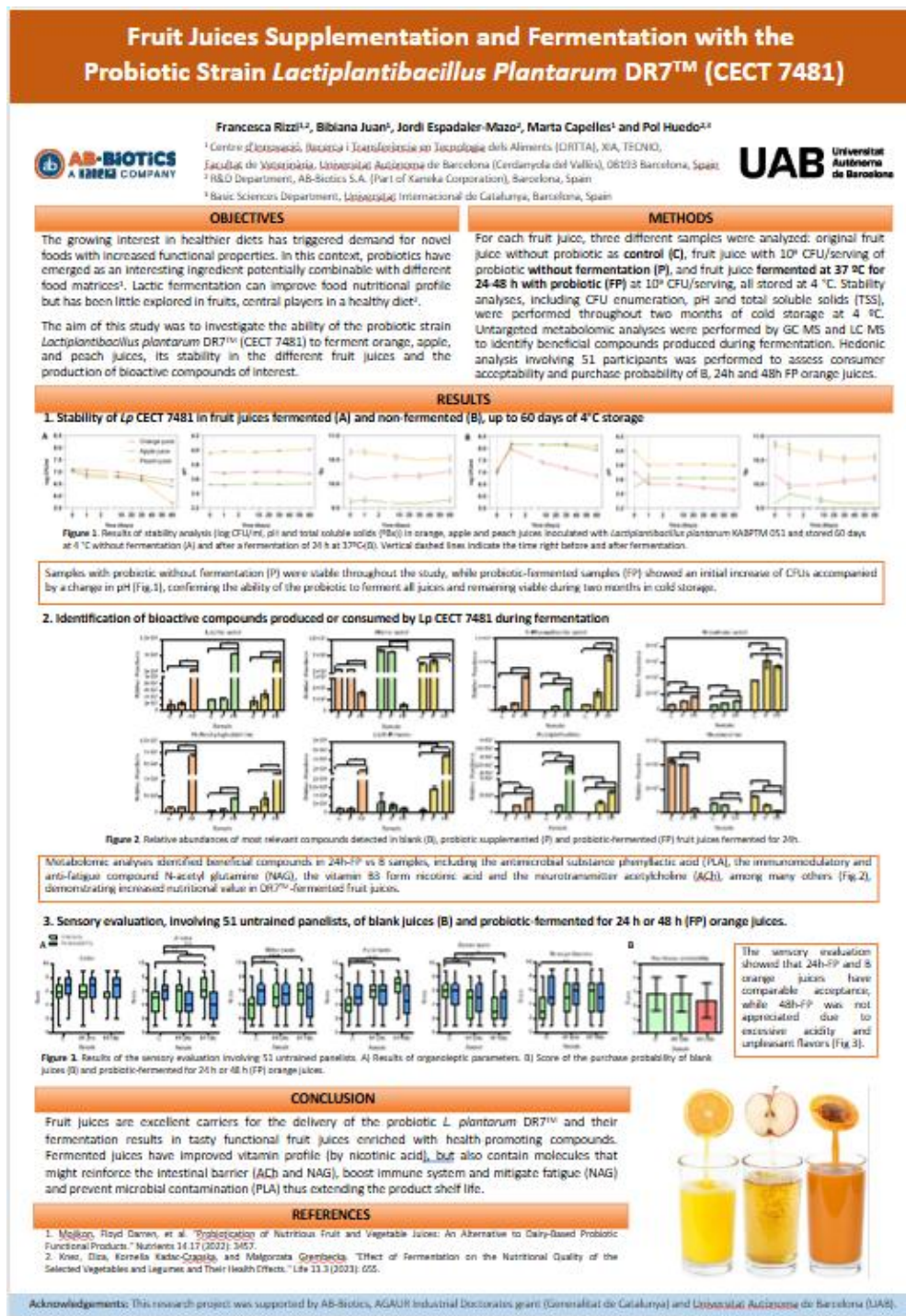
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ANNEXES

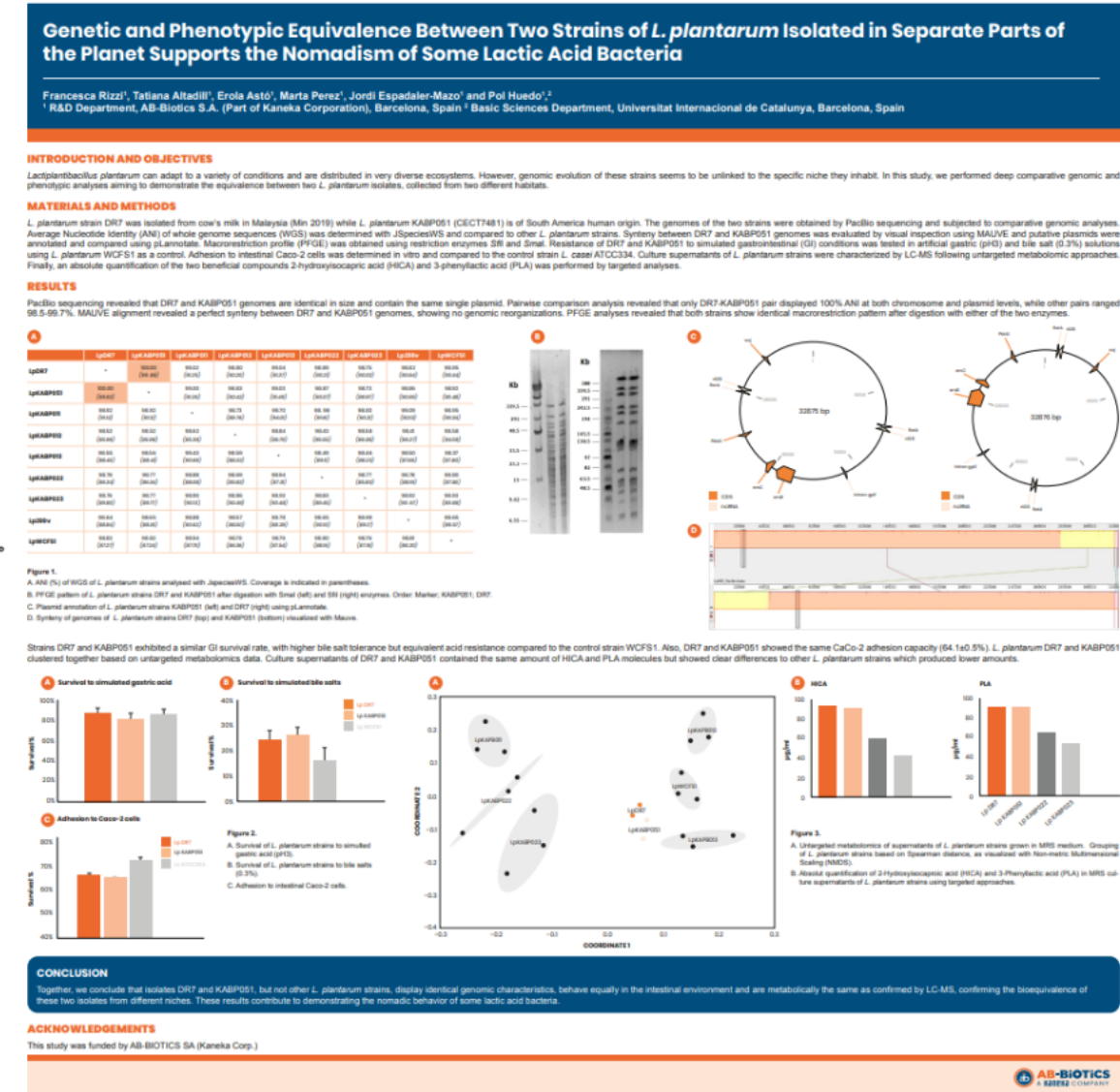
1. POSTER: FRUIT JUICES SUPPLEMENTATION AND FERMENTATION WITH THE PROBIOTIC STRAIN *LACTIPLANTIBACILLUS PLANTARUM* DR7™ (CECT 7481)

The poster was presented at the congress Probiotics, Prebiotics and New Foods (Rome, Italy, 2023).



2. POSTER: GENETIC AND PHENOTYPIC EQUIVALENCE BETWEEN TWO STRAINS OF *L. PLANTARUM* ISOLATED IN SEPARATE PARTS OF THE PLANET SUPPORTS THE NOMADISM OF SOME LACTIC ACID BACTERIA

The poster was presented at the congress Discover BMB (San Antonio, Texas, USA, 2024). DOI:<https://doi.org/10.1016/j.jbc.2024.105833> (Journal of Biological Chemistry).



KNOWLEDGE TRANSFER

1. **Poster:** Fruits Juices Supplementation and Fermentation with the Probiotic Strain *Lactiplantibacillus plantarum* DR7™ (CECT 7481) → Congress *Probiotics, Prebiotics & New foods* (September 17-19,2023; Rome, Italy)
2. **Poster:** Genetic and Phenotypic Equivalence Between Two Strains of *L. plantarum* Isolated in Separate Parts of the Planet Supports the Nomadism of Some Lactic Acid Bacteria → Congress *Discover BMB* (March 23-26, 2024; San Antonio, Texas)
3. **Oral presentation:** Coltivare la Calma: nutrire il Microbioma infantile fin dal primo giorno → Congress *Spazio Nutrizione* (October 17-18, 2025; Milano, Italy)
4. **Oral presentation:** Postbiotics: An Innovative Approach to Health → Webinar *Nuevas fronteras en aplicaciones de la microbiología: La era de los postbióticos* (May 28, 2025; Carinsa, online)
5. **Scientific article:** *Lactiplantibacillus plantarum* KABP051: Stability in Fruit Juices and Production of Bioactive Compounds During Their Fermentation → Scientific journal *Foods* (Published: 28 November 2024)

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GRAZIE.