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Characterization and *In Vivo* Evaluation
of the Synbiotic Potential of Probiotic
Lactic Acid Bacteria Isolated from
Traditional Lithuanian Fermented Foods

DOCTORAL DISSERTATION

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Tradiciniuose lietuviškuose
fermentuotuose maisto produktuose
išskirtų probiotinių pieno rūgšties
bakterijų charakterizavimas ir
sinbiotinio potencialo vertinimas *in vivo*

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ABBREVIATIONS

5-HT – Serotonin
AD – Alzheimer's disease
AMR – Antimicrobial resistance
CD – Control diet
CFS – Cell-free supernatants
CVD – Cardiovascular disease
DPPH – 2,2-diphenyl-1-picrylhydrazyl
EFSA – European food safety association
ELISA – Enzyme-linked immunosorbent assay
EPM – Elevated plus maze
FAO – Food and Agriculture Organization
FF – Fermented foods
FOS – Fructo-oligosaccharide
FST – Force swim test
GABA – Gamma-aminobutyric acid
GIT – Gastrointestinal tract
GLP-1 – Glucagon-like peptide-1
GM – Gut microbiota
GOS – Galacto-oligosaccharides
GTT – Glucose tolerance test
HFD – High-fat diet
IBD – Inflammatory bowel disease
IEC – Intestinal epithelial cells
IL – Interleukin
ISAPP – International Scientific Association for Probiotics and Prebiotics
LA – Lactic acid
LAB – Lactic acid bacteria
LD – Light and dark box
LPS – Lipopolysaccharide
MGB – Microbiota-gut-brain axis
MLST – Multilocus sequence typing
MRS – de Man, Rogosa, and Sharpe media
ND – Neurodegenerative disease
NOR – Novel object recognition
OA – Organic acids
OF – Open field
PBS – Phosphate-buffered saline
PCoA – Principal coordinate analysis

QPS – Qualified Presumption of Safety
SCFA – Short chain fatty acids
SIT – Social interaction test
SPT – Sucrose preference test
T2D – Type 2 diabetes
TEAC – Trolox equivalent antioxidant concentration
TNF – Tumor necrosis factor
TST – Tail suspension test
VF – Virulence factors
VRW – Voluntary running wheel
WGS – Whole-genome sequencing
WHO – World health organization
YM – Y-maze

INTRODUCTION

Fermented foods (FF) have been part of human diets for centuries (Leech et al. 2020). They are valued not only for their taste, aroma, flavor, and longer shelf life, but also for their numerous health benefits (Castellone et al. 2021). During fermentation process, microorganisms produce many beneficial metabolites such as organic acids (OA), vitamins, bioactive peptides, exopolysaccharides, and polyphenols (Praveen and Brogi 2025b). These compounds improve both food quality and human health. A major role in fermentation is played by lactic acid bacteria (LAB), which include genera such as *Lactiplantibacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, and *Enterococcus* (Praveen and Brogi 2025b). These bacteria are widely used in the fermentation of dairy products, vegetables, fruits, and cereals, and they have a long history of safe use in food production (Santa et al. 2025).

LAB and *Bifidobacterium* strains that confer a health benefit on the host when administered in adequate amounts are classified as probiotics (Fijan 2014), with documented effects reaching beyond the gut to host metabolism, immune function, and brain activity via the microbiota-gut-liver-brain axis (Abouelela and Helmy 2024; Leeuwendaal, Stanton, O'toole, et al. 2022a; Szota et al. 2026). Because these effects are highly strain-specific, no genus- or species-level generalisation is sufficient: each candidate strain must be characterized individually for its ability to survive the gastrointestinal tract (GIT), adhere to intestinal epithelial cells, inhibit pathogens, and, where relevant, act synergistically with prebiotic substrates as part of a synbiotic formulation before it can be considered for safety evaluation and further application (Megur et al. 2023; Swanson et al. 2020; Churin et al. 2026).

Traditional Lithuanian FF, including fermented vegetables, fruits, dairy products, and grain-based drinks, are produced through natural fermentation under conditions of low pH, salt stress, and low oxygen tension that may select for LAB with improved GIT resilience and metabolite output (Dimidi et al. 2019; Leeuwendaal, Stanton, O'toole, et al. 2022a). Despite this potential, they remain a poorly studied source of novel probiotic strains (Bartkiene et al. 2020; Grujović et al. 2022; Slapkauskaite et al. 2016). , and it is not known whether a candidate strain from this source could be advanced through safety evaluation under European Food Safety Authority (EFSA) Qualified Presumption of Safety (QPS) criteria (Kwon et al. 2021; Roe et al. 2022) and

translate into measurable benefit in a model of diet-induced metabolic and neuroinflammatory dysfunction.

Moreover, to function as probiotics, LAB strains must survive the harsh conditions of the GIT. This includes resistance to stomach acid, pepsin, bile salts, and pancreatin. After surviving digestion, the bacteria should be able to attach to intestinal epithelial cells (IEC), which helps them colonize the gut, compete with harmful bacteria, and interact with the immune system (Megur et al. 2023a). These properties can be tested *in vitro* using human IEC lines.

Another important probiotic property is antimicrobial activity. LAB can inhibit pathogenic bacteria by producing OA, hydrogen peroxide, and bacteriocins (Lingling Wang et al. 2025). In addition to probiotics, prebiotics are non-digestible food components that are selectively utilized by beneficial microorganisms, thereby promoting their growth and activity in the gastrointestinal tract (Glenn R Gibson et al. 2017; Swanson et al. 2020). Common prebiotics include fructooligosaccharides (FOS) and galactooligosaccharides (GOS), which stimulate the growth of beneficial bacteria and enhance the production of health-promoting metabolites (De Jesus Raposo, De Moraes, and De Moraes 2016; Mitmesser and Combs 2017; Sako and Tanaka 2011b). Synbiotics are formulations that combine probiotics and prebiotics to improve the survival, colonization, and functional activity of beneficial microorganisms in the host, thereby enhancing their health benefits (Swanson et al. 2020).

An additional objective of this thesis was to investigate the synbiotic potential of selected LAB strains by evaluating their ability to utilize prebiotic substrates and produce L-tryptophan. L-tryptophan is an essential amino acid that serves as a precursor for several biologically important metabolites, including serotonin (5-HT) and melatonin (Friedman 2018). The presence of tryptophan biosynthesis-related genes (*trpA* and *trpB*), together with HPLC-MS metabolite profiling, provided evidence of the capacity of selected strains to synthesize L-tryptophan under specific growth conditions. These findings contribute to the functional characterization of the isolated strains and support their potential application as probiotic or synbiotic candidates with potential relevance to the microbiota-gut-brain axis (MGB). A two-way biochemical communication between the gut, its microbiota diversity and the brain, known as MGB.

The final part of this dissertation describes a 25-week animal study using female C57BL/6J mice fed a high-fat diet (HFD). The aim was to evaluate the effects of the probiotic strain *Lactocaseibacillus paracasei* 11W, isolated from fermented pear, alone or combined with 5% GOS in a model of metabolic syndrome. HFD causes obesity, glucose intolerance, fatty liver disease, the gut microbiota (GM) imbalance, and neuroinflammation, which resemble human metabolic disorder (Abbasi and Khodadadi 2025; Do et al. 2018). Female mice were specifically selected because they remain underrepresented in many preclinical probiotic studies. Focusing on female mice provides valuable information on probiotic efficacy in this sex; however, the use of a single sex limits direct comparisons of sex-specific responses, which should be addressed in future studies (Qiao et al. 2025; Stapleton et al. 2023).

This study investigates how novel LAB strains isolated from traditional Lithuanian FF influence metabolism, gut health, inflammation, microbiota composition, and brain-related functions. The findings connect traditional Lithuanian food culture with modern probiotic science and support the development of new synbiotic therapies for metabolic and neuroinflammatory conditions associated with neurodegeneration.

Aim and Objectives

The aim of this doctoral thesis was to isolate, identify, and characterize novel probiotic LAB from traditional Lithuanian FF and to evaluate the effects of a selected probiotic candidate on metabolism, behavior, inflammation, and GM composition in a mouse model of metabolic dysfunction.

To achieve this aim, the following objectives have been carried out.

1. To isolate morphologically diverse LAB from traditional Lithuanian FF and characterize their probiotic potential through *in vitro* GIT survival, adhesion, and antimicrobial assays.
2. To assess the synbiotic potential of selected LAB isolates through prebiotic substrate utilization (FOS and GOS) and bioactive metabolite production.
3. To perform WGS of promising LAB candidates and identify probiotic-associated functional genes encoding stress tolerance, adhesion, bile salt hydrolysis, and tryptophan biosynthesis.
4. To evaluate the safety profiles of isolated LAB strains through phenotypic and genomic screening for VF and transferable antimicrobial resistance, in accordance with EFSA QPS guidelines.

5. To investigate the effects of the best-performing candidate, alone and in synbiotic combination with GOS, on metabolic, behavioral, inflammatory, and GM parameters in an HFD-induced metabolic dysfunction in a female mouse model.

Scientific Novelty and Practical Value of the Study

Of 150 LAB isolates recovered from traditional Lithuanian FF, comprehensive *in vitro* screening across two independent isolation campaigns (Batch I and Batch II) narrowed the collection to ten promising probiotic candidates. In contrast to the single dominant species typically reported from screening studies of a single fermented food, the ten candidates spanned five LAB genera, *Lacticaseibacillus*, *Lactiplantibacillus*, *Pediococcus*, *Leuconostoc*, and *Limosilactobacillus*, recovered from a range of traditional Lithuanian FF, including fermented pear, cherry tomato, cucumber, sauerkraut, kombucha, and fermented green beans, demonstrating that Lithuanian FF harbor a taxonomically and functionally diverse probiotic reservoir rather than a single recurring genus.

Whole-genome sequencing (WGS) of all ten candidates provided molecular confirmation of species identity and revealed strain-specific probiotic gene profiles, including genes for acid tolerance (*gadC*), mucus and epithelial adhesion (*fbp*, *pdeK*), and tryptophan biosynthesis (*trpS2*). Five strains met the full set of EFSA QPS safety criteria, showing no hemolytic activity, no gelatin or mucin degradation, and no transferable antimicrobial resistance or virulence factor genes.

Among the candidates, *Lacticaseibacillus paracasei* 11W was distinguished by the combination of robust survival across all four simulated GIT barriers, the strongest auto-aggregation and HCT116 adhesion in its batch, a probiotic gene profile that included the adhesion gene *pdeK*, detected in no other Batch I isolate, and the highest synbiotic L-tryptophan output (16.63 μ M) of any strain tested, obtained in the presence of 4% GOS. Advanced to a 25-week HFD-induced mouse model, *L. paracasei* 11W combined with GOS produced measurable improvements across metabolic, behavioral, colonic and hippocampal inflammatory, and GM outcomes, providing an integrated *in vitro*-to-*in vivo* validation pipeline for a Lithuanian-FF-derived LAB strain. Collectively, these findings identify traditional Lithuanian FF as an underused source of taxonomically diverse, functionally characterized LAB, and position

L. paracasei 11W, alone or as a synbiotic formulation with GOS, as a translatable candidate for future human trials targeting diet-induced metabolic dysfunction and its neuroinflammatory and behavioral comorbidities.

Defending Statements

1. Selected LAB strains isolated from traditional Lithuanian FF exhibit characteristics required for probiotic functionality, including GIT survival, antimicrobial activity, and adhesion to IEC.
2. Selected LAB strains demonstrate synbiotic potential through their utilization of prebiotic substrates, leading to enhanced growth and modulation of L-tryptophan production.
3. Selected LAB strains possess genetic determinants associated with probiotic functionality, including stress tolerance, adhesion, bile salt hydrolysis, and tryptophan biosynthesis.
4. Selected LAB strains satisfy key EFSA QPS safety requirements for probiotic application, including the absence of hemolytic activity, virulence factors, and transferable antimicrobial resistance determinants.
5. *Lacticaseibacillus paracasei* 11W and GOS modulate metabolic, behavioral, inflammatory, and GM dysbiosis induced by an HFD.

1. LITERATURE OVERVIEW

1.1. Fermented food

FF have been consumed and have played an important role in human diet for more than a millennium for their beneficial properties (Leeuwendaal, Stanton, O'Toole, et al. 2022; Siddiqui et al. 2023). There is evidence of fermentation practice that date back over 13,000 years to early civilizations such as Vedic, Natufians, Egyptian, and Chinese (L. Liu et al. 2018). FF emerged as spontaneous processes driven by microbial metabolism and evolved into practices that persevere in cultures across the globe (I. Park and Mannaa 2025a). Food fermentation is known for remodeling food storage abilities, transforming flavors, and altering textures (Siddiqui et al. 2023). But has recently gained increased public and scientific attention for its potential to convey health-promoting characteristics to foods (Park and Mannaa 2025a). However, the possible bridge between consumption of FF, the roles of microorganisms that facilitate their transformations, and human health outcomes remain incompletely understood but are a topic of active investigation (Leeuwendaal, Stanton, O'Toole, et al. 2022). The field of FF research is broad, consisting of a wide variety of food types, health outcomes, and methodologies ranging from observational to interventional research (Porrás-García et al. 2023a). Large cohort studies consistently associate the consumption of FF with reduced risk of metabolic disorders, certain cancers, and neurodegenerative impairment (Porrás-García et al. 2023a; Sampsell et al. 2025a).

According to the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement, FF are foods made through desired microbial growth and enzymatic conversion of food components (Marco et al. 2021). FF production involves a variety of starting materials and microbial strains, each driving distinct biochemical transformations (Park and Mannaa 2025a; Sampsell et al. 2025a). These microbial processes not only contribute to taste and preservation but also generate functional compounds with potential health benefits (Praveen and Brogi 2025a). Not all fermented products contain viable microbes at the point of consumption or have health-promoting properties (Praveen and Brogi 2025a). Although this does not mean that such foods, or even microbe-free products derived from microbial fermentation, are without potential benefit (Fitsum, Gebreyohannes, and Sbhatu 2025a). The fermentation process usually occurs under anaerobic or

aerobic conditions, where microorganisms utilize sugars as energy sources and produce metabolites such as OA, alcohol, carbon dioxide, and bioactive peptides (Gangakhedkar et al. 2025). These metabolites contribute to preservation by lowering the pH, inhibiting the growth of pathogenic microorganisms, and hence enhancing shelf life (Yuan et al. 2024).

FF are increasingly recognized as functional foods because they provide health benefits beyond basic nutrition (Arshad et al. 2025; Leeuwendaal, Stanton, O'Toole, et al. 2022). This is due to the presence of live microorganisms and bioactive compounds (Arshad et al. 2025). The microbial diversity in FF depends on raw material, environmental conditions, and fermentation practices (Gonçalves et al. 2026). Recent research has shown that FF plays role in modulating the GM and its composition and functioning, linking to human health (Leeuwendaal, Stanton, O'Toole, et al. 2022). FF acts as a carrier of beneficial bacteria, which survive GIT transit and its harsh conditions, and exert favorable effects on the host (Soemarie, Milanda, and Barliana 2021).

In addition to microbial benefits, fermentation of the food sources enhances the nutritional value by increasing the availability of vitamins, amino acids, and bioactive compounds like polyphenols (Park and Mannaa 2025a). Moreover, fermentation also reduces anti-nutritional factors, such as tannins and phytates, which are naturally occurring compounds that can impair digestion, absorption, or utilization of nutrients (Abbaspour 2024), thereby increasing mineral bioavailability and absorption (Liu et al. 2026). Recent studies highlight the role of FF in metabolic health, immune regulation, and mental wellbeing through the microbiota-gut-liver-brain axis (Garnås 2023). Making FF important in research related to anti-diabetic, anti-inflammatory, and psychobiotic effects (Cheng et al. 2019; Shamsudin et al. 2022; Zulkawi et al. 2018) Figure 1.1.

1.1.1. Health benefits fermented foods

Consumption of FF on daily basis has been shown to exhibit beneficial effects on health. There is numerous FF that have positive effects when consumed all around the globe including natto, miso, tempeh, boza, injera, kimchi, buttermilk and amazake (Santa et al. 2025). All these FF have a protective effect and can be used as a natural intervention for treatment and management of various health conditions, including metabolic disorders, gastrointestinal

disturbances, inflammation, oxidative stress, and microbiota-related imbalances (Essa et al. 2023). They may also help mitigate the adverse effects associated with chronic diseases by improving gut health, enhancing immune function, and regulating physiological processes (Leeuwendaal, Stanton, O'toole, et al. 2022a; Marco et al. 2017; Sampsell et al. 2025b; Valentino et al. 2024). The intake of Moroccan fermented dairy products, lben and raib, has been linked to reducing the risk of colorectal cancer through improvement of the gut barrier integrity (El Kinany et al. 2020). Kimchi is mostly consumed FF in East Asia (El Kinany et al. 2020). Kimchi is regarded as a healthy food due to its nutritional value and several bioactive compounds like phenolic compounds, flavonoids, vitamins, dietary fiber, carotenoids, and sulfur-containing compounds and as a potentially probiotic food due to its high content of various LAB (Patra et al. 2016). Whereas administration of kimchi powder to rats injected with a carcinogen concluded to a protective effect by acidification of the intestinal environment, and upregulation of protective genes (Oh et al. 2005). In a clinical trial it found that consuming 100 g of fermented kimchi daily for 10 weeks significantly improved fecal microbiota diversity, boosting beneficial genera. This highlights that LAB present in kimchi helps enhance the GM balance (J. M. Park et al. 2021). In another research, a 12-week randomized controlled trial demonstrated that a daily intake of 210 g of kimchi improved symptoms of IBS, including reduced abdominal pain and bloating, by modulating intestinal inflammation (H. Y. Kim et al. 2022). A comprehensive prospective cohort study revealed a significant link between higher kimchi consumption and reduced body weight and obesity risk (Jung et al. 2024). These outcomes were observed that kimchi is linked to the anti-inflammatory effects and GM supporting properties of LAB and dietary fibers present in kimchi (Tan et al. 2023). On the other FF note, regular consumption of koumiss has been associated with improved lipid profiles, including reduced total cholesterol and triglyceride levels and increased HDL, likely due to the combined action of fermentation products on lipid metabolism (Dönmez et al. 2014).

Shifting on the immunomodulatory effects, a study with dahi, refers to a traditional Indian FF product that is not widely known globally, typically characterized by fermentation-associated microorganisms and potential health-promoting properties (Saha et al. 2025). In the study it was exhibited that regular consumption of dahi in malnourished children improved both pro-inflammatory and anti-inflammatory (IL-4, IL-10) cytokine levels (Dewan

Pooja 2009). This indicated a more balanced and functional immune response during nutritional rehabilitation (Dewan Pooja 2009)(Figure 1.1).

Tempeh consumption improved the global cognitive scores of adults aged > 60 years with mild cognitive impairment after 6 months of intervention together with improved language function (Handajani et al. 2020). Moreover, regular consumption of fermented soy products like miso and natto is consistently linked to reductions in visceral fat, better insulin sensitivity, low prevalence of sarcopenia and decreased risks of Type 2 diabetes (T2D) and cardiovascular disease (CVD) (Nozue et al. 2021)(Takahashi et al. 2021)(Siddiqui et al., 2023). Other than these health benefits there are numerous other effects by which FF can be used for positive effects on patients with diseases (Santa et al. 2025). These health benefits are mostly executed by the beneficial LAB residing in the FF, which has a direct influence on the host GM modulation and overall physiological function (Jyoti Prakash Tamang 2022)(Figure 1.1).

1.1.2 Metabolic pathways of fermentation microorganisms

Fermentation is a complex microbial process in which microorganisms convert carbohydrates, proteins, and other food components into various metabolites (Praveen and Brogi 2025b). In FF, the metabolic activity is mainly driven by LAB, *Bifidobacterium*, yeasts, and other microorganisms that utilize different biochemical pathways depending on the available substrates, environmental conditions, and microbial species present (Kanimozhi, Nagalakshmi, and Sukumar 2026). The major fermentation pathways occurring in FF include homofermentative and heterofermentative LA fermentation, alcoholic fermentation, acetic acid fermentation, propionic acid fermentation, and proteolytic fermentation (Mannaa et al. 2021). These pathways involve specific metabolic enzymes that regulate the conversion of substrates into OA, alcohols, gases, vitamins, peptides, and other bioactive compounds (Mannaa et al. 2021)(Anumudu, Miri, and Onyeaka 2024a).

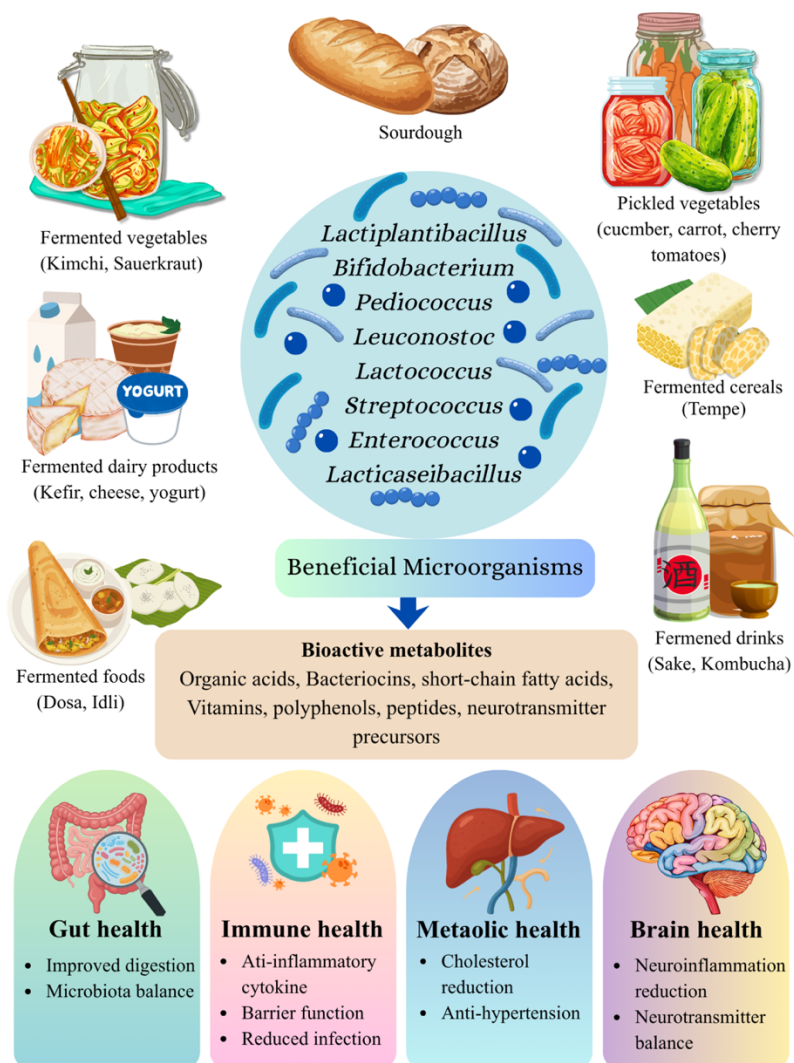


Figure 1.1 Schematic overview of common fermented foods, representative beneficial microorganisms, their bioactive metabolites, and associated health benefits. The bacterial genera shown are representative examples and are not intended to indicate that each genus is present in every FF listed. (The figure was made using canva.com)

1.1.2.1. Lactic acid fermentation pathways

LA fermentation is the dominant metabolic pathway in many FF, including yogurt, kefir, cheese, kimchi, sauerkraut, and sourdough (Anumudu, Miri, and Onyeaka 2024a). This pathway is mainly performed by LAB belonging to genera such as *Lactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus*,

Pediococcus, *Leuconostoc*, *Lactococcus*, and *Streptococcus* (Anumudu, Miri, and Onyeaka 2024a). LAB metabolize carbohydrates, mainly glucose, lactose, and plant-derived sugars, through glycolytic pathways to produce LA (Abedi and Hashemi 2020)

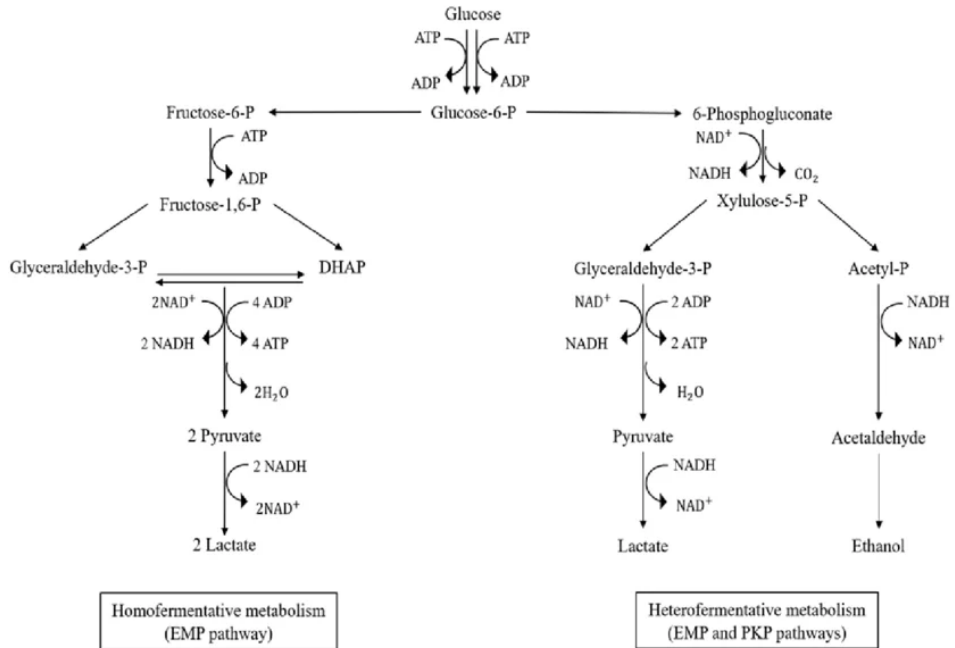


Figure 1.2. Metabolism for LAB (homofermentative and heterofermentative pathways) (Siang Aun Cheah et al. 2022).

In homofermentative LA fermentation, glucose is metabolized through the Embden-Meyerhof-Parnas's pathway (Abedi and Hashemi 2020). During this pathway, glucose is phosphorylated by hexokinase/glucokinase and converted into fructose-6-phosphate (Siang Aun Cheah et al. 2022b). The key regulatory enzyme phosphofructokinase converts fructose-6-phosphate into fructose-1,6-bisphosphate, which is subsequently cleaved into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Siang Aun Cheah et al. 2022b). Through multiple enzymatic reactions involving glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, and pyruvate kinase, pyruvate is generated (Siang Aun Cheah et al. 2022b). Finally, the enzyme lactate dehydrogenase converts pyruvate into LA by regenerating NAD⁺ required for glycolysis (Siang Aun Cheah et al. 2022b)(Figure 1.2).

Homofermentative LAB such as *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactiplantibacillus plantarum* mainly produce LA as the

final metabolite (Abedi and Hashemi 2020; Bintsis 2018). The accumulation of LA reduces food pH, inhibiting pathogenic and spoilage microorganisms and improving food safety and preservation (Z. Gao et al. 2019).

In contrast, heterofermentative LAB utilize the phosphoketolase pathway, which is linked to the pentose phosphate pathway (Bintsis 2018). Microorganisms such as *Leuconostoc mesenteroides*, *Lactobacillus brevis*, and *Lactobacillus fermentum* metabolize glucose into LA, ethanol/acetic acid, and carbon dioxide (Abedi and Hashemi 2020). The key enzyme involved in this pathway is phosphoketolase, which converts xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetyl-phosphate (Dalby, Ward, and Hailes 2012). Depending on the availability of electron acceptors, acetyl-phosphate is converted by acetate kinase into acetic acid or by alcohol dehydrogenase into ethanol (Dalby, Ward, and Hailes 2012). This pathway is particularly important in vegetable fermentations such as kimchi and sauerkraut, where CO₂ production contributes to anaerobic conditions and flavor development (Abedi and Hashemi 2020).

1.1.2.2. Acetic acid fermentation

Acetic acid fermentation is carried out mainly by acetic acid bacteria, including *Acetobacter* and *Gluconobacter* (K. Zhang et al. 2023). These microorganisms oxidize ethanol produced by yeasts into acetic acid under aerobic conditions (Guillamón and Mas 2011). The first step involves oxidation of ethanol to acetaldehyde by alcohol dehydrogenase (Guillamón and Mas 2011; K. Zhang et al. 2023). Subsequently, acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase (Guillamón and Mas 2011). This pathway is important in vinegar production and contributes antimicrobial activity because acetic acid reduces pH and inhibits undesirable microorganisms (K. Zhang et al. 2023).

1.1.2.3. Propionic acid fermentation pathway

Propionic acid fermentation occurs mainly in Swiss-type cheeses and is performed by *Propionibacterium freudenreichii* (Fröhlich-Wyder et al. 2017). During this pathway, lactate produced by LAB is converted into propionate, acetate, and carbon dioxide through the Wood–Werkman cycle (Fröhlich-Wyder et al. 2017; Rossi et al. 2025). The pathway involves enzymes including lactate dehydrogenase, methylmalonyl-CoA carboxyltransferase,

and propionyl-CoA transferase (Fröhlich-Wyder et al. 2017). The production of CO₂ contributes to the formation of cheese holes, while propionic acid contributes to characteristic flavor development (Abeijón Mukdsi et al. 2014)(Figure 1.3).

Figure 1.3. Propionic acid fermentation via the Wood–Werkman cycle in *Propionibacterium freudenreichii* (Antone et al. 2023).

1.1.2.4. Short-chain fatty acid production pathways

Acetate production occurs primarily through heterolactic fermentation and the bifidobacterial phosphoketolase pathway (Ward 2014). Heterofermentative LAB, including *Leuconostoc mesenteroides*, *Limosilactobacillus fermentum*, and *Lactobacillus brevis*, metabolize glucose via the pentose phosphate-phosphoketolase pathway (Yaqi Wang et al. 2021). In this pathway, glucose

is converted to xylulose-5-phosphate, which is cleaved by the key enzyme xylulose-5-phosphate phosphoketolase into glyceraldehyde-3-phosphate and acetyl-phosphate (Posthuma et al. 2002). Acetyl-phosphate is subsequently converted into acetate-by-acetate kinase, generating ATP as an additional energy source for the cell (Posthuma et al. 2002). This pathway contributes significantly to acetate accumulation in vegetable fermentations such as kimchi, sauerkraut, and fermented cereals (I. Park and Manna 2025b).

Another important route of acetate production is the bifid shunt pathway; characteristic of *Bifidobacterium* species frequently used in dairy fermentations and probiotic products (O’Callaghan and van Sinderen 2016). The key enzyme fructose-6-phosphate phosphoketolase converts fructose-6-phosphate into erythrose-4-phosphate and acetyl-phosphate, ultimately producing acetate and lactate as the principal fermentation end products (Rivière et al. 2016). This pathway yields more ATP than classical lactic acid fermentation and is considered one of the most efficient carbohydrate utilization pathways among food-associated microorganisms (Pokusaeva, Fitzgerald, and Van Sinderen 2011).

Propionate is produced mainly by *Propionibacterium freudenreichii*, a microorganism widely used in Swiss-type cheese fermentation (Thierry et al. 2011). Unlike LAB, propionibacteria utilize lactate generated by primary fermenters and convert it into propionate through the Wood–Werkman cycle (Rossi et al. 2025). The central enzymes involved include methylmalonyl-CoA carboxyltransferase, methylmalonyl-CoA mutase, and propionyl-CoA transferase, which catalyze the conversion of lactate through succinate and methylmalonyl-CoA intermediates to propionate (Petushkova, Mayorova, and Tsygankov 2021). Simultaneously, carbon dioxide is generated, contributing to the formation of characteristic cheese eyes and flavor compounds (X. Zheng, Shi, and Wang 2021a).

Butyrate production is less common in conventional FF but may occur in mixed-culture fermentations involving anaerobic bacteria (Snodgrass and Velayudhan 2026). The pathway begins with glycolytic conversion of carbohydrates to pyruvate, followed by formation of acetyl-CoA via pyruvate oxidoreductase (Snodgrass and Velayudhan 2026). Two molecules of acetyl-CoA are condensed by thiolase (acetyl-CoA acetyltransferase) to form acetoacetyl-CoA (V. Singh et al. 2023). Subsequent reactions catalyzed by 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA

dehydrogenase generate butyryl-CoA, which is converted to butyrate by either butyrate kinase or butyryl-CoA CoA-transferase (Anand, Kaur, and Mande 2016). Although butyrate concentrations in FF are generally lower than acetate or propionate, this metabolite is highly relevant because of its recognized role in intestinal health and anti-inflammatory activity (Recharla, Geesala, and Shi 2023).

Collectively, these pathways illustrate how fermentation microorganisms transform dietary carbohydrates into SCFA through highly specialized enzymatic reactions (Portincasa et al. 2022). Beyond their role in microbial energy metabolism, SCFA contribute to food preservation, flavor development, and the functional properties of FF, thereby linking microbial metabolism with both food quality and potential health benefits (Fitsum, Gebreyohannes, and Sbhatu 2025b; Sankarganesh et al. 2025).

1.1.3. Lactic acid bacteria and *Bifidobacterium* residing in FF

The taxonomy of the LAB group has undergone substantial revision in recent years. The former genus *Lactobacillus* was reclassified in 2020 into 25 genera, including *Lactiplantibacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, and *Levilactobacillus*, based on whole-genome phylogenetic and phenotypic analysis, together with the related genera *Leuconostoc*, *Pediococcus*, *Weissella*, and *Fructilactobacillus* within the family Lactobacillaceae (Zheng et al. 2020). All LAB genera fall within the phylum Bacillota (formerly Firmicutes) and the order Lactobacillales, and are Gram-positive, non-spore-forming, acid-tolerant, fermentative bacteria (Zheng et al. 2020). This reclassification is reflected throughout this thesis: strains previously assigned to *Lactobacillus* are referred to by their current genus names (e.g., *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*).

Traditional FF comprises of diverse microorganisms and unique microbial communities. The food and beverage fermentation can be distinguished based on the prevailing microorganisms, as well as their primary end products (Hui 2004). These foods consist of LAB, yeasts, and other microorganisms that thrive in specific environments. The LAB mediates the fermentation process of milk, meat, vegetables, and some grains to produce yogurt, kefir, cheese, kimchi and sourdough breads (Venturini Copetti 2019). These microorganisms also create stable ecological niches where these microbes can

interact and produce beneficial compounds that influence food safety to flavor and even nutritional value (W. Liu et al., 2025).

Microbial interactions within FF ecosystems play a crucial role in shaping microbial community structure and metabolic activity (Figure 1.4). These interactions include mutualism, in which both microorganisms' benefit; commensalism, where one microorganism benefits without affecting the other (Tshikantwa et al. 2018); competition, in which microorganisms compete for nutrients or ecological niches; and amensalism, where one microorganism inhibits another without itself being affected (Veiga 2016). Such interactions influence microbial growth, metabolite production, fermentation performance, and ultimately the quality and functionality of FF (Figure 1.4).

One of the most widely consumed FF is fermented cabbage, or sauerkraut, popular in European cuisines (Jyoti P. Tamang, Watanabe, and Holzapfel 2016). Sauerkraut is produced through spontaneous LAB fermentation for shredded cabbage in the presence of salt (Di Cagno et al. 2013)(Di Cagno et al. 2013). During the process of fermentation, microbial succession occurs step wise. Firstly, *Leuconostoc* species initiated the fermentation, following to *Lactobacillus plantarum*, and then *Lactobacillus brevis*, dominating the later stages due to their acid tolerance (Di Cagno et al. 2013). These bacteria produce lactic acid (LA), acetic acid, carbon dioxide, and the antimicrobial compounds which help with preservation of the food and inhibition of growth of pathogenic bacteria (Zabat et al. 2018).

The microorganisms that proliferate in cheese strongly affect their texture, appearance, and flavor, and lastly its safety (Mayo et al. 2021). These microorganisms are added directly as starter cultures with specific know strains, or mixture of unknown or novel strains (Coelho, Malcata, and Silva 2022). The microbial community in the cheese is strongly influenced by the environmental conditions, this is linked to the amount of salt added during the making, temperature, humidity, time and place of aging (X. Zheng et al., 2021). The early fermentation of cheese is carried out by LAB strains such as *Lactococcus lactis* and *Lactococcus cremoris* (Ferroukhi, Chassard, and Mardon 2024). These bacteria produce LA, which is used up by molds as a nutrient source (X. Zheng et al., 2021). During aging, fungi produce enzymes that break down proteins and fats, this changes the cheese texture and taste (Coelho, Malcata, and Silva 2022). In blue cheese making, oxygen is needed for mold growth. This includes the method by adding bacteria such as

Leuconostoc, which produce carbon dioxide and create small holes that help oxygen to move inside the cheese (Melkonian et al. 2023; X. Zheng, Shi, and Wang 2021b).

Another popular FF across Europe is kefir (Bourrie, Willing, and Cotter 2016). Kefir is a fermented milk beverage produced by kefir grains in combination with LAB and acetic acid bacteria (Prado et al. 2015). The grains of kefir represented highly stable microbial flora that can be maintained for years through repeated fermentation techniques (Leech et al. 2020). The microbial diversity in kefir contributes to the production of OA, vitamins, and bioactive peptides (Bengoa et al. 2019). These compounds make kefir a functional fermented beverage with health benefit (Ahmed et al. 2013; Bengoa et al. 2019; Leech et al. 2020; Prado et al. 2015).

Sourdough bread fermentation also harbors an important microbial community, where LAB and wild yeast coexist in a symbiotic relationship (Pérez-Alvarado et al. 2022). In the fermentation process of sourdough, LAB produces organic acid that lowers the pH and improves mineral bioavailability, while the yeasts produce carbon dioxide that leads to bread leavening (Arendt, Moroni, and Zannini 2011). The microbial composition in sourdough completely relies on the four influential factors, first the temperature, second the fermentation time, third the local environmental microbiota, fourth region-specific microbial communities (Minervini et al. 2014).

These traditional FF acts as a microbial niche as they provide nutrients, moisture, anaerobic conditions, and selective salt concentration, which allows specific microorganisms to grow and evolve in the ecosystems (Jyoti Prakash Tamang et al. 2020). The microbial population in the FF is often stable and self-regulated, leading to complex ecological networks including competition, cooperation, and metabolic cross-feeding (Walsh et al. 2023).

Although *Bifidobacterium* is frequently discussed alongside LAB in the food-fermentation and probiotic literature, the two groups are taxonomically distinct: LAB belong to the phylum Bacillota (order Lactobacillales), whereas *Bifidobacterium* belongs to the phylum Actinomycetota (family Bifidobacteriaceae) and ferments carbohydrates via the bifid shunt (fructose-6-phosphate phosphoketolase pathway) rather than the classical Embden–Meyerhof or heterofermentative pathways used by true LAB. Because

Bifidobacterium nonetheless produces lactic acid and is functionally and industrially classified together with LAB as a LA-producing, food-fermenting group, the two are frequently discussed jointly in the probiotic and fermented-food literature.

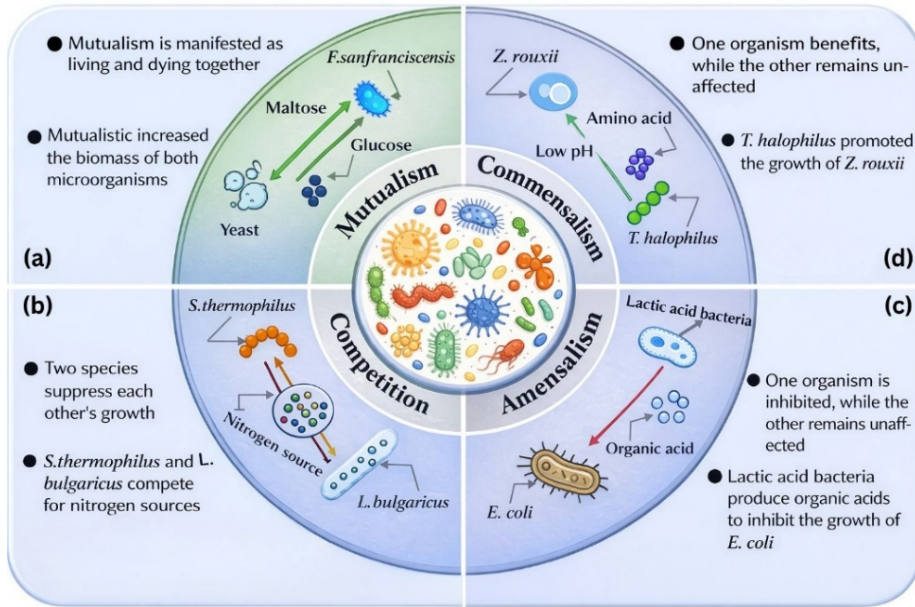


Figure 1.4. Forms of microbial interactions. (a) Mutualism. (b) Commensalism. (c) Amensalism. (d) Competition. “Green arrow” means that the growth of these microorganisms was promoted; “Red arrow” means that the growth of these microorganisms was inhibited. *F. sanfranciscensis*: *Fructilactobacillus sanfranciscensis*; *Z. rouxii*: *Zygosaccharomyces rouxii*; *T. halophilus*: *Tetragenococcus halophilus*; *E. coli*: *Escherichia coli*; *S. thermophilus*: *Streptococcus thermophilus*; and *L. bulgaricus*: *Lactobacillus delbrueckii* subsp. *Bulgaricus* (W. Liu et al. 2025b).

1.2. Functional metabolites generated during fermentation

During the process of fermentation, microorganisms produce a wide range of metabolites that contribute to food preservation, produce unique flavor/texture, enhance nutritional value, and provide health-promoting properties like antioxidant and antimicrobial effects (Şanlıer, Gökçen, and Sezgin 2019). The key functional metabolites include organic acid, bioactive compounds and peptides, extracellular polysaccharides, vitamins and nutrients, and modified polyphenols (Jyoti Prakash Tamang et al. 2020).

1.2.1 Organic acids

OA have received considerable attention in industrial applications as food additives, and in pharmaceuticals and cosmetics (Zabat et al. 2018). These are one of the primary metabolites produced during the fermentation process (Baruah, Ray, and Halami 2022; Bi et al. 2023; Castellone et al. 2021; N. Guan and Liu 2020; Şanlıer, Gökçen, and Sezgin 2019). These include LA, acetic acid, propionic acid, and butyric acid (Dittoe, Ricke, and Kiess 2018). LAB converts carbohydrates into LA using homofermentative or heterofermentative metabolic pathways, resulting in acidification of food (Akpogheli et al. 2025b). The acidification and reduction of the pH inhibit the proliferation of the pathogenic and spoilage microorganisms, thereby improving food preservation and safety (Atasoy et al. 2024). OA also improve mineral absorption by increasing mineral solubility (Hotz and Gibson 2007). This may contribute to gut health by acting as substrates for GM (Akpogheli et al. 2025b; Atasoy et al. 2024).

1.2.2. Bioactive peptides

These are short amino acid sequences released during protein hydrolysis by microbial proteases during the fermentation process (Korhonen and Pihlanto 2006). These peptides produced are inactive within the protein but become biologically active once released through enzymatic hydrolysis (Akbarian et al. 2022; Hartmann and Meisel 2007). Bioactive peptides derived from fermentation of milk and cereals products have been shown to improve antihypertensive activity (Q. Guo, Chen, and Chen 2023). This is usually due to angiotensin-converting enzyme inhibition (Q. Guo, Chen, and Chen 2023). Moreover, antioxidant activity through free radical scavenging, antimicrobial activity, and immune system modulation (Bruce, Boateng, and Boateng 2025). These peptides contribute significantly to the functional properties of FF (Samaranayaka and Li-Chan 2011).

1.2.3. Exopolysaccharides

These are extracellular polysaccharides produced by LAB and other microorganisms during fermentation (Chensha Sun et al. 2026). EPS produced by LAB contributes to the texture, viscosity stability of the FF, particularly milk products and sourdough bread (Werning et al. 2022). EPS is also known to have health benefits such as prebiotic activity, cholesterol reduction,

immune modulation, and antioxidant activity (Kusmiati et al. 2025). EPS also contributes to probiotic bacteria survival in GIT by forming protective biofilm and enhancing adhesion to IEC (Angelin and Kavitha 2020).

1.2.4. Vitamins

Numerous LAB organisms which play a role in fermentation have the capability to synthesis vitamins, particularly B group vitamins like riboflavin, (Angelin & Kavitha, 2020) lamin (Angelin and Kavitha 2020) folate, niacin, and cobalamin (Angelin and Kavitha 2020; Mladěnka et al. 2022). Fermentation by LAB can therefore increase the vitamin content of the foods and improve vitamin B2 bioavailability (Knez, Kadac-Czapska, and Grembecka 2023). This is particularly important in plant-based diets, vitamin availability may otherwise be limited (Knez, Kadac-Czapska, and Grembecka 2023). Vitamins production during fermentation contributes to the classification of FF as functional foods with enhanced nutritional value (Negrete-Romero et al. 2021).

1.2.5. Polyphenols

Polyphenols are large group of plant secondary metabolites (Bas 2026). These include phenolic acid, flavonoids, tannins, ligans, and stilbenes, widely present in fruit, vegetables, coffee, tea and plant-based FF (Saad et al. 2025). In plant-based foods these polyphenols are mostly present in bound forms like glycosides, esters, or polymers that are poorly absorbed in the human GIT (Bolat et al. 2024). These are broken down by the LAB and yeasts by the enzyme's esterases, glucosidases, phenolic acid decarboxylases into free phenolic compounds (Saad et al. 2025). Another major transformation during the fermentation process is the conversion of complex polyphenols into simpler phenolic metabolites like gallic acid, ferulic acid, vanillic acid (Bolat et al. 2024; Prabhu et al. 2021). These compounds are mainly produced through microbial hydrolysis, reduction, and dihydroxylation (N. Kumar and Goel 2019). These phenolic acids show strong antioxidant activity and have a contribution to prevent oxidative stress-related diseases (A. Kumar et al. 2025). In the gut the polyphenols are also metabolized in urolithins, phenyl propionic acids, which exert anti-inflammatory and cardioprotective effects (Saad et al. 2025).

1.2.6. Tryptophan-derived metabolites

Tryptophan is an essential aromatic amino acid that can be transformed by fermentation microorganisms into several bioactive metabolites with relevance to gut and brain health (H. Kaur, Bose, and Mande 2019)(K. Gao et al. 2020). During fermentation, LAB and other microorganisms may influence tryptophan availability either by producing tryptophan, metabolizing it into indole derivatives, or modulating pathways linked to serotonin and kynurenine metabolism (Y. Hou, Li, and Ying 2023). These metabolites are particularly important because tryptophan is a precursor for serotonin, melatonin, indole compounds, and other molecules involved in immune regulation, intestinal barrier function, and MGB axis signaling (K. Gao et al. 2020; Lukić et al. 2022; Ye et al. 2022).

Microbial tryptophan metabolism may generate indole, indole-3-lactic acid, indole-3-acetic acid, indole-3-aldehyde, and related compounds (H. Kaur, Bose, and Mande 2019)(K. Gao et al. 2020). These metabolites can interact with host receptors such as the aryl hydrocarbon receptor (AhR), contributing to epithelial barrier maintenance, mucosal immune regulation, and anti-inflammatory responses (Ye et al. 2022). Therefore, tryptophan-derived metabolites represent an important class of functional fermentation products, especially in the context of psychobiotic and immunomodulatory probiotic strains (K. Gao et al. 2020; H. Kaur, Bose, and Mande 2019; Lukić et al. 2022).

1.2.7. Bacteriocins and antimicrobial compounds

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, including many LAB strains involved in food fermentation (C. C. G. Silva, Silva, and Ribeiro 2018). These compounds inhibit the growth of closely related or pathogenic microorganisms and therefore contribute to food safety, microbial stability, and preservation (Cleveland et al. 2001; O'Connor et al. 2015). LAB-derived bacteriocins may act by disrupting bacterial cell membranes, forming pores, inhibiting cell wall biosynthesis, or interfering with essential metabolic processes (Chikindas et al. 2018; Cotter, Ross, and Hill 2013).

In FF, bacteriocin-producing LAB can suppress spoilage organisms and foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*,

Salmonella enterica, and *Escherichia coli* (Gálvez et al. 2007; C. C. G. Silva, Silva, and Ribeiro 2018). In addition to bacteriocins, LAB also produce other antimicrobial substances, including OA, hydrogen peroxide, ethanol, reuterin, and antifungal metabolites (Chikindas et al. 2018; Gálvez et al. 2007; Mduduzi Paul Mokoena 2017). These compounds lower pH, disrupt pathogen metabolism, and create an unfavorable environment for microbial contaminants (Mduduzi Paul Mokoena 2017).

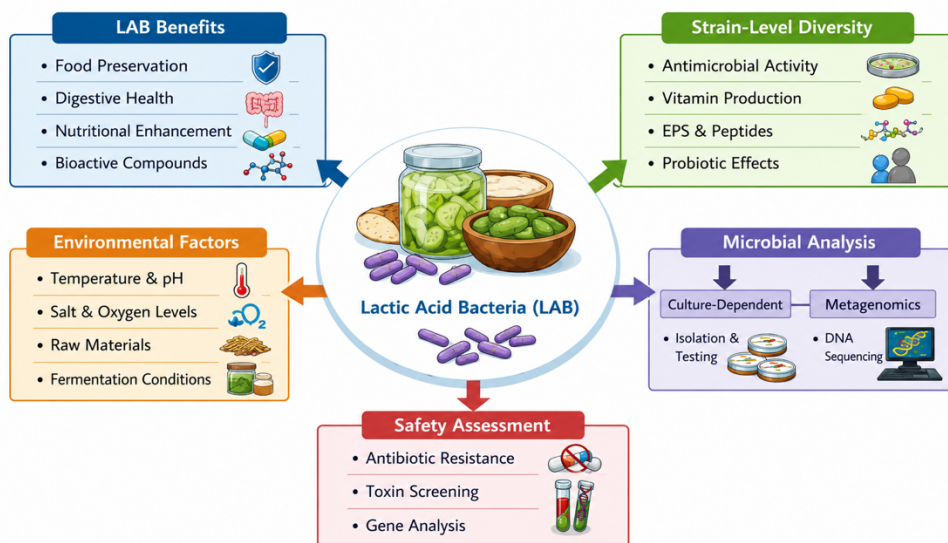


Figure 1.5. Overview of the role of beneficial bacteria LAB in FF and its selection criteria. The figure illustrates the role of LAB in FF and summarizes their fictional importance, diversity, environmental factors, safety assessments, microbial analysis and strain-level diversity. These factors highlight the main aspects related to LAB in FF. LAB benefits include food, preservation, digestive health, nutritional enhancement of food through vitamin and mineral production, and bioactive compound. The environmental factors demonstrate how temperature, pH, salt concentration, oxygen levels, raw material of the FF, and fermentation conditions have a direct influence on the microbial community composition during fermentation. The safety assessments highlight the importance of LAB strain selection and screening with antibiotic resistance, toxin, and in-depth gene analysis, to ensure safe use in food. The microbial analysis section has culture development methods, where laboratories and testing of microorganisms take place. The other is metagenomics, a culture-dependent method, where genomic analysis of entire microbial communities takes place. The strain- level diversity section exhibits that different LAB strains possess numerous beneficial functional properties and probiotic effect (The figure was made using canva.com).

The functional and technological properties of LAB are influenced by strain-level diversity and environmental factors associated with the fermentation process (Figure 1.5). Therefore, comprehensive characterization of potential probiotic LAB requires both microbial identification and safety assessment. Culture-dependent methods and molecular approaches, including DNA sequencing, can be used for the isolation and taxonomic identification of LAB, whereas genomic analyses can provide further insights into strain-specific functional characteristics. In addition, safety assessment, including the evaluation of antimicrobial resistance, potential toxin-related traits, and relevant genetic determinants, is essential when selecting LAB strains for potential probiotic and food applications (Figure 1.5).

1.3. Fermented food microbiota and their impact on host metabolism, immunity, and brain health

FF contains a wide range of microorganisms, but among them, LAB are the most important beneficial bacteria linked to food fermentation (Marco et al. 2017; Marcone, Belton, and Fitzgerald 2017; Parlindungan et al. 2021; Sampsell et al. 2025b). These beneficial LAB in FF help to maintain intestinal microbial endotoxemia (Medina-Vera et al. 2019). A condition in which microbial byproducts such as lipopolysaccharide (LPS) enter circulation and lead to low-grade inflammation and insulin resistance (Mohr et al., 2022). Certain LAB strains also produce short-chain fatty acids (SCFA) and other beneficial metabolites that improve energy balance and support metabolic signaling in host tissues (Shakya et al., 2025). Hence, FF is being studied as supportive dietary requirement in obesity, T2D, and cardiometabolic disorders (Sampsell et al., 2025a). The magnitude of the benefits depends completely on the specific microorganism, food composition, and the amount consumed (Zhang, 2022) Figure 1.6.

FF also has anti-inflammatory benefits because of beneficial bacteria residing in it (Sun, Zhu, et al., 2026; Zhang, 2022). These bacteria modulate immune response and reduce inflammatory signaling in the gut and other organs (Sun, Zhu, et al., 2026; Zhang, 2022) (Illikoud, et al., 2022). Research suggests that these bacteria and their metabolites strengthen the intestinal barrier and limit passage of pro-inflammatory cytokines into the bloodstream (Hou et al., 2022). Some strains from FF can downregulate inflammatory cytokines like tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (Hou et al., 2022; Sampsell et al., 2025c; C. Sun, Zhu, et al., 2026). This highlights that chronic

inflammation is recognized as a common biological driver in disorders such as obesity, metabolic syndrome, inflammatory bowel disease (IBD), and autoimmune conditions (Rohm et al., 2022). Hence, beneficial bacteria in FF may function as nutritional modulators that can influence the inflammatory tone of the host (C. Sun, Zhu, et al., 2026).

The FF also exhibits neurological benefits that have gained major interest because of the gut and brain connection through MGB (Porrás-García et al., 2023). This connection network involves microbiota, immune system, endocrine signaling, and neural pathways (Ullah et al., 2023). Recent studies have indicated that FF may enhance cognitive and neurological health by improving metabolites that have effects on brain function. During aging the microbial diversity is lost and increased intestinal permeability are associated with the risk of ND (O’Riordan et al., 2025)(Loh et al., 2024). FF helps in reversing these changes by introducing beneficial microbes and biologically active compounds that lead to modulation of microbiota and could reduce neuroinflammatory processes (Porrás-García et al., 2023). Numerous experimental data have linked FF intake with potential benefits in NDs like AD and PD, multiple sclerosis, depression, anxiety, and cognitive impairment (B. Kim et al., 2016). The evidence is still evolving and is getting stronger for some outcomes than others (B. Kim et al., 2016; Loh et al., 2024) Figure 1.6.

Although not all microorganisms in the FF are considered beneficial, and not every fermented product has been clinically proven to improve health benefit (Loh et al., 2024). The beneficial impact usually depends on the strain, the dose, the fermentation process, and lastly the health status of the consumer (Valentino et al., 2024). Even so, the current literature supports the ingestion of FF and its microbial metabolites that can lead to metabolic regulation, anti-inflammatory balance, and neurological well-being (Figure 1.6) (Bester et al., 2026; B. Kim et al., 2016; Loh et al., 2024). For this reason, FF has gained attention not only as traditional dietary components, but also as functional foods with a role in preventive nutrition and beneficial support for chronic disease management (Bester et al., 2026). The complex relationships between fermented food components, modulation of the GM, and their potential effects on host health are summarized in Figure 1.6, providing an integrated overview of the mechanisms discussed above.

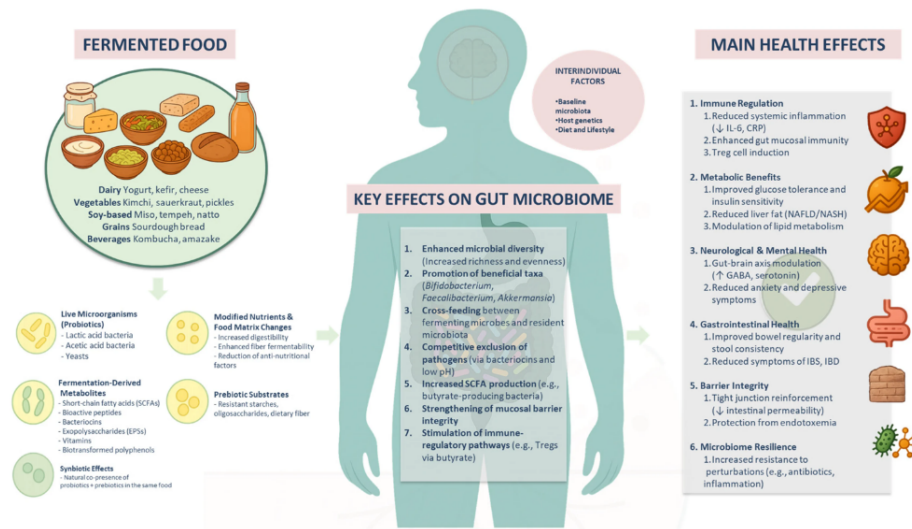


Figure 1.6. Overviews of the mechanisms through which FF influence the GM and host health. Different types of FF including dairy, vegetables, grain based, beverages deliver live beneficial microbes and fermentation derived metabolites modified nutrients, and prebiotic substances. These compounds interact with host microbiota by enhancing microbial diversity by promoting the growth of beneficial taxa and suppressing the growth of pathogen and produce SCFA production, mucosal integrity and immune modulation (I. Park & Mannaa, 2025b).

1.4. Human gut microbiota and its role in health and disease

Human GM is a complex ecosystem comprising bacteria, fungi, viruses, and protozoa that plays an important role in human health and disease (Ross et al. 2024). The GM is complexly involved in multiple aspects of host physiology throughout life, from the development and maturation of immune responses to stress response and overall behavior (Lundgren et al. 2018) (Burokas et al. 2015b). During birth and infant development, factors such as mode of delivery, feeding practices, antibiotic exposure, and environmental influences play important roles in shaping the composition and development of GM (Lundgren et al. 2018; Ross et al. 2024). In adulthood, the GM is generally characterized by relative temporal stability; however, its composition and function can still be influenced by external factors, particularly diet, medication use, lifestyle, and disease (Cryan et al. 2019; S. Salminen et al. 1998). This complex interplay among nutrients, the GM and the immune system serve as critical regulatory mechanism for preserving homeostasis and defending against external pathogens (R. K. Singh et al. 2017). Studies have shown that both short-term and long-term FF dietary regimes alter the

composition and functionality of the GM, demonstrating the power of FF in influencing human health (David et al. 2014)(Leeuwendaal, Stanton, O'toole, et al., 2022).

The ability of FF to modulate the GM has been documented by several studies with varying degrees of success (Daliri et al. 2025). Studies comparing consumers and non-consumers of plant-based FF suggest that their consumption is associated with differences in gut microbial composition, including alterations in microbial beta diversity and metabolomic profiles. FF consumption for longer duration directly modulates the GM by enhancing the performance of the host and modulation GM by increasing beneficial bacteria (Anyimadu et al. 2026). There is substantial evidence that GM dysbiosis is associated with the onset and progression of numerous adverse health conditions, including obesity, T2D, metabolic syndrome, CVD, IBD, colorectal cancer, non-alcoholic fatty liver disease, and various neurological and neuropsychiatric disorders such as depression, anxiety, AD, Parkinson's disease, and autism spectrum disorder. (Meng et al. 2020). Recent studies have stated that GM dysbiosis is also associated with metabolic syndromes like T2D and Obesity (Sasidharan Pillai et al. 2024; Sasidharan Pillai and Ashraf 2026; Shehata et al. 2026).

As GM functioning has a direct influence on host physiology, it also can affect brain functioning (Suganya and Koo 2020). It is observed that GM can positively modulate the functioning of the brain by improving cognition, behavior, neuronal development, and neuroprotective effects (Mitra et al. 2023)(Cryan 2025). This bidirectional communication is known as MGB (Suganya and Koo 2020). Studies have demonstrated that approximately 90% of the body's 5-HT is synthesized in the GIT, primarily by enterochromaffin cells. Although peripheral 5-HT does not readily cross the blood-brain barrier, GM can influence serotonergic signaling through modulation of 5-HT synthesis, tryptophan availability, and microbial metabolite production. These mechanisms may affect gut physiology, immune responses, vagal nerve signaling, and MGB communication, thereby indirectly influencing brain function and behavior (Guzel and Mirowska-Guzel 2022). In conclusion GM has a pivotal role to play in host physiology and brain functioning, stating that GM influences overall functioning of the body.

1.5. The microbiota-gut-brain axis

As aforementioned, GM is an ecological community of commensal microorganisms that live symbiotically and pathogenically in our body (Mancuso and Santangelo 2018) and plays a vital role in regulatory functions in health and disease (Burokas et al. 2015a; Burokas and Mela 2025). At the level of bacterial strains, GM demonstrates tremendous diversity and variation in microorganisms related to the age of the person and can be different in individuals (Quigley 2017).

Numerous studies indicate that GM can have an influence in synthesizing various neurotransmitters and neuromodulators, which affect gut-brain communication and brain function (Y. Chen, Xu, and Chen 2021; Taj et al. 2025). Signal transduction is complex and can have the propensity to include neural, endocrine, immune, and metabolic pathways (Che et al. 2026; Taj et al. 2025). However, its detailed mechanism and signals still must be elucidated (Taj et al. 2025). Clinical and preclinical studies have shown that gut microorganisms can produce metabolites, which affect brain functioning (Che et al. 2026; Megur et al. 2021, 2022a).

Bacteria such as *Escherichia*, *Lactobacillus*, *Saccharomyces*, and *Bacillus* can synthesize amino acids including gamma-aminobutyric acid, dopamine, butyrate, histamine, and 5-HT, which can play a significant role in emphasizing the brain activity of the individuals (Jameson and Hsiao 2018). These microbial metabolites are increasingly recognized as important mediators of MGB communication. However, the effects of these compounds on host physiology do not necessarily depend on their direct absorption into the bloodstream or transport to the brain. Instead, gut microorganisms can influence neural function through multiple mechanisms, including modulation of enteroendocrine cell activity, vagal nerve signaling, enteric nervous system interactions, immune regulation, and alterations in host neurotransmitter synthesis and metabolism (Dicks 2022). It was found that the microbiota of aged individuals with AD have a lower level of bacteria that resulted in decreased butyrate levels, which, in turn, could lead to increased inflammation in the brain and the progression of cognitive loss (Zhu et al. 2023). These findings suggest that microbiota performs numerous vital functions in our body, including releasing biochemical by-products such as SCFA and gases (Wachamo and Gaultier 2025). Moreover, animal studies conducted on pigs and rats showed an effect on memory due to microbiota, *Bacillus* and

Saccharomyces (Xu & Lu, 2025). Interestingly, a recent study has shown that microbiota transfer from human subjects with obesity led to reduced memory scores in mice, aligning this trait in humans with that of recipient mice, where RNA sequencing the medial prefrontal cortex of those mice uncovered that short-term memory is associated with aromatic amino acid pathways, inflammatory genes, and clusters of bacterial species (Arnoriaga-Rodríguez et al. 2020).

As the GIT of humans are inhabited by numerous microorganisms essential for by-product formation, it has been recently reevaluated in functional terms and different important mechanisms have been established in the bidirectional connection with the brain known as MGB (Wachamo and Gaultier 2025). MGB refers to a crosstalk between the brain and the gut involving multiple overlapping pathways, including the autonomic, neuroendocrine, vagus nerve, the immune system, or the metabolic processes of GM and immune system as well as bacterial metabolites and neuromodulatory molecules (Yassin et al. 2025). The MGB axis mirrors the constant connection between the CNS and the GIT. Several rodent studies suggest potential involvement of GM in behavioral changes (Paul and Natarajan 2025). The sympathetic and parasympathetic arms of the autonomic nervous system, including the neuroendocrine and neuroimmune systems, are known to be vital pathways in MGB (Burokas et al., 2015). The precise mechanism that arbitrates gut-brain interplay is not fully comprehended, yet it is suggested that it entails immune, endocrine, and neural pathways, leading to a possible alteration in AD patients (Paul & Natarajan, 2025). The results from a rat study showed that *Bifidobacterium infantis*, an intestinal resident microorganism, has a link to immune response in the brain (Lei et al. 2025). An augmentation in the number of *Lactobacillus casei*, *Bacteroides fragilis*, and *Streptococcus thermophilus* in the rodent intestine showed a positive effect on brain activity and performance (Lei et al. 2025). On the other hand, *Eubacterium rectale*, *Porphyromonas gingivalis*, and *Lactobacillus rhamnosus* can play a vital role in the onset of AD (Chandra, Sisodia, and Vassar 2023).

Consideration of the human GM as a substantial correspondent to nutrition, health, and disease is a relatively contemporary study, and currently, peer-reviewed studies relating modifications in GM to the etiopathology of human diseases are few (Seo and Holtzman 2024). Claims on the potential involvement of the GM in brain function are made, in part, due to the well-described pathways of communication between the brain and the GIT which

have been intensively studied around food intake, satiety, and regulation of the digestive tract (C. Chen et al. 2025).

Incorporation of certain microorganisms, such as probiotics, in diet intake can be used as a therapeutic strategy to reduce neurological disorders (Ojha et al. 2023). *Bifidobacterium* and *Lactobacillus casei* are two microorganisms which have a beneficial effect on neurological disorders (Calzada-Gonzales et al. 2025).

1.6. Concept and definition of probiotics

The WHO/FAO (Food and Agriculture Organization of the United Nations) definition of probiotics, which has been broadly acknowledged, was formulated at a joint FAO/WHO expert consultation in 2001 (Probiotics in food: health and nutritional properties and guidelines for evaluation 2006). Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO) (Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance 2012). This definition emphasizes that there are three key points: Microorganisms must be viable, present in a viable form in the product and delivered in adequate numbers at the target site, and they must beneficially affect the host (Reid, Gadir, and Dhir 2019).

This definition was reiterated by the ISAPP in their 2014 consensus statement with a small change in the wording for readability: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). ISAPP rounded off the criteria by clearly stating only strains with proven health benefits can be called probiotics (Hill et al. 2014; Marco et al. 2021). They also added that traditional fermented products with live microbes usually do not satisfy the strict requirements for the definition of probiotics, except when they contain strains already characterized with evidence (Marco et al. 2021).

Recent publications have continued to adopt the FAO/WHO definition of probiotics, emphasizing that probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts (Siddiqui et al., 2023). The importance of clear criteria that include live microbial characterization, proper dosage, and *in vivo* demonstrated health outcomes, including controlled clinical trials (Binda et al. 2020). ISAPP has also

developed consensus definitions for several related concepts, including prebiotics, synbiotics, and postbiotics. Prebiotics are substrates that are selectively utilized by host microorganisms and confer a health benefit, whereas synbiotics are mixtures comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confer a health benefit. Postbiotics are preparations of inanimate microorganisms and/or their components that confer a health benefit on the host. Together, these concepts represent complementary approaches to modulating GM and promoting host health. (The ISAPP quick guide to probiotics for health professionals: History, efficacy, and safety A resource prepared by the International Scientific Association for Probiotics and Prebiotics 2019). All these terms share a requirement for proven health benefit but differ in chemical structure, composition, and mechanisms (Marco et al. 2021).

1.7. Lactic acid bacteria with probiotic potential

As aforementioned, LAB represents a diverse group of microorganisms characterized by their potential to ferment carbohydrates primarily into LA (Akpogheli et al. 2025a). LAB comprises a functionally defined group of Gram-positive, non-spore-forming, acid-tolerant bacteria, predominantly belonging to the phylum Bacillota (formerly Firmicutes) and the order Lactobacillales. Representative LAB genera include *Lactiplantibacillus*, *Lacticaseibacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus*, many members of which are associated with FF and GIT. However, the genera *Enterococcus* and *Streptococcus* also include opportunistic and pathogenic species and strains; therefore, their presence in these genera does not inherently indicate probiotic potential or safety (Akpogheli et al. 2025a; Sionek et al. 2023a). Isolation of LAB from such foods has led to the discovery of novel strains with remarkable probiotic features (Nandha and Shukla 2023). These strains features include tolerance to GI stress conditions and production of bioactive metabolites (Uhegwu and Anumudu 2025). Indeed, recent research has demonstrated the LAB isolated from FF displays strong antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (Lee et al., 2021). Moreover, screening of Lithuanian FF has revealed multiple LAB strains capable of surviving static GIT conditions while also demonstrating functional probiotic traits (Megur et al., 2023). These strains also exhibit resistance to low pH, bile sats, and digestive enzymes, which are crucial properties for

survival within the GIT (Megur et al., 2023). Furthermore, the selected isolates have exhibited significant antimicrobial activity and adhesion ability towards intestinal epithelial cell lines, suggesting their potential for gut colonization (Megur et al., 2023).

1.7.1 *Pediococcus pentosaceus*

Several *Pediococcus pentosaceus* strains are already used commercially as probiotics and food-fermentation starters, valued for their antimicrobial activity, GIT tolerance, and generally favourable safety profile, though not every strain performs consistently *in vivo* and safety screening remains necessary on a strain-by-strain basis.

Pediococcus is a genus of LAB which is Gram-positive, nonmotile, and non-spore forming (Qi et al. 2021). It belongs to the family of *Lactobacillaceae* (Jiang et al. 2021; Qi et al. 2021). The morphology of these bacteria are mostly cocci, often occurring in pairs or in tetrads, formed when cell division is in two perpendicular planes (Qi et al. 2021). *P. pentosaceus* colonies range in size (1.0-2.5 mm in diameter), with smooth, round, and greyish white morphology (J. S. Lee et al. 2021; Qi et al. 2021). They are mostly facultative anaerobes, catalase-negative, and homofermentative. The optimum pH of growth of this genus is 6.0-6.5 (Jiang et al. 2021). They primarily produce LA as the dominant fermentation byproduct (Jiang et al. 2021). The genus includes only four species that play an important role in fermentation: *Pediococcus damnosus*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, and *Pediococcus inopinatus* (Lin et al. 2019). All these species grow at 30°C, but the temperature range is 25-40°C (Lin et al. 2019; Mullan 2014). Within these strains *P. pentosaceus* is one of the most favorable strains for probiotic and industrial application (Lin et al. 2019; Mullan 2014).

Pediococcus species are found in various traditional FF across vegetables, fruits, cereal, meat and dairy products (Raccach 2014). They play an essential role in fermentation of kimchi, sauerkraut, cheese and sourdough (Y. Zhang et al. 2025). They contribute to acidification which preserves these foods by spoilage bacteria and can enhance flavor and texture development. The *P. pentosaceus* strain is frequently isolated from fermented vegetables, fruits and dairy products like kefir (Balakrishnan and Agrawal 2014). This strain has also been isolated from meat fermentation and cereal based FF. This isolation

from different niches highlights *P. pentosaceus* broad ecological niche in FF microbes (Raccach 2014).

P. pentosaceus attracts attention for its antagonist activity towards the pathogenic bacteria (Adnan et al. 2021). It possesses antimicrobial mechanisms including bacteriocin secretion, organic acid production which reduces the expression of VF (Megur, Simoliunas, and Burokas 2025). It also competes with the pathogenic bacteria for epithelial adhesion sites, and aggregates with pathogens that reduce their ability to colonize the host and food sources (Adnan et al. 2021). These mechanisms of the *P. pentosaceus* explain its use as a biopreservative in foods (Megur, Simoliunas, and Burokas 2025).

P. pentosaceus has been studied a lot in laboratories. It is reported to have beneficial effects on hosts in many ways (Hu et al. 2021). It suppresses the inflammation by making our gut healthier, modulating the bacteria composition in our gut, exhibiting protective effects (Hu et al. 2021). For example, the strain *P. pentosaceus* LI05 administered to mice with colitis, helped them by modulating immunological profiles, GM and elevated SCFA (Bian et al. 2020). Other types of *P. pentosaceus* have been shown to help animals fight bacteria like *Clostridioides difficile*, *Salmonella* and fish-pathogen infections (Bian et al. 2020; Hu et al. 2021; Lan et al. 2020). This shows that different types of *P. pentosaceus* can have strain specific effects on the host (Balakrishnan and Agrawal 2014; Bian et al. 2020; Hu et al. 2021; Lan et al. 2020).

There is limited research on humans but still notable. A randomized, double blinded placebo-controlled trial administered *P. pentosaceus* LP28 to subjects who were overweight. This strain administered helped subjects to lose weight. This beneficial effect suggests translational potential beyond food application (Higashikawa et al. 2016). However, not every strain that looks promising *in vitro* will be beneficial *in vivo* applications. One study on mice showed that the strain *P. pentosaceus* 40 made a *Salmonella* infection worse (Silva et al., 2017). This is important to remember: not all types of *P. pentosaceus* strains are beneficial and there is a need for careful selection and safety evaluation (Silva et al., 2017).

1.7.2. *Lactiplantibacillus plantarum*

Lactiplantibacillus plantarum is one of the most extensively commercialised LAB species in probiotic and FF applications worldwide, valued for its broad environmental adaptability, antimicrobial and antioxidant activity, and reported metabolic and immune benefits, although strain-to-strain variability in antibiotic resistance and safety profile means individual isolates still require dedicated evaluation.

Among the different types of LAB, *L. plantarum* is one of the most extensively studied bacteria (Z. Zhang et al. 2025). It is considered as a Gram-positive, rod-shaped, facultatively anaerobic bacteria. These possess broad pH and temperature tolerance and have relatively large genome for LAB species (Yilmaz et al. 2022). The strains are metabolically versatile and can utilize many types of carbohydrates (Yilmaz et al. 2022). This explains their presence in versatile environments including complex plants, food, and host-microbiome (Echegaray et al. 2023). Food-derived *L. plantarum* strains have shown extensive resistance and adhesion in GIT and excellent antioxidant and antimicrobial properties (Echegaray et al. 2023). *L. plantarum* has a QPS from EFSA. In this hostile environment, numerous strains of *L. plantarum* have been shown to be potential probiotics due to their pH and bile tolerance achieved through various strategies such as maintenance of intracellular pH homeostasis (Yadav, Puniya, and Shukla 2016). It rapidly recycles damaged proteins, and induction of several stress response pathways, including bile salt hydrolase activity and mechanisms involved in maintaining the proton motive force (Yadav, Puniya, and Shukla 2016). *L. plantarum* MA2 strain and B23 strain showed excellent tolerance and could survive at low pH (2.5–3). The strain *L. plantarum* GCC 19M1 displayed significant tolerance to low pH conditions (Xiong et al. 2025). Moreover, this strain exhibited survival rates that ranged between 93.48% and 96.97% when exposed to simulated gastric juice (pH=3) (Xiong et al. 2025). The strain GCC 19M1 was also found to be significantly tolerant to 0.3% bile, 0.5% pancreatin, and 5% NaCl (Nath et al., 2020). Another strain, *L. plantarum* SJ14 shown superior tolerance to acid and bile-like human GI tract conditions (Metrouh et al. 2022). Strains of *L. plantarum* isolated from fermented vegetables, pickles and kimchi (KACC11451 and Wikim0112) demonstrated excellent GI environment resistance (Jeong et al. 2021).

Adherence to the mucus of the epithelial cells or adherence to the components of the extracellular matrix of the GIT are desired characteristics in probiotic microorganism because they will favor the colonization of the probiotic in the host (Gorreja and Walker 2022). Moreover, the adhesion of the bacteria in the intestinal tract also contributes to the inhibition of pathogens colonization and improves overall mucus layer healing (Gorreja and Walker 2022). In this context, various research has focused on studying the adherence capacity of *L. plantarum* in *in vitro* conditions. In a study where the cell adhesion assay on human IEC revealed that the extent of adhesion to HT-29 IEC of *L. plantarum* DKL3 and JGR2 was similar to that of the probiotic strain *Lactacaseibacillus rhamnosus* GG (Surve, Shinde, and Kulkarni 2022). Thus, both *L. plantarum* strains showed adherence extents of 82.8% and 79.6%, respectively. These findings further demonstrate the strain-dependent nature of adhesion among *L. plantarum*, with the tested strains exhibiting relatively high adherence compared with other strains reported in the literature (Surve, Shinde, and Kulkarni 2022). In other studies, it demonstrated through the analysis of self-coagulation that *L. plantarum* DMDL 9010 strain had good adhesion to the intestinal epithelium, which could be related to the presence of genes that encode different proteins attributed to adhesion to different extracellular matrices and intestinal mucus (D. M. Liu, Huang, and Liang 2022).

L. plantarum also exhibits antioxidant activity, reducing the damage caused by oxidation reactions (Jing Wang et al. 2021). These bacteria display antioxidant agents through different mechanisms, among which are the metal ion chelating ability, antioxidant enzymes system, antioxidant metabolites, mediation of antioxidant signaling pathways, regulation of enzymes that produce reactive oxygen species and through the modulation of the GM (Jing Wang et al. 2021). For instance, *L. plantarum* DMDL 9010 showed an excellent antioxidant capacity through the superoxide anion scavenging activity method, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with the supernatants showing a higher antioxidant capacity than bacterial precipitates (D. M. Liu, Huang, and Liang 2022). Another strain *L. plantarum* MA2 had a high antioxidant capacity through the cellular antioxidant activity test, highlighting the antioxidant capacity of cell-free extract of the logarithmic phase compared to cell-free extract of the stationary phase and fermentation supernatant of the logarithmic phase (W. Tang et al. 2018).

For the food industrial application *L. plantatrum* produced bacteriocin, to produce antimicrobial activity against foodborne pathogens and spoilage

bacteria (Mduduzi P. Mokoena, Omatola, and Olaniran 2021). Many strains of *L. plantarum* have shown to be capable of producing bacteriocins (Y. Dai, Wang, and Xu 2026). *L. plantarum* generates a bacteriocin normally referred to as plantaricin, which belongs to class I (lantibiotic) and class II (non-lantibiotic) bacteriocins (Y. Dai, Wang, and Xu 2026). Given that bacteriocins produced from certain *L. plantarum* strains have been researched to have antimicrobial activities against foodborne spoilage and pathogens bacteria, this microorganism may have probiotic potential and deserve further investigation to be able to increase their use in the food industry (Y. Dai, Wang, and Xu 2026).

Clinical and preclinical studies have shown that *L. plantarum* benefits are strain specific (Chatsirisakul et al. 2025). In a systematic review and meta-analysis, *L. plantarum* supplementation improved periodontal health, reduced GIT disorders, reduced cholesterol and LDL in CVD (Chatsirisakul et al. 2025). The consumption of *L. plantarum* improves the function of the intestinal epithelial barrier and reduces the symptoms of IBS. *L. plantarum* plays a main role in modulating visceral sensitivity, which is the main cause of abdominal pain in IBS patients (Yucui Zhao et al. 2024). Moreover, *L. plantarum* may influence intestinal motility through interactions with the enteric nervous system and enteroendocrine cells, leading to altered production of neuroactive compounds such as 5-HT and short-chain fatty acids SCFA, which regulate smooth muscle contraction, gut transit, and gut-brain signaling pathways (Berding and Cryan 2022; Cowan and Cryan 2021; Dinan, Stanton, and Cryan 2013). *L. plantarum* 299V improved symptoms of pain and bloating in IBS (Ducrotté, Sawant, and Jayanthi 2012). A study on *L. plantarum* LP01 in combination with *B. breve* BR 03 or *L. acidophilus* LA 02 to improve IBS symptoms showed that the combination of these strains led to a significant reduction in symptoms (Saggioro 2004).

There has been an increasing knowledge about the strain-specific diversity, probiotic properties, and health benefits of different *L. plantarum* strains can be of great importance.

1.7.3. *Lacticaseibacillus paracasei*

Members of the *Lacticaseibacillus paracasei* group are among the most widely commercialised LAB probiotics, used across fermented dairy and synbiotic products for their documented immunomodulatory, gut-barrier-

supportive, and metabolite-producing properties, with an overall safety record considered favourable by EFSA QPS status, though individual strains still require case-by-case screening for transferable resistance and virulence determinants.

L. paracasei belongs to the genus *Lactobacillus*. It is a Gram-positive, rod-shaped, and facultative anaerobic bacteria that performs LA fermentation (“Supragingival Microbes,” 2015). This species has flexible metabolic ability, which allows it to utilize a wide range of carbohydrates (Roy 2019). It can produce by product LA from hexoses, lactate and acetate from pentoses and its optimal growth temperature range (10-37 °C), which makes it a perfect strain for human intestine survival (Bouki et al. 2020). Due to its wide range of carbohydrate utilization ability, *L. paracasei* is a species identified in various FF (C. H. Huang et al. 2018). And it has been widely explored for its novel health benefits, making it a popular genus among gut commensal bacteria (Syahniar, Wahid, and Yasmon 2026). *L. paracasei* is second most employed probiotic species in human applications (Colautti et al. 2024) (Syahniar, Wahid, and Yasmon 2026). It comprises diverse unique strains with a long history of safe use in food uses that have been studied for their health-promoting characteristics (C. H. Huang et al. 2018).

According to various studies, *L. paracasei* can help break down food by digesting long chain carbohydrates, improve nutritional absorption in the food as well as gut, and potentially relieve diarrhea symptoms (Syahniar, Wahid, and Yasmon 2026). *L. paracasei* can also improve gut barrier function, elevate mucus layer, and reduce intestinal permeability, which is critical for preventing dangerous bacteria translocation causing inflammation triggered diseases (N. Li et al. 2025). Moreover, *L. paracasei* administration can assist in controlling the immune system, perhaps lowering inflammation and boosting the body’s ability to fight infectious diseases (Bosco et al. 2011; C. Li et al. 2023; N. Li et al. 2025; Tian et al. 2025). Some strains of *L. paracasei* have shown to increase antibody (IgA) production and promote the development of regulatory T cells, which help to modulate the immunological response in the host (Bosco et al. 2011; C. Li et al. 2023; Tian et al. 2025). One of the strains of *L. paracasei* subsp. *paracasei* NTU 101 is known to attenuate skin inflammation and symptoms of allergic reactions in human trials (C. F. Liu et al. 2024). Some strains of *L. paracasei* have the potential to lower cholesterol levels, for example, *L. paracasei* TISTR 2593 (Khongrum et al. 2023). Administration of the *L. paracasei* TISTR 2593 reduces obesity

via altering adipogenesis (Khongrum et al. 2023). Whereas in another study *L. paracasei* 8700:2 supplementation has shown to lower triglycerides, reduce the severity of metabolic syndrome, and delay weight gain (Syahniar, Wahid, and Yasmon 2026). Another research demonstrated that the *L. paracasei* NL41 strain might have the potential to reduce insulin resistance and oxidative stress while also maintaining beta cell function (Zeng et al. 2019).

L. paracasei also has attracted increasing attention for its beneficial effect on the MGB signaling. It improves the behavior, cognition, and the stress response system (Ansari et al. 2023). In randomized controlled trials *L. paracasei* Lpc-37 and *L. paracasei* K56 reduced stress-related biomarkers (Y. Guan et al. 2025). The application of the *L. paracasei* promotes a healthier intestinal environment, strengthens the gut barrier, and reduces inflammation, all of which are important for proper signaling of gut-brain axis (Ansari et al. 2023). Some studies in recent years have also indicate that *L. paracasei* strains can affect the hypothalamic-pituitary-adrenal axis, which plays a central role in the body's response to stress (Patterson et al. 2020). Specific strains of *L. paracasei* modulates neurotransmitter-related pathways which include 5-HT, gamma-aminobutyric acid (GABA), glutamate receptors, suggesting a possible mechanism for neuroactive effects (Patterson et al. 2020). In preclinical studies the supplementation with *L. paracasei* strains directly improves the anxiety-like and depressive-like behaviors, supporting its role in mental well-being (Karen et al., 2021). Although human studies and application of *L. paracasei* is still limited, but some strains have the potential to reduce stress, improve sleep quality, and psychological comfort. These effects are mostly strain specific (Wu et al. 2025). Not all strains of *L. paracasei* produce gut-brain benefits. To confirm its potential in humans, large-scale clinical trials are needed to know its efficiency and investigate its mechanisms involved.

Other than *L. paracasei* mental benefits, it also may have beneficial effects on diabetes, especially in treatment of T2D (S. Chen and Zhang 2023). In clinical and preclinical trials, it can improve glycemic control, have effect on insulin resistance, inflammation, and gut barrier function (S. Chen and Zhang 2023; S. Li et al. 2024; Zeng et al. 2021). In one randomized, double-blind, placebo-controlled clinical trial, administration with *L. paracasei* HII01 for 12 weeks significantly reduced fasting blood glucose and lowered pro-inflammatory markers in patients with T2D, while also increasing beneficial GM and improving SCFA (Toeijing et al. 2021). In another clinical study in diabetic

participants reported that *L. paracasei* HII01 supplementation improved hyperglycemia by favorably modifying the GM and reducing endotoxemia, which supports the idea that part of its antidiabetic action may occur through the gut-liver axis (Toeijing et al. 2021). Preclinical studies in diabetic mice, *L. paracasei* TD062 improved fasting and postprandial blood glucose, glucose tolerance, hepatic glycogen, lipid metabolism, antioxidant capacity, and expression of glucose-metabolism genes, including pathways linked to PI3K/Akt signaling (Dang et al. 2018). In another study, *L. paracasei* N1115 and L14 have shown protective effects in diabetic rodent models by reducing insulin resistance, improving glucose tolerance, lowering oxidative stress, and restoring GM balance (Zeng et al. 2023). In more recent study it also found that *L. paracasei* Glu-07 improved glucose uptake in HepG2 cells and reduced hyperglycemia and hyperlipidemia in diabetic mice (W. Xu et al. 2025). These findings suggest that *L. paracasei* may support gut health, diabetes and brain health management through multiple mechanisms.

1.7.4. *Limosilactobacillus fermentum*

Several *Limosilactobacillus fermentum* strains are already marketed or studied as probiotics in fermented dairy and plant-based products, recognised for antimicrobial activity, antioxidant capacity, and adhesion to intestinal epithelium, with safety concerns generally limited to strain-specific antibiotic-resistance carriage rather than the species as a whole.

L. fermentum is a Gram-positive, non-spore-forming, catalase-negative type of LAB that belongs to the family *Lactobacillaceae* (Roy 2019). It is now reclassified within the genus *Limosilactobacillus* after taxonomic reorganization based on genomics (Wei et al. 2025). It is a rod-shaped, acid-producing bacteria that can grow in diverse ecological niches (de Luna Freire et al. 2024). Mostly *L. fermentum* strains are isolated from different types of FF, human milk, the GIT, saliva, feces of animals and humans, and the female urogenital tract (Asan-Ozusaglam and Gunyakti 2019; Yan Zhao et al. 2022). A key microbiological feature of *L. fermentum* is its ability to tolerate the harsh GIT environment (Yan Zhao et al. 2022). The *L. fermentum* CECT5716 strain, showed survival even under very low pH conditions and demonstrated strong GIT persistence, adhesion to IEC, and production of metabolites such as LA, SCFA, and antioxidant compounds (Rodríguez-Sojo et al. 2021). Different strains of *L. fermentum* also vary in their probiotic properties, which includes bile salt tolerance and antimicrobial activity (Yan Zhao et al. 2022)(Klayraung

and Okonogi 2009). It also produces beneficial molecules such as glutathione, folate, and riboflavin, making them highly strain-specific in probiotic characterization properties (Yan Zhao et al. 2022).

L. fermentum has been associated with health benefits which show antimicrobial, anti-inflammatory, antioxidant, immunomodulatory and anti-metabolic effects (Yan Zhao et al. 2019). It produces organic compounds, bacteriocin-like activity, and adhesion to IEC, supporting barrier functioning and elimination of pathogenic bacteria (H. Kim et al. 2022). *L. fermentum* has antimicrobial effect on pathogens like *Salmonella*, *S. aureus*, *Listeria*, *E. coli*, *C. jejuni*, and *C. albicans* (Y. Zheng et al. 2025). Strains *L. fermentum* ME-3 and CECT5716 show robust antioxidant effects which can participate in glutathione-related redox cycling and help reduce oxidative stress (Rodríguez-Sojo et al. 2021). *L. fermentum* strains also possess immunomodulatory effect by influencing cytokine expression, mucosal barrier support, support T-regulatory cell activity and IgA responses (Garcia-Castillo et al. 2019). These mechanisms can link potential benefits in IBD, respiratory infections, CVD risk, and metabolic disorders such as obesity and T2D (Garcia-Castillo et al. 2019). The strains of *L. fermentum* isolated from human milk have a possible role in supporting immune development and maintaining gastrointestinal health through modulation of the GM, enhancement of intestinal barrier function, and regulation of host immune responses (Asan-Ozusaglam and Gunyakti 2019).

In preclinical studies *L. fermentum* CECT5716 reduced inflammatory mediators and restored mucin production (Rodríguez-Sojo et al. 2021). Moreover, it improved tight-junction proteins such as occludin and ZO-1, and corrected dysbiosis in models of colitis (Rodríguez-Sojo et al. 2021). In metabolic syndrome and diabetic models, the strain *L. fermentum* CECT5716 improved glucose and insulin levels, reduced hepatic steatosis, normalized lipid profiles, and enhanced glucagon-like peptide-1 (GLP-1)-related pathways (Rivero-Gutiérrez et al. 2017). The strain *L. fermentum* CECT5716 used in LPS challenged models revealed that the mice showed lower TNF- α , better glutathione status, and less organ injury after LPS exposure (Ozen, Piloquet, and Schaubeck 2023). In another study with strain *L. fermentum* NCMR 2826 and T2D rats lowered serum total cholesterol, reduced fatty liver changes, and modulated GM by increasing *Lactobacillus* and *Bifidobacterium* while decreasing *E. coli* (Q. Li et al. 2025).

Among the clinically investigated strains, *L. fermentum* CECT5716 has been primarily studied for its potential immunomodulatory effects, particularly in infants. Clinical studies have reported associations with reduced respiratory infections, enhanced immune responses following vaccination, and a favorable safety profile in formula-fed infants (Ozen, Piloquet, and Schaubeck 2023). These findings distinguish CECT5716 from strains investigated predominantly for gastrointestinal or metabolic outcomes and further demonstrate the strain-specific nature of probiotic effects. In another study, *L. fermentum* ME-3 has been associated with improved antioxidant effect and a better CVD and diabetes risk profile, including reduced total cholesterol (Kullisaar et al. 2016). The strain *L. fermentum* JDFM216 administration in older adults improved cognitive behavior, immune responses and GM composition (M. R. Park et al. 2020). These trials confirm the safety of *L. fermentum* as a probiotic strain across groups of infants and adults. Effects of numerous isolates of *L. fermentum* remain strain-specific, and larger trials are needed for its applications.

1.7.5. *Leuconostoc mesenteroides*

A number of *Leuconostoc mesenteroides* strains are already applied commercially as starter cultures and probiotic candidates in fermented vegetables and dairy products, valued primarily for exopolysaccharide and dextran production, heterofermentative organic-acid output, and a generally favourable safety profile, although concerns around limited GIT survival and strain-dependent antibiotic resistance have been raised in some studies.

L. mesenteroides is Gram-positive spherical to ovoid shaped cells measuring 0.5-0.7 μm in diameter and 0.7-1.2 μm in length (Soetaert et al., 1995). It is facultatively anaerobic, non-motile, non-spore-forming, and thriving optimally at 20-30°C (Dekker and Frank 2002). *L. mesenteroides* metabolize glucose via phosphoketolase pathway, producing lactate, ethanol, CO₂ and acetate (K. W. Lee et al. 2020). Its genome averages 1.9 Mbp with 37.8% GC content (Dekker and Frank 2002; K. W. Lee et al. 2020; Zeid et al. 2025). Usually, *L. mesenteroides* strains are found on plant surfaces and in FF like kimchi, sauerkraut, and cheese, known for its pH lowering properties to inhibit food spoilage microorganisms (K. W. Lee et al. 2020; Zeid et al. 2025).

In GM *L. mesenteroides* leads to production of SCFA like acetate, propionate which enhances microbiota diversity with *Bacteroidetes* and

Verrucomicrobia. It leads to increase in *Akkermansia* by gut modulation, improving glucose tolerance, insulin sensitivity and reducing obesity markers (Hayeeawaema et al. 2025; H. S. Jo, Cho, and Hong 2025). Heat-Killed *L. mesenteroides* H40 alleviates scopolamine-induced cognitive impairment in mice by reducing neuroinflammation, boosting brain-derived neurotrophic factor, and antioxidants (N. K. Lee et al., 2025). In another study *L. mesenteroides* GBUT-21 strain inhibited SARS-CoV-2 replication and inflammation *in vitro*, suggesting antiviral potential (Alzahrani et al. 2022). Similarly, *L. mesenteroides* WHH1141 strain ameliorated food allergy in OVA-sensitized mice by normalizing Th2 cytokines, IgE, and GM with beneficial microorganisms (K. Gao et al. 2023). In a HFD induced obesity in male mice, supplementation with *L. mesenteroides* subsp. *Mesenteroides* SD23 improved metabolic parameters (Castro-Rodríguez et al. 2020). The KS-TN11 strain exhibited antibacterial against fish pathogens, α -glucosidase inhibition for antidiabetic potential (Paray et al. 2018).

Human clinical trials specifically on *L. mesenteroides* as a probiotic are limited, with most evidence from observational cases of infections rather than intentional interventions (N. K. Lee, Lee, Won, et al. 2025). Although in randomized, double-blind, placebo-controlled trial in patients with Japanese cedar pollinosis for 16-week supplementation with NTM048 subsp. Strain RCT improved nasal blockage symptoms without significantly raising salivary s-IgA, suggesting localized immunomodulation (Yamamoto et al. 2023). The psychological status hinted at benefits at higher doses (Yamamoto et al. 2023).

1.8. Prebiotics

Prebiotics are a class of nutritional compounds categorized together, not necessarily by structural affinity, but by the potential to promote the growth and/or activity of specific beneficial bacteria in the GM. The concept of prebiotics came into recognition due to Glenn Gibson and Marcel Roberfroid in 1995 (G R Gibson and Roberfroid 1995). A prebiotic is known as “a non-digestible food constituent that beneficially influences the host by selectively promoting the growth and/or activity of one or a restricted number of bacteria in the colon and thus improving the host health”(Seppo Salminen et al. 2021). In 2004, prebiotics were upgraded to include four criteria: (1) resistance to hydrolysis by mammalian enzymes, gastric acidity, and GIT absorption; (2) they should only be fermented by GM; (3) induce systemic or luminal effects

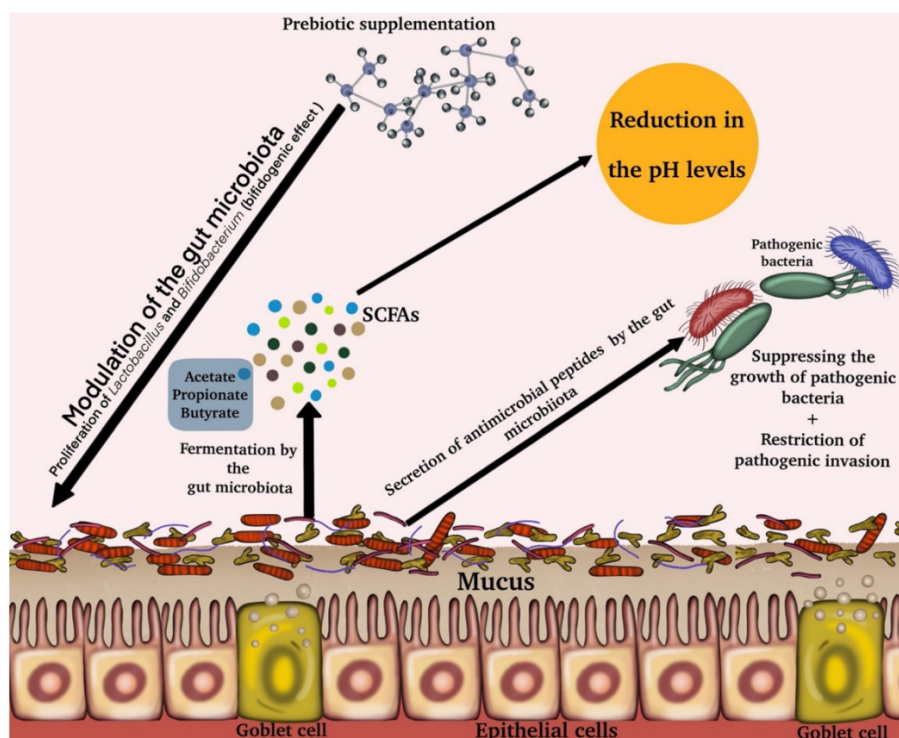


Figure 1.7. Mechanism of action of prebiotic supplementation. Prebiotic administration in a regular diet increases bacterial growth and functionality of specific species or genera, leading to modulation of the GM and showing a strong bifidogenic effect. Goblet cells play a key role in the production of mucus, which helps to protect the mucous membrane and form a layer in the colon that helps to reduce the inflammation caused by the bacterial interaction with IEC. The modulated GM ferments prebiotics to form SCFA (acetate, propionate, and butyrate), from which health benefits can be accrued. The production of antimicrobial agents and the reduction in the pH levels of the intestine due to prebiotic supplementation can suppress and restrict the growth of pathogenic bacteria, which can lead to positive health effects (Megur et al., 2022b).

that are beneficial to host health; and (4) selectively stimulate the growth and activity of GM associated with health and well-being (Holscher 2017). The health benefits of prebiotics are diverse and include immune modulation through increased immune-regulatory interleukins and intestinal-specific immunoglobulins; reduction of pro-inflammatory interleukins (Shokryazdan et al. 2017)(Pluta et al. 2020); and production of SCFA such as acetate, propionate, and butyrate (Macfarlane and Macfarlane 2011)(Figure 1.6). SCFA are carboxylic acids with aliphatic tails of one to six carbons that are

produced by anaerobic fermentation of dietary fibers in the intestine by the GM (Venegas et al. 2019). SCFA are an important indicator of bacterial fermentation in the colon and are known to improve gut health by maintaining intestinal barrier integrity (Peng et al. 2009), mucus production (Gaudier et al. 2009), protection against inflammation, and reduction in colorectal cancer and obesity (O’Keefe 2016).

1.8.1. Inulin

Inulin-type prebiotics are members of an immense group called “fructans”. Fructans constitute a group of compounds that confine all naturally occurring plant oligosaccharides and polysaccharides in which one or more fructosyl–fructose linkages form most glycosidic bonds (Cardoso et al. 2021). Hence, they are the primarily polymers of fructose units. Fructans can also be characterized by the DP, which refers to the number of repeated units in a polymer or oligomer chain (Kelly 2009). The category of fructans consists of inulin and oligofructose (FOS) (C. K. Yeung et al. 2005) .

Inulin is a collective term that comprises all linear fructans with β (2→1) fructosyl–fructose glycosidic bonds (Ibrahim 2018); this specific type of glycosidic bond gives inulin its distinctive physiological and structural properties. Inulin-type fructans resist enzymatic hydrolysis by small intestine digestive enzymes and human salivary enzymes because of the beta configuration bonds between fructose monomers (Roberfroid, 2007). Chemically, the linear chain of inulin is either an α -D-glucopyranosyl-[β -D-fructofuranosyl](n-1)- β -D-fructofuranoside (GpyFn) or $\alpha\beta$ -D-fructopyranosyl-[β -D-fructofuranosyl](n-1)- β -D-fructofuranoside (FpyFn) (Cui and Roberts 2009). Unlike digestible carbohydrates, inulin selectively stimulates beneficial members of the GM, particularly bacteria capable of utilizing fructans as an energy source. The most consistently reported effect of inulin supplementation is a bifidogenic response, characterized by increased abundance of *Bifidobacterium* species, including *Bifidobacterium adolescentis*, *B. longum*, and *B. breve*. In addition, inulin-type fructans can promote the growth of other beneficial SCFA producing bacteria, including members of the genera *Anaerostipes*, *Faecalibacterium*, and *Bacteroides* (Aldubayan et al. 2023; Nagy et al. 2023). A systematic review and meta-analysis demonstrated that chicory-derived inulin-type fructans significantly increased fecal *Bifidobacterium* abundance and improved bowel function, confirming their role as microbiota-targeted dietary components (Nagy et al.

2023). The metabolic effects of inulin are strongly associated with microbiota remodeling and SCFA production. Inulin supplementation has been reported to improve glucose homeostasis, insulin sensitivity, lipid metabolism, and inflammatory status, suggesting potential applications in metabolic disorders such as obesity and type 2 diabetes. A study involving individuals with overweight, or obesity showed that inulin-type fructan supplementation altered GM composition and influenced cardiometabolic parameters, demonstrating the interaction between dietary fibers, microbial metabolism, and host physiology (Aldubayan et al. 2023). The prebiotic properties of inulin are particularly relevant for probiotic development because it can enhance the survival, growth, and functional activity of beneficial microorganisms. When combined with probiotic strains, inulin can act as a selective substrate that supports probiotic colonization and increases the production of beneficial metabolites, forming a synbiotic combination (Patel et al. 2025). Clinical studies have demonstrated that synbiotic formulations containing inulin-type fructans together with probiotic strains such as *Lactobacillus* and *Bifidobacterium* can increase beneficial bacterial populations, favor SCFA-producing microbial communities, and improve markers related to gut immune function (De Giani et al. 2022). Therefore, inulin is not only a structural dietary fiber but also a functional prebiotic component capable of modulating microbiota composition, enhancing probiotic activity, and supporting metabolic and immune health.

1.8.2. Fructo-oligosaccharide

Another type of fructans i.e., FOS, is a natural component that can be found in plants (Chatterjee and Ojiambo 2014). FOS are commercially prepared from chicory in a hydrolysis reaction using inulinase and may also be derived in an enzymatic synthetic reaction via the transfer of fructosyl units from sucrose molecules (Korczak & Slavin, 2018). When presented structurally, FOS consist of a sucrose molecule linked by a chain of 3–30 fructosyl units. FOS are oligomeric linear fructans with β -(2–1) or β -(2–6) fructosyl-fructose linkages with the first monomer of the chain either being α -D- glucopyranosyl or β - D -fructopyranosyl residue (Ibrahim 2018). The DP of inulin is up to 60 and the DP of FOS is less than 10 (Yun, 1996).

The primary biological effect of FOS is the selective stimulation of beneficial saccharolytic bacteria, particularly members of the genus *Bifidobacterium* (Dou et al. 2022). Several human intervention studies have demonstrated that

FOS supplementation consistently increases the abundance of *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium catenulatum*, which possess carbohydrate-active enzymes enabling efficient fructan utilization (Mao et al. 2018). In addition, FOS can promote the growth of other beneficial microbial groups, including *Lactobacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Faecalibacterium prausnitzii*, and *Anaerostipes* species, which contribute to intestinal homeostasis and SCFA production (Scott, De Paepe, and Van de Wiele 2022). A randomized controlled trial showed that daily consumption of 5 g FOS for 4 weeks significantly increased fecal *Bifidobacterium* levels compared with placebo, confirming the bifidogenic activity of FOS at relatively low dietary doses (Liu et al., 2022).

Following microbial fermentation, FOS serves as a substrate for bacterial production of SCFA, mainly acetate, propionate, and butyrate. *Bifidobacterium* species primarily produce acetate and lactate, which can subsequently be cross-fed to butyrate-producing bacteria such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Anaerostipes caccae* (Markowiak-Kopeć and Ślizewska 2020). This microbial cross-feeding mechanism enhances butyrate production, which is essential for maintaining intestinal epithelial integrity, regulating immune responses, and reducing inflammatory signaling (M. T. Siddiqui and Cresci 2021). Butyrate acts as the primary energy source for colonocytes and promotes expression of tight-junction proteins, thereby improving intestinal barrier function (Saban Güler et al. 2025).

FOS-induced microbiota modulation is also associated with metabolic benefits. Increased abundance of SCFA-producing bacteria after FOS supplementation contributes to improved glucose regulation, lipid metabolism, and appetite control through activation of free fatty acid receptors (FFAR2 and FFAR3), stimulation of gut hormones such as GLP-1 and peptide YY, and reduction of systemic inflammation (Nogal, Valdes, and Menni 2021). In individuals with overweight or metabolic disturbances, supplementation with 10–15 g/day FOS has been reported to improve insulin sensitivity, increase beneficial microbial taxa, and reduce markers associated with metabolic dysfunction (Mahalak et al. 2023). Additionally, FOS supplementation may decrease intestinal colonization by potentially harmful bacteria by increasing competitive exclusion through beneficial microbes and lowering intestinal pH via organic acid production.

The prebiotic properties of FOS are highly relevant for probiotic applications because they can enhance probiotic survival, persistence, and functionality within the gastrointestinal tract (Rodrigues et al. 2011). FOS can act as a selective growth substrate for probiotic strains belonging to *Lactobacillus* and *Bifidobacterium*, improving their metabolic activity and increasing production of beneficial compounds (Mahalak et al. 2023). Synbiotic combinations of FOS with probiotic strains such as *Lactobacillus rhamnosus* GG, *Lactocaseibacillus paracasei*, and *Bifidobacterium animalis* subsp. *lactis* have demonstrated enhanced modulation of GM compared with probiotics alone (Castro-Herrera et al. 2021). For example, supplementation with synbiotic formulations containing FOS (approximately 5–10 g/day) together with probiotic bacteria has been associated with increased abundance of beneficial bacteria, improved intestinal barrier function, and enhanced SCFA production (Procopciuc, Hangan, and Lucaciu 2026; Roupar et al. 2022).

1.8.3. Galacto-oligosaccharide

GOS are the product of lactose extension and are included among non-digestible oligosaccharides. They are arranged in two subgroups: (i) with excess galactose at C₃, C₄ and C₆; and (ii) manufactured from lactose through enzymatic trans-glycosylation (Sako & Tanaka, 2011). The mixture of the product depends on the reaction conditions and the enzymes used. β -galactosidase of various origins, such as *Aspergillus oryzae*, *Bacillus circulans*, and *Cryptococcus laurentii*, is used for the industrial production of GOS (Crittenden and Playne 1996). The general constituents of this oligosaccharide are from tri- to penta-saccharide with β (1→6), β (1→3), and β (1→4) linkages (Figure 1.8). This category of GOS is known as trans-GOS (Megur et al., 2022). Culture studies of *Bifidobacteria* and most of *Lactobacilli* and enterobacteria, including some streptococci-metabolized trans-oligosaccharide, with *Bifidobacteria* showed robust growth. There are some GOS derived from the isomers of lactose, due to influential factors such as the source of the enzyme, temperature, pH, and substrate concentration. They are also considered as prebiotics (Mahoney 1998a).

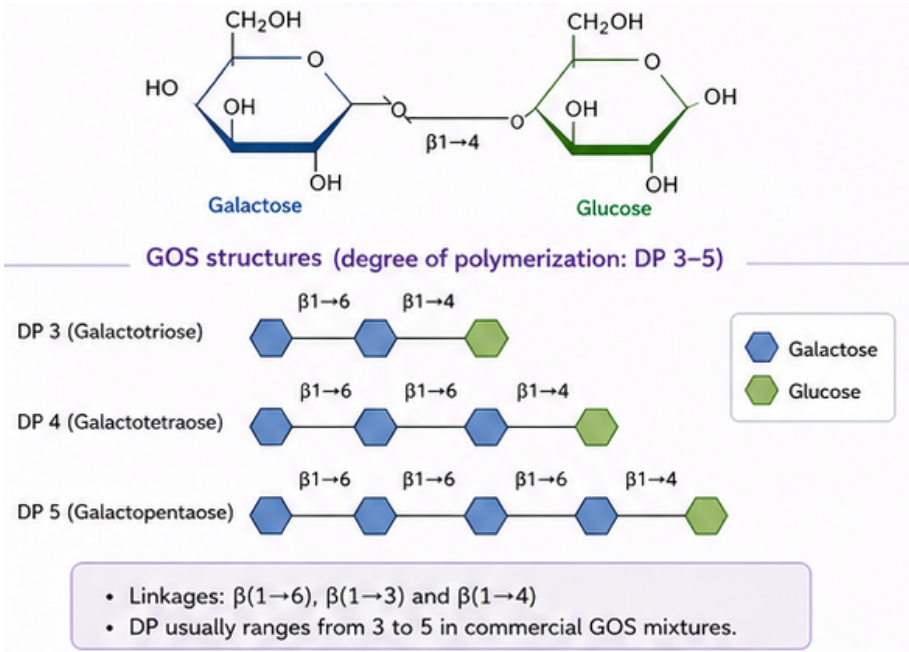


Figure 1.8. Structure of GOS. GOS are non-digestible carbohydrates composed of one or more D-galactose residues linked to a terminal lactose molecule through β -glycosidic bonds, predominantly $\beta(1 \rightarrow 6)$, $\beta(1 \rightarrow 3)$, and $\beta(1 \rightarrow 4)$ linkages. Commercial GOS mixtures typically consist of oligosaccharides with a degree of polymerization ranging from 3 to 5.

The prebiotic effects of GOS are primarily attributed to their selective utilization by beneficial GM. Among the most responsive taxa are members of the genus *Bifidobacterium*, including *Bifidobacterium longum*, *B. adolescentis*, *B. breve*, and *B. bifidum*, which possess specialized glycosidases and carbohydrate transport systems that enable efficient GOS metabolism (Ignatova et al. 2025). Supplementation with GOS has also been shown to stimulate the growth of several lactobacilli, including *Lactocaseibacillus paracasei*, *Lactiplantibacillus plantarum*, and *Lactocaseibacillus rhamnosus*, thereby promoting a more beneficial gut microbial community (Yongchao Wang et al. 2025). Human intervention studies have consistently demonstrated that GOS supplementation induces a bifidogenic effect, resulting in increased abundance of *Bifidobacterium* species and, to a lesser extent, lactobacilli (Harthoorn et al. 2025). Daily supplementation with 5–15 g GOS has been reported to increase beneficial bacterial populations while reducing the relative abundance of potentially pathogenic taxa through competitive exclusion and environmental acidification (Angima et al. 2025; Mei, Yuan, and Li 2022).

Fermentation of GOS by gut microorganisms results in the production of SCFA, particularly acetate, propionate, and butyrate (Mahoney 1998b). *Bifidobacteria* primarily generate acetate and lactate through the fructose-6-phosphate phosphoketolase pathway, while cross-feeding interactions allow butyrate-producing bacteria such as *Faecalibacterium prausnitzii*, *Anaerostipes spp.*, and *Eubacterium rectale* to utilize these metabolites for butyrate synthesis (Wilson and Whelan 2017). Consequently, GOS supplementation promotes an increase in total SCFA concentrations and enhances metabolic activity within the intestinal ecosystem (Chang et al. 2022). The increased production of SCFA contributes to several beneficial physiological effects. Acetate and propionate participate in lipid and glucose metabolism through activation of free fatty acid receptors (FFAR2 and FFAR3), whereas butyrate serves as the primary energy source for colonocytes and promotes intestinal barrier integrity through upregulation of tight-junction proteins (Y. Du et al. 2024). Furthermore, SCFA exert anti-inflammatory effects by regulating immune cell activity and reducing the expression of pro-inflammatory cytokines (X. F. Liu et al. 2023).

Beyond GM modulation, GOS supplementation has been associated with improvements in metabolic health, including enhanced insulin sensitivity, improved glucose homeostasis, and reduced low-grade inflammation (Mistry et al. 2020). These effects are thought to arise from the combined actions of microbiota remodeling, increased SCFA production, and improved intestinal barrier function (Morrison and Preston 2016). Emerging evidence also suggests that GOS may influence MGB axis signaling through microbial metabolite production and modulation of tryptophan metabolism (X. F. Liu et al. 2023; Mistry et al. 2020; Morrison and Preston 2016).

1.8.4. Prebiotics for the Treatment of Obesity and Diabetes

Globally, the population of diabetes patients is increasing, imposing a great social and economic burden on public health (Guariguata et al. 2014). T2D is a chronic metabolic syndrome of abnormal lipid and glucose metabolism that leads to neuropathy, retinopathy, leg ulcers, and gangrene (Nathan 1993). The factors that could have an impact on T2D development are obesity, genetics, smoking, age, hypertension, and sedentary lifestyle (Sherwin et al. 2003). In recent studies, it has been proposed that the remodeling of the GM composition from obesity could lead to the pathogenesis of T2D (Ley et al. 2006a, 2006b; Moya-Pérez et al. 2017; Rabot et al. 2010; Yde et al. 2021).

The two dominant bacteria groups in human GIT are *Bacteroidetes* and *Firmicutes* (Ley et al. 2006b). A link between obesity and GM composition has been reported in humans, showing an increase in the number of *Firmicutes* and a decrease in the diversity of *Bacteroidetes* (Snellson et al. 2021) (Figure 2).

Prebiotics have gained a considerable place in the management of obesity and diabetes due to their ability to modulate GM composition, thereby affecting the status of GIT and exerting anti-diabetic effects (Tajabadi-Ebrahimi et al. 2017)(H.-Y. Li et al. 2021). As prebiotics consist of different forms, their supplementation can be considered as a dietary therapy for the prevention and treatment of T2D (Vezza et al. 2020), and in the fight against obesity by affecting food intake and appetite and metabolic activities (Kang et al. 2023; Maioli et al. 2021) (Figure 2).

FOS have numerous desirable characteristics such as low calories, safety for diabetics, no carcinogenicity, and bifidus-stimulating functionality (Yun 1996b). Due to these properties, FOS are considered a functional food ingredient that improves health status (Y. A. Kim, Keogh, and Clifton 2018). Increasing studies demonstrated the functional properties of FOS including the reduction of blood glucose levels, cholesterol levels, and lowering of blood pressure (Dehghan, Pourghassem Gargari, and Asghari Jafar-abadi 2014). Moreover, Clinical studies using 5–15 g/day FOS have demonstrated improvements in gut microbial composition, SCFA production, glucose metabolism, lipid profiles, and inflammatory markers, supporting its application in obesity and metabolic syndrome management (Yu, Sun, and Fu 2025).

Meanwhile, inulin as a prebiotic has shown mixed results on the glycemic scale (Aliasgharzadeh et al. 2015). A study carried out on 54 middle-aged (between 35 and 65 years) healthy adults (men and women) as a double-blind, randomized, placebo-controlled parallel groups with 10 g of inulin supplementation for 8 weeks did not show any significant changes in the body weight (Jackson et al. 1999). A decrease in plasma insulin level was observed after 4 weeks of treatment and remained lower up to the 8th week, along with lower plasma triglyceride concentrations. The total cholesterol was lower in the inulin-supplemented group when compared to the placebo group. The study concluded that inulin supplementation may influence the degradation of triglyceride-rich lipoprotein particles Jackson et al., 1999). In human trials

conducted on obese women treated with inulin, greater proportions of *Bifidobacterium* and *Faecalibacterium* were observed, an effect that coincided with reduced fat mass and serum LPS (Dewulf et al. 2013). The important role of *Bifidobacterium* in the fight against obesity has recently been demonstrated by *B. longum* both in preclinical obesity models and in humans (Schellekens et al. 2021). Inulin supplementation has consistently been associated with enrichment of *Bifidobacterium adolescentis*, *B. longum*, *Faecalibacterium prausnitzii*, and *Anaerostipes* species (Moens, Weckx, and De Vuyst 2016). These microbial changes increase acetate and butyrate production through primary fermentation and cross-feeding mechanisms (Morrison and Preston 2016). Increased SCFA concentrations have been linked to improved insulin sensitivity, enhanced secretion of GLP-1 and peptide YY, reduced metabolic endotoxemia, and improved glucose regulation in overweight and obese individuals (Münte and Hartmann 2025). Intervention study administering 10–16 g/day inulin for 6–12 weeks ported increased bifidobacterial abundance together with improvements in inflammatory and metabolic markers (Baba et al. 2023).

In summary, prebiotics show efficiency not only in modulating or restructuring and stabilizing the host microbiome, but also in the regulation of many mechanisms associated with the development and metabolic consequences of obesity. Furthermore, prebiotics should be enriched in popular foods, increasing the chances of consistent consumption and improving overall health. At least, dietary prebiotic supplementation represents a safe, well-tolerated, and inexpensive therapeutic approach and should be considered as a potential therapy for the treatment and prevention of T2D and obesity (Figure 1.9).

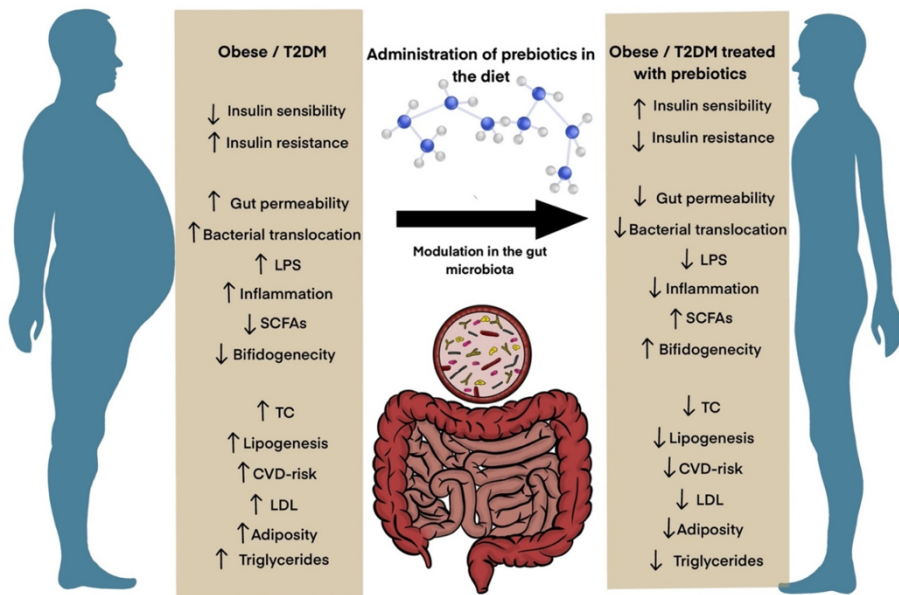


Figure 1.9. An overview of the improvement in the health of obese/T2D patients treated by modulating their GM using prebiotics supplementation in a regular diet. Administration of prebiotics has the potential to modulate GM composition in patients suffering from T2D and obesity and can be used as a therapeutic approach to cure the adverse effects of metabolic diseases. The daily intake of prebiotics in a designed diet has a major influence on GM by decreasing gut permeability, bacterial translocation, and reducing LPS-induced inflammation. However, this diet increases SCFA and bifidogenecity in the gut, leading to lower total cholesterol levels, lipogenesis, LDL triglycerides, and adiposity, eventually resulting in lower risk of CVD (Megur et al. 2022a).

1.9. Synbiotics

Synbiotics are defined as combination of probiotics and prebiotics that selectively modulate and enhance metabolic activity of the beneficial bacteria in the gut, thereby conferring health benefits to the host (Parhi, Liu, and Choo 2024). This combination concept evolved from simple approach to a more targeted strategy. Synbiotics was highlighted by the ISAPP, emphasizing functional synergy between compounds (Swanson et al. 2020). Synbiotic administration exerts their effect on the host through multiple mechanisms, including modulation of GM composition, elevated production of SCFA, immunomodulation, and regulation of metabolic pathways (Smolinska, Popescu, and Zemelka-Wiacek 2025). Synbiotics enhance the integrity of the intestinal barrier by stimulating the production of tight junction proteins and

mucus production. Hence, thereby preventing the translocation of harmful bacteria and endotoxins into the bloodstream (Smolinska, Popescu, and Zemelka-Wiacek 2025).

Preclinical studies have provided an important insight into the efficiency of synbiotic administration. In a study using Sprague–Dawley rats, synbiotic supplementation of a blend consisting of 1 billion CFU of probiotic bacteria combined with 100 mg of fucose (a type of FOS) demonstrated significant protective effects against antibiotic-induced dysbiosis (Sarangarajan et al. 2024). The results show restoration of GM balance, improvement in intestinal morphology, confirming the safety and therapeutic potential of synbiotics in GM disruption models (Sarangarajan et al. 2024). Similarly, a study on broiler chickens revealed that synbiotic formulations of 2×10^9 CFU g⁻¹ LAB of *Lactobacillus* spp., 2×10^7 CFU g⁻¹ of *Saccharomyces cerevisiae* yeast and 2% inulin significantly altered GM composition, increased beneficial bacterial populations, and enhanced the production of SCFA (Ślizewska et al. 2020). These changes by the supplementation are crucial for gut health and metabolic regulation, ultimately improving growth performance and metabolic efficiency (Ślizewska et al. 2020). In a study conducted on canine models, synbiotic supplementation of 50 mg of inulin and 20 billion live total CFU, administration resulted in significant shifts in GM diversity demonstrating synbiotics potential in actively restructuring the GM even in healthy subjects (Tanprasertsuk et al. 2021). Moreover, systematic analyses of animal models on age-related disorders suggests that synbiotics administration contribute to improved muscle mass, reduced frailty, and better overall metabolic outcomes, supporting their role in modulating systemic physiological processes beyond just gut modulation (Lashkarbolouk et al. 2025).

In clinical trials, synbiotic administration has shown potential in reducing disease and a reliable therapeutic application. In a randomized double blinded, placebo-controlled trial, multi-strain synbiotic, a combination of five *Lactobacillus* and *Bifidobacterium* strains alongside short-chain fructooligosaccharides, administration for 12 weeks significant reduction in IBS symptom severity, their relief and improved abdominal pain (Sommermeier et al. 2024). In multiple randomized clinical trial synbiotic combination of formulations containing *Lactobacillus* or *Bifidobacterium* paired with FOS, application showed potential in early metabolic disease management. It improved glucose metabolism, reduced insulin resistance (X. Wang et al. 2021). Another study in pregnant women with gestational diabetes

synbiotic of five probiotics with inulin application provided metabolic benefits by improving glycemic control and reducing insulin resistance markers (Zhou et al. 2021).

In conclusion, synbiotics represent a promising therapeutic approach to improving human health through targeted modulation of GM. By combining the beneficial effects of probiotics and prebiotics, synbiotics offer a synergistic strategy that enhances microbial survival, promotes beneficial metabolic activity, and supports host physiological functions.

1.10. Diet-induced metabolic syndrome

Diet plays a major role in physiological functioning and metabolism in rodents and human studies (Rodríguez-Correa et al. 2020). Diet-induced metabolic syndrome displays a cluster of cardiometabolic risk agents by overconsumption of energy-dense diets (Rodríguez-Correa et al. 2020). These diets are particularly rich in saturated fats, refined sugars, and fructose (Gancheva, Galunska, and Zhelyazkova-Savova 2017). These diet consumptions manifest as a central obesity, dyslipidemia, hypertension, and insulin resistance, often leading to T2D, obesity, and CVD in preclinical and clinical cohorts (Alam, Kim, and Jang 2022). Recent studies emphasize the role of GM modulation, dysbiosis, and adipose tissue remodeling as a key driver (Dasriya et al. 2024). This makes it crucial for the investigation into dietary intervention (Pandit, Meganathan, and Vedagiri 2025).

1.10.1. High-fat-diet

HFD is typically defined as food sources providing 45-75% of total calories from fat (saturated fats like palmitate and lard) (Fan et al. 2024). Chronic consumption of a HFD induces profound metabolic disturbances in rodent models, including obesity, insulin resistance, glucose intolerance, dyslipidemia, and metabolic syndrome, typically developing within 4–18 weeks depending on diet composition and study design. HFD models are widely used because they reproduce several metabolic abnormalities observed in humans consuming Western-style diets characterized by high fat and energy intake, including increased visceral adiposity, impaired glucose homeostasis, and progression toward T2D (Akowuah et al. 2023; Burokas et al. 2018; Espinosa-Carrasco et al. 2018). These adverse effects include multi-organ crosstalk, which involves lipid overload, oxidative stress, inflammation, and

non-beneficial GM shifts (Akowuah et al., 2023; Beltran et al., 2025; Fan et al., 2024). All these considerations make HFD a major culprit in diet-induced metabolic diseases and ND (Plantera et al. 2025; Uranga et al. 2010).

Consumption of HFD surpasses the physiological fat intake typical of standard diets, eventually leading to overload in intestinal absorption through the transporters CD36 and FATP4 (Maulana and Ridwan 2021). This overload of the HFD results in acute elevation of chylomicrons in the postprandial phase and an immediate accumulation of hepatic triglycerides (X. Jia et al. 2023). The saturated fats, like palmitic acid, make up to 30-50% of the lipids in HFD, while decreasing the abundance of polysaturated fatty acids, which include omega-3 fatty acids like docosahexaenoic acid and eicosapentaenoic acid (Denisenko et al. 2020). These adverse shifts affect the membrane fluidity and disruption of the balance of eicosanoids (Kahle et al. 2015). HFDs consumption over the short period, inhibit the phosphorylation of hypothalamic AMPK α (B. Wang and Cheng 2018), which in turn results in an increase in orexigenic neuropeptides neuropeptide Y and agouti-related peptide, diminishing satiety signals from pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript (Beck 2006; Maulana and Ridwan 2021). This alteration leads to an increase in caloric intake 20-50%, promotes hyperphagia (X. Jia et al. 2023). Additionally, enteric neurons detect lipids through G protein-coupled receptor 40 and G protein-coupled receptor 120, prolonging gastric emptying (Nikolla et al. 2025). This mechanism also inadvertently enhances pathways through the downregulation of dopamine D2 receptors in the nucleus accumbens (Oladun, Mall, and Kim 2025).

These interconnected metabolic, neuroendocrine, and inflammatory alterations illustrate the complexity of HFD-induced metabolic dysfunction and provide the pathophysiological basis for the experimental model used in this dissertation. Increasing evidence also indicates that the GM contributes to HFD-associated metabolic disturbances, providing a rationale for investigating microbiota-targeted interventions, including probiotics, prebiotics, and synbiotics.

This dissertation demonstrates that traditional Lithuanian FF represent a valuable source of probiotic LAB with desirable functional and safety characteristics. Selected strains exhibited synbiotic interactions with prebiotic substrates and enhanced production of bioactive metabolites. Furthermore, *Lacticaseibacillus paracasei* 11W, particularly in combination with GOS,

improved metabolic, inflammatory, behavioral, and GM outcomes in a high-fat diet mouse model. Collectively, these findings support the development of novel synbiotic approaches derived from traditional Lithuanian FF for the management of metabolic and neuroinflammatory disorders.

2. MATERIALS AND METHODS

This work involves *in vitro* and *in vivo* studies. In the *in vitro* section, isolation of probiotic LAB from FF, characterization and genomic analysis, as well as through safety assessment of selected LAB strains were conducted. The strains genome was compared using previously published different types of LAB genomes in NCBI database. Desirable strains were selected and proceeded with preclinical testing in mice model. After *in vitro* selection of the novel probiotic strain, the studies were then proceeded to *in vivo* application. The preclinical trial was based on mice, where HFD induced obesity and diabetes were induced. Then the probiotic and synbiotic combination was administrated to investigate which supplementation has a therapeutic benefit on mice. The details of the materials, reagents, equipment and biological materials and mentioned in supplementary materials section.

2.1. Sample isolation, collection and incubation

Strains were isolated in 2021 from different FF collected at Halės Turgus market (Halle Market, Vilnius, Lithuania) and local homemade FF. The FF selected were traditional Lithuanian sauerkraut, kombucha, cheese, yogurt, and cucumber pickles. Each sample (1 g or 1 mL) was carefully placed into separate sterile test tubes containing 9 mL of sterile 0.1% peptone water (Sigma-Aldrich, Poznań, Poland). Aliquots of 10 µL from appropriate 10⁸ CFU/mL dilution were pentagonally streaked on the pre-solidified de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Wesel, Germany) and incubated at 35 °C for 48–72 h under aerobic conditions. Representative colonies of LAB were randomly picked and purified by repeated streak plating on MRS agar until pure colonies were obtained. Pure bacterial cultures were maintained on MRS agar plates and subcultured every 3 weeks until imperative for characterization. Cell morphology and colonial characterization were observed on MRS agar. These isolates were stored and preserved in a –80 °C deep freezer (Froilabo, Livingston, United Kingdom) at the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Centre, Vilnius University, Vilnius, Lithuania.

2.1.1. Bacterial and mammalian cell culture conditions

Bacterial isolates were routinely maintained on MRS agar and in MRS broth (Oxoid, Wesel, Germany) at 35–37 °C under aerobic conditions and

subcultured every 3 weeks, as described in Section 2.1; for individual assays, working cultures were prepared as 18–24 h subcultures as specified in the corresponding subsection.

The human colonic cell lines HCT116 were procured from the Department of Biological Models, Vilnius University, Lithuania. The cells were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; ThermoFisher Scientific, Lithuania) supplemented with 10% (v/v) heat-inactivated (30min, 56°C) fetal bovine serum (Thermo Fisher Scientific, Lithuania). The cells in a medium were also supplemented with a 1% (v/v) penicillin streptomycin (Thermo Fisher Scientific, Lithuania) solution to a final concentration of 100U/mL–1 penicillin and 100µg/mL–1 streptomycin. The incubation was at 37°C in an atmosphere of 5% CO₂ and 95% air. The cells were nourished with complete DMEM every alternate day until the cells reached 75–80% confluency.

2.2. Survival *in vitro* gastrointestinal conditions

2.2.1 Resistance to low pH

The resistance of LAB to low pH was studied. Briefly, LAB cultures incubated at 37 °C for 24 h were centrifuged at 10,000 g for 10 min. The pellets were suspended in sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, Poland) and adjusted to a pH of 2.0 using 1 M HCl. The mixture was then incubated at 37 °C for 4 h. Aliquots were taken at 0 and after 4 h. The samples were serially diluted in peptone water, and the viable cells were determined by the spread plate method using MRS agar. The plates were incubated at 37 °C for 24 h, and the percentage survival of the bacteria was calculated as follows:

$$\% \text{ Survival} = (\text{CFU of viable cells survived}) / (\text{CFU of initial viable cells inoculated}) \times 100$$

2.2.2 Resistance to pepsin

To test the viability in the presence of pepsin, simulated gastric juice was prepared by suspending 3 mg/mL pepsin (Sigma- Aldrich, Poland) in sterile peptone water (w/v) and adjusted to pH 2.0. The fluid was inoculated with active cultures at an inoculum size of 1% (v/v) and incubated at 37 °C for 4 h. The spread plate method determined the viable cells before (T1) and after

incubation (T2) (Megur et al., 2023). The percentage survival of the bacteria was calculated according to resistance to low pH.

$$\% \text{ Survival} = (\text{CFU of viable cells survived}) / (\text{CFU of initial viable cells inoculated}) \times 100$$

2.2.3 Resistance to bile salts

Resistance to intestinal juices was tested as reported (Megur et al., 2023). Briefly, 0.3% (w/v) bile salt (Sigma- Aldrich, Poland) was dissolved in sterile peptone water (w/v) and adjusted to pH 8 cell-free culture supernatants. The fluid was inoculated with 1% (v/v) LAB cultures and incubated at 37 °C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated according to the equation below.

$$\% \text{ Survival} = (\text{CFU of viable cells survived}) / (\text{CFU of initial viable cells inoculated}) \times 100$$

2.2.4 Resistance to pancreatin

Resistance to intestinal juices was tested as reported (Wu et al., 2023). Briefly, 1mg/mL (Sigma-Aldrich, Poland) of pancreatin was dissolved in sterile peptone water (w/v) and adjusted to pH 7.2 cell-free culture supernatants. The fluid was inoculated with 1% (v/v) LAB cultures and incubated at 37 °C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated according to the equation below.

$$\% \text{ Survival} = (\text{CFU of viable cells survived}) / (\text{CFU of initial viable cells inoculated}) \times 100$$

2.3 DNA extraction and lactic acid bacteria genome identification

LAB strains were grown overnight in MRS broth (Oxoid, Wesel, Germany) at 35 °C. The QIAGEN DNeasy PowerSoil Pro Kit (Promega, France) was used to isolate DNA from the bacteria following the manufacturer's protocol. The quantity of DNA in the samples was measured using the QuantiFluor® dsDNA System with the GloMax Plate Reader System (Promega, France). To

prepare the DNA libraries, Nextera XT DNA Library Preparation Kit (Illumina, Poland) was used coupled with IDT Unique Dual Indexes. An amount of 1 ng DNA was used as input. The genomic DNA was fragmented using Illumina Nextera XT fragmentation enzyme and a unique dual index was added to the sample. The library was then constructed using 12 cycles of PCR. The DNA library was purified using AMPure magnetic beads and eluted in QIAGEN EB buffer. The quantity of DNA in the library was measured with a Qubit 4 fluorometer and a Qubit dsDNA HS Assay Kit. The library was then sequenced with the Illumina NovaSeq platform with 2×150 bp reads.

2.4. Gut colonization ability of lactic acid bacteria isolates

2.4.1 Probiotic antimicrobial activity

Antibacterial activity was determined using the agar well diffusion test as previously described (Chaichana et al., 2025). *Staphylococcus aureus* ATCC 29213, *S. Typhimurium* ATCC 14028, *Streptococcus pyogenes* ATCC 12384, *Streptococcus pyogenes* ATCC 19615, and *Klebsiella pneumoniae* ATCC 13883 were obtained from the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Center, Vilnius University and were used as indicator strains for the detection of antimicrobial activity. The LAB was cultured in 3 mL MRS broth medium and incubated for 24 h at 37 °C. The MRS broth tubes were centrifuged (10000 g for 10 min) to prepare cell-free culture supernatants (CFS). The pH values of the supernatants were adjusted to approximately 7.2 by adding NaOH. A suspension of 100 µL of 10^7 CFU/mL of each pathogenic strain was then prepared and spread onto the nutrient agar, into which 5-mm-deep wells had been dug. Approximately 100 µL of CFS was poured into each well, and nutrient agar plates were incubated for 24 h to 48 h at 37 °C. Finally, the inhibition zone diameter was measured in millimeters (mm).

2.4.2. Auto-aggregation

The overnight selected LAB culture was centrifuged at 10000 g for 10 min to harvest the cell pellets. Pellets were washed thrice with PBS (pH 7.4) and re-suspended in PBS, and the initial absorbance was noted at 600 nm. The bacterial suspension was incubated at 37 °C for 24 h, and the final absorbance of the supernatant was measured at 600 nm at three different times: 4 h, 12 h, and 24 h. The formula measured the percentage of cellular auto-aggregation:

$$\% \text{ Survival} = (\text{CFU of viable cells survived}) / (\text{CFU of initial viable cells inoculated}) \times 100$$

2.4.3 Co-aggregation

Equal volumes of cells (1.5 mL) of the different LAB and pathogen strains were mixed, vortexed for 10s, and incubated at 37 °C. The absorbance was determined by the mixture and the bacterial suspensions alone. Coaggregation was calculated as follows:

$$\text{Coaggregation (\%)} = \{[(\text{Apat} + \text{Aprobio})/2 - \text{Amix}] / (\text{Apat} + \text{Aprobio})\} \times 100$$

where Apat and Aprobio represent A600 nm of the separate bacterial suspensions in control tubes and Amix represents the absorbance of the mixed bacterial suspension at 2 h.

2.4.4 Percentage adhesion on HCT116 cells

The human colonic cancer cell lines HCT116 were obtained from the Department of Biological Models, Institute of Biochemistry, Life Sciences Center, Vilnius University, Lithuania. The cell concentration in the monolayer was determined by trypsinizing the adhered cells with 3 mL of 0.25% trypsin–EDTA solution for 5–10 min at 37 °C. The final cell count in suspension was quantified with the help of a hemocytometer (Sigma-Aldrich, Poznan, Poland). For the adhesion assay, HCT116 cells were seeded individually in each well of standard 12-well tissue culture plates at a concentration of 1×10^7 cells/mL and incubated for 48 h or more, or until a complete monolayer was formed. The medium was changed every 24–48h. Before the adhesion assay, the spent medium was completely removed after 24 h and cells were fed with DMEM lacking antibiotics. The LAB isolates intended for adhesion assay were propagated in MRS broth and cultures obtained after 18h of growth at 37 °C were centrifuged at 6000×g for 10 min. The pellet was rinsed once with PBS (pH 7.4). The cell density was adjusted to the desired levels by measuring the absorbance at 600 nm. The precise number of viable bacteria used in the assay was determined by plate counting on MRS agar.

The adhesion of LAB isolates was measured as described previously (Megur et al. 2023a) with few modifications. The HCT 116 cells in a monolayer were washed twice with 3mL of PBS (pH 7.6). The 2 mL of DMEM without serum and antibiotics was added to each well and incubated at 37 °C for 40 min before inoculation of bacteria. Different LAB isolates with a concentration of approximately 1×10^7 CFU suspended in 1 mL DMEM without serum and antibiotics were used to inoculate each well of tissue culture plates. The plates were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air for 3 h.

The monolayer was washed five times with sterile PBS (pH 7.4) to remove non-adherent bacteria. To enumerate the viable adhered bacteria, the cells from the monolayer were detached by trypsinization. Each well was treated with 1mL of 0.25% trypsin–EDTA solution and incubated for 15 min at room temperature. The suspension of lysed cells and LAB was serially diluted with saline solution and plated on MRS agar. The enumeration was done after 48h of incubation at 37 °C in an anaerobic atmosphere by plate counting method. The adhesion was expressed as the percentage of the number of bacteria adhered to the total bacteria used for the experiment and calculated as:

$$\% \text{ Adhesion} = (\text{Initial count of bacteria})/(\text{Final count of bacteria}) \times 100$$

2.4.5. Trolox equivalent antioxidant capacity

The scavenging effect of the CFS on a DPPH radical was performed. Briefly, the scavenging ratio of the sample and Trolox (Abcam, Cambridge, United Kingdom) on DPPH (Abcam, Cambridge, UK) at the same time was tested, and then a suitable concentration range of the Trolox and its scavenging percentage was found. Then, a linear regression equation between the Trolox concentration and its scavenging percentage was built, and the Trolox equivalent antioxidant capacity (TEAC) was calculated through the equation. A higher TEAC value meant higher DPPH scavenging activity. Meanwhile, the scavenging percentage on the DPPH radical of the sample solution was tested following the treatment of the Trolox solution. The scavenging effect on the DPPH radical of the samples could be calculated as the Trolox equivalent's antioxidant capacity from the calibration curve: $y = -0.0298x + 0.9995$.

2.5 Influence of prebiotics on the growth of strains

Prebiotic influence on LAB growth was tested as described previously with slight modifications (Megur et al., 2023). Briefly, LAB cultures incubated at 37 °C for 24 h were administered in the MRS broth containing prebiotic 5% GOS and 5% FOS, i.e., the lowest concentration that elicited a significant increase in the growth of LAB. The LAB growth was noted at every 4 h interval at 37 °C by measuring absorbance at 600 nm. The optical densities were measured using a spectrometer (Eppendorf Bio spectrometer®, Hamburg, Germany). The initial optical density value of the media was deducted from the final value for each test sample.

2.6 Cell-free culture supernatant preparation

The following protocol was modified from previous studies (Kaewchomphunuch et al. 2022)(Yang et al. 2021). The LAB strains were inoculated in MRS broth and grown at 37 °C while shaking overnight. Then, the inoculated MRS broth was transferred to 2 mL microcentrifuge tube and performed centrifugation for 3 min at 5000×g (Denville Micro 260D Microcentrifuge, Denville Scientific, Inc., Metuchen, NJ, USA). The supernatant was collected by sterile syringe with a needle and then filtered with a sterile polyethersulfone membrane filter with a pore size 0.22 µm (Guangzhou Jet Bio-Filtration Co., Ltd., Guangzhou, China).

2.7 Metabolite profiling of LAB

The metabolite profiling of LAB was executed and monitored in collaboration with Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry as previously described (Vaitekūnas et al., 2020). In the prebiotic-supplemented LAB CFS samples, metabolite concentrations were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS). First, the CFS of LAB were mixed with an equal volume of acetonitrile and centrifuged for 10 min at 10,000 rpm. The samples were analyzed using the Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (Shimadzu, Kyoto, Japan) and LCMS-2020 mass spectrometer (Shimadzu, Kyoto, Japan) with an electrospray ionization (ESI) source. The chromatographic separation was conducted using a YMC Pack Pro C18 column (3 × 150 mm; YMC, Kyoto, Japan) at 40 °C and a mobile phase that consisted of 0.1% formic acid water

solution (solvent A) and acetonitrile (solvent B) delivered in the 5–95% gradient elution mode. Mass scans were measured from m/z 50 up to m/z 2,000 at a 350 °C interface temperature, 250 °C desolvation line (DL) temperature, $\pm 4,500$ V interface voltage, and neutral DL/Qarray, using N₂ as nebulizing and drying gas. Mass spectrometry data were acquired in both positive and negative ionization modes. The data were analyzed using LabSolutions software (Shimadzu, Kyoto, Japan). Production of tyrosine and GABA was detected by the mass spectrometry module of HPLC-MS system and quantified by the calibration curves of the standard compounds. The presence or absence of tyrosine and GABA was verified by spiking the samples with the corresponding compounds.

2.8. Safety assessment of LAB strains

2.8.1. Hemolytic activity

Hemolytic activity was tested for LAB isolates and the positive control, *S. aureus* ATCC 29213. These isolates were inoculated on a blood agar medium containing 5% sheep blood (Sigma-Aldrich, Poznan, Poland) for 48 h at 37 °C. After incubation, the plates were observed for hemolysis zones around the colonies.

2.8.2. Antibiotic susceptibility test

Antibiotic susceptibility tests were performed as previously described (Sirichoat et al. 2020). The panel spans the clinically and regulatorily relevant antibiotic classes — cell-wall synthesis inhibitors (penicillin G, ampicillin, vancomycin), protein synthesis inhibitors (erythromycin, lincomycin, kanamycin, gentamicin, streptomycin, tetracycline), and RNA-polymerase inhibition (rifampicin) — rather than being an arbitrary selection, consistent with the antibiotic panel recommended under EFSA QPS guidance and the ISO 10932/IDF 223 standard for LAB susceptibility testing. Briefly, the test has been performed by disc diffusion method against rifampicin, erythromycin, penicillin G, ampicillin, lincomycin, kanamycin, gentamycin, streptomycin, vancomycin, and tetracyclin (Carl Roth, Karlsruhe, Germany).

2.8.3. Mucin degradation

The mucin degradation ability of LAB isolates was assessed. Briefly, the LAB isolates were grown in MRS broth supplemented with 0.5% (w/v) glucose and 0.5% (w/v) mucin from the porcine stomach (Sigma-Aldrich, St. Louis, Missouri, USA). After inoculation, cultures were incubated at 37 °C for 48 h. Bacterial growth was measured every 6 h at an absorbance of 600 nm. The strain *E. coli* ATCC 25922 was used as positive control and grown in Tryptic soy broth (Carl Roth, Karlsruhe, Germany) containing 0.5% (w/v) glucose supplemented with or without 0.5% (w/v) mucin (Sigma-Aldrich, Poznan, Poland) and cultured at 37 °C for 48 h under aerobic conditions. The optical densities were measured using an Eppendorf Biospectrometer (Eppendorf, Hamburg, Germany). The initial optical density of the medium was deduced from the final value of each test sample.

2.8.4. Gelatin degradation

The gelatin degradation ability of the LAB was investigated using MRS media containing 3% (w/v) gelatin (Sigma- Aldrich, Poznań, Poland). The strain *Staphylococcus aureus* ATCC 6538 was used as a reference for quality control and was grown on tryptone soya broth containing 3% (w/v) gelatin. Gelatin degrading ability was evaluated by the presence of a clear zone around the bacteria colony.

2.8.5. Search for antimicrobial resistance genes, virulence factors, and plasmid

In *in silico* analysis, it was conducted to identify genes associated with tryptophan, tyrosine, GABA, AMR and VF within the assembled genomes. Abricate software was employed to screen the genomes against reference databases, specifically Resfinder (<https://genepi.food.dtu.dk/resfinder>) for AMR genes and VFDB (<https://www.mgc.ac.cn/VFs/>) for VF genes (Florensa et al. 2022). A gene was considered present if its sequence exhibited greater than 90% nucleotide identity and aligned over 60% of the reference gene's length with the assembled genome. Additionally, MUMmer software was used to calculate the average nucleotide identity (ANI) between isolates. Finally, genome annotation of the assembled isolates was performed using the Prokka Annotation Pipeline. Multilocus sequence typing (MLST) was achieved by comparing de novo assemblies against the PubMLST database using BLAST

(Megur, Lastauskienė, and Burokas 2024; Megur, Simoliunas, and Burokas 2025).

2.9. Experimental strains selected

L. paracasei 11w was isolated from the fermented pear procured from Hales Turgus, Vilnius. *L. paracasei* 11w was selected from the previous study and was cultured at 37°C for 24 h in MRS broth (de Man, Rogosa and Sharpe, BD Life Science, USA). The cell pellet was obtained by centrifugation at $5,000 \times g$ for 10 min at 4 °C and resuspended in PBS (Corning, USA) by 1×10^7 CFU/mL.

2.10. *In vivo* study

2.10.1. Animals

All animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council and approved by the Lithuanian State Food and Veterinary Service (Project No G2-174). For this study, 36 young female C57BL/6J mice, aged 7 weeks, were utilized. The mice were housed in groups of 4-5 per cage under controlled conditions (temperature maintained at 22 ± 1 °C, humidity at $55 \pm 3\%$), with food and water provided *ad libitum*. Mice were kept on a 12-h light/12-h dark cycle with lights on at 07:00.

2.10.2. Diet and dietary supplements

In this study, the impact of dietary regimen and dietary supplements on female mice was investigated. Further, the mice were categorized into four groups, each consisting of 8 - 10 mice. During the span of 25 weeks, the female mice were provided with either a CD or HFD. The groups included: CD (control diet); HFD (HF *ad libitum*); HFD + *L. paracasei* 11W strain (HF+11W); and HFD+11W+5% GOS (synbiotic administration), as illustrated in Figure 2.1. The CD was formulated to contain 3.87 kcal/g, while the HFD comprised two types of saturated fatty acids (stearic acid and palmitic acid), from which 60% of calories originated from fats, 24% carbohydrates, and 16% proteins (5.21 kcal/g) (Altromin; Germany). For the prebiotic intervention, GOS was administered at a concentration of 5% (KingPrebiotics®; New Francisco

(Yunfu City) Biotechnology Corporation Limited; China) mixed in water for the housing bottles, with the bottles being replaced biweekly.

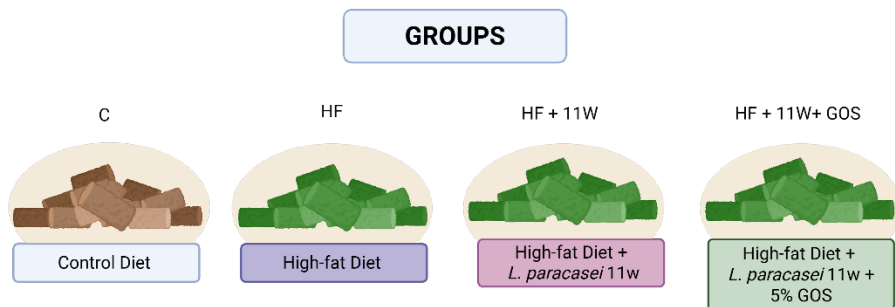


Figure 2.1 Diet administration for the female mice divided into four different groups.

2.10.3. Glucose tolerance test (GTT)

A GTT was conducted to assess glucose metabolism impairment according to established protocols (Golubeva et al. 2017a)(Burokas et al. 2017a). Following a 14 h fast with *ad libitum* access to water, basal blood glucose levels were measured (0-min timepoint). Animals then received an intraperitoneal (i.p.) injection of glucose at a dose of 2 g/kg. Blood glucose concentrations were subsequently determined at 15, 30, 45, 60, and 120-min intervals. For each measurement, a 5-microliter blood sample was obtained via tail snip and analyzed using a blood glucose monitoring system (CONTOUR®NEXT GEN, United States).

2.10.4 Animal behavior experiments

The animals underwent a 1h acclimatization period in the respective experimental rooms prior to behavioral testing. A one-day rest period was provided thereafter. Following the GTT, animals were rested for seven days before the culling procedure. The experimental timeline presented in Fig. 2.2.

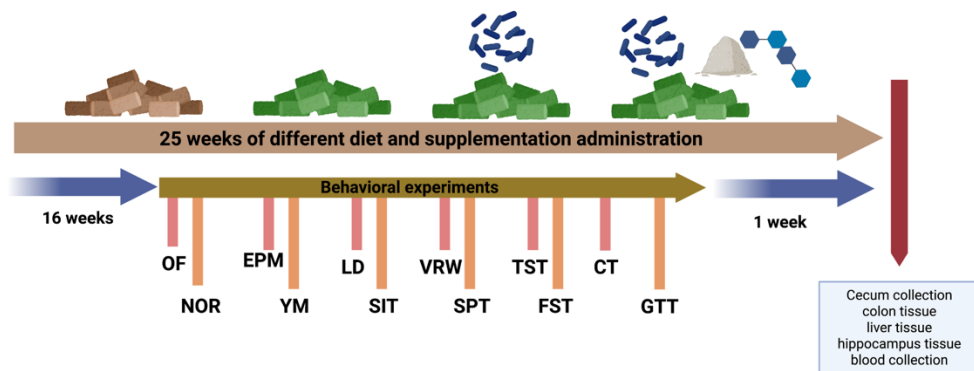


Figure 2.2. Experimental timeline of the animal study (n=36). The behavioral experiments were conducted after 16 weeks of diet administration, and the tests were arranged from less stressful to most stressful experiments. In the end of the time point, the red arrow indicates time points for sample collection from cecum or tissues. OF- Open field; NOR- Novel object recognition; EPM- Elevated plus maze; YM- Y maze; LD- Light and dark box; SIT- Social Interaction test; VRW- Voluntary running wheel; SPT- Sucrose preference test; TST- Tail suspension test; FST- Force swim test; CT- Carmaine test; and GTT- Glucose tolerance test.

2.10.4.1. Open field test (OF)

To assess anxiety-like behavior in mice, the subjects were positioned in the center of an open field arena. The arena, constructed from white opaque Plexiglas, measured 40 cm in length, 40 cm in width, and 30 cm in height (Vijaya et al., 2024). The test was conducted under illumination of 500 lux, measured at floor level in the arena center, for a duration of 10 min. Behavior was recorded via video for subsequent analysis. The percentage of time spent and the number of entries into a virtually defined central zone, measuring 10 cm × 10 cm, were quantified. Video recordings were analyzed using the Viewer software (Biobserve, Germany).

2.10.4.2. Light and dark box (LD)

Anxiety like behavior was evaluated in female mice using LD test as described previously (Ziegler et al. 2024). The experimental apparatus comprised a dimly lit enclosure (25 lux at maze floor level) constructed of black, opaque Plexiglas (20 cm L × 20 cm W × 35 cm H), designated the "dark box." This was connected via a short, narrow tunnel (7 cm L × 7 cm W × 7 cm H) to a brightly lit enclosure (500 lx at maze floor level) constructed of white, opaque

Plexiglas (30 cm L × 20 cm W × 35 cm H), designated the "light box." Sessions were video recorded. At the start of each 5min observation session, individual mice were positioned within the dark box, oriented toward the tunnel. The number of entries into and the percentage of time spent within the light box were quantified under the experimental conditions.

2.10.4.3. Elevated plus maze (EPM)

The EPM was performed as previously described in other studies. The mice were positioned in the center of a black, opaque Plexiglas elevated plus-maze, consisting of four arms (35 cm long × 6 cm wide), with two open and two enclosed arms. The apparatus was elevated 30 cm above the floor and illuminated (30–35 lux in the open arms and 4–6 lux in the closed arms; light intensity was measured at the maze level). Behavior was recorded via video for the duration of the experiment. Each animal underwent a single five-min test session, during which the percentage of time spent in and the number of entries into the open arms served as indices of anxiety.

2.10.4.4. Novel object recognition (NOR) test

The V-shaped maze test was conducted as previously reported (Burokas et al., 2014). The V-shaped maze was constructed from black opaque Plexiglas, featuring two perpendicular arms each measuring 25 cm in length, 5 cm in width, and 15 cm in height. Illumination was maintained at a low level (15 lux, measured at the maze's top). The experimental protocol comprised three phases: acclimatization (day 1), training (day 2), and testing (day 2). A three-hour interval was imposed between the training and testing phases to assess short-term memory. This test utilizes the spontaneous preference of rodents to explore a novel object over a familiar one, quantified as a discrimination index (DI). The DI was calculated using the following formula:

$$DI = ((TR - TL)) / ((TR + TL))$$

where TR represented the exploration time devoted to the new object, and TL represented the exploration time devoted to old object.

2.10.4.5. Tail suspension test (TST)

The procedure was developed based on the previously researched protocol (Kunevičius et al. 2025). The mice were suspended by the tail with a clip positioned approximately 2 cm from the tail's tip, which was attached to a metal bar elevated 30 cm above the surface. A single 6-min session was conducted, during which the duration of immobility was recorded. Immobility was defined as passive hanging. All sessions were videotaped for subsequent manual analysis by an experimenter blinded to the experimental conditions.

2.10.4.6. Y- maze

To evaluate the spatial memory in mice the Y-maze procedure was developed based on the previously researched protocol (Abdulai-saiku et al. 2025). The mice were kept in a grey, opaque Plexiglas apparatus with 3 identical arms (40 cm long × 9 cm wide × 15 cm high). The apparatus was a closed Y-shaped maze and was illuminated from the top (15 lux, measured at the end of the arms)(Kunevičius et al. 2025). Test sessions of 10 min were conducted, during which the number of arms entered in alternation by the mice was tallied and documented. An arm entry was defined as the placement of all four limbs within the zone of an arm.

2.10.4.7. Voluntary running wheel (VRW)

The voluntary physical activity of female mice was evaluated following previously described protocol (Reuser et al. 2022). The animals were individually housed in standard cages equipped with a running wheel (12 cm metal wheel diameter), with *ad libitum* access to their respective diet and water (Vijaya et al., 2024). Voluntary physical activity was quantified by recording the number of running wheel rotations over a 24 h period, comprising a 12 h light cycle (80–100 lux at cage level) and a 12 h dark cycle. Activity data were acquired using a specialized recording system with PowerLab hardware and analyzed with LabChart-8 software (ADInstruments Ltd, UK) to measure wheel rotations.

2.10.4.8 Force swim test (FST)

The FST in mice is employed to evaluate depressive-like behavior by placing subjects in an inescapable water-filled apparatus and quantifying immobility duration as a measure of behavioral despair (Burokas et al. 2017a). In this procedure, mice were individually placed into a clear glass cylinder (24 cm height × 21 cm diameter) containing water to a depth of 15 cm (25 ± 0.5 °C). The water was replaced between subjects to eliminate olfactory cues. The 6-min test session was recorded with a stationary digital camera. Behavioral data were subsequently scored in duplicate from the recorded videos (Video Media Player software) and averaged by an experimenter blinded to experimental conditions. Latency to the first bout of immobility was recorded. Total immobility time (s) was measured during the final 4 min of the test, where immobility was defined as the absence of active movement except for minor motions necessary to keep the head above the water surface.

2.10.4.9. Social Interaction test (SIT)

The SIT was conducted in a V-shaped maze comprising two perpendicular arms terminating in a compartment separated by a metal bar (Kunevičius et al. 2025). The experiment involved three consecutive phases: habituation, sociability testing, and social novelty testing, each lasting 5 mins. In the habituation phase, animals explored an empty maze. During sociability testing, experimental animals explored either a neutral object or an unfamiliar mouse, with the unfamiliar mice matched for sex, age, and weight. In the social novelty testing phase, the neutral object was replaced by a novel, unfamiliar animal. The sociability index (SI) was calculated using the same method as the DI.

$$SI = ((TA - TO))/((TA + TO))$$

where TA represented the exploration time devoted to the unfamiliar animal, and TO represent the exploration time devoted to an inanimate object.

2.10.4.10. Sucrose preference test (SPT)

The SPT evaluates anhedonia in female mice by quantifying their preference for a sucrose solution over plain water (Bura et al. 2010; M.-Y. Liu et al. 2018). To mitigate biases associated with group housing, the procedure was performed in individual home cages. Female mice were singly housed for 48-

72 h for acclimation, with *ad libitum* access to two bottles of plain water to preclude side bias. On the first day, each singly housed mouse remained in its home cage with two water bottles. On the second day, both bottles were replaced with one containing water and a second containing a 2% sucrose solution. Both bottles were weighed prior to placement in the cage. On the third day, bottles were weighed to determine liquid consumption during the preceding 24 h period. They were subsequently refilled, weighed, and returned to the cage with the positions of the sucrose and water bottles alternated to control for place preference. On the fourth day, the bottles were weighed again. The sucrose preference index was calculated as the average sucrose consumption across the final two-day period divided by the average total liquid consumption (the sum of the average water and average sucrose solution consumed).

2.10.5. Tissue collection

Animals were sacrificed in a randomized manner with respect to treatment and testing condition, with sampling conducted between 9:00 a.m. and 1:00 p.m. Trunk blood was collected in tubes containing potassium EDTA (Ethylene Diamine Tetra Acetic Acid) and centrifuged for 15 min at 4000 g. Plasma was procured and stored at -80°C for subsequent analysis of Insulin. The cecum was removed, weighed, and stored at -80°C for future microbiota analysis. The brain was rapidly excised, dissected, and each region was snap-frozen on dry ice before storage at -80°C for further analysis.

2.10.6 ELISA assays

Quantification of pro-inflammatory cytokines (IL-6, TNF- α , IFN- γ , IL-5) and insulin levels in soluble and insoluble fractions was conducted. Frozen colon samples were homogenized on ice in 1 mL of PBS containing a complete protease inhibitor cocktail (Roche), 1 mM PMSF (Sigma), and 2 mM sodium orthovanadate (Sigma) using a Dounce homogenizer. The homogenates were vortexed and then centrifuged at $14,000 \times g$ for 30 min at 4°C . The resulting supernatant, designated the soluble fraction, was collected and stored at -80°C . The remaining pellet was resuspended in 0.4 mL of 70% formic acid, agitated, and centrifuged at $14,000 \times g$ for 30 min at 4°C to solubilize the insoluble fraction. This supernatant was subsequently neutralized with 1 M Tris-base buffer (pH 9). Protein concentrations for both fractions were

determined using a Bradford Protein Assay (Thermo Fisher Scientific, United States). Prior to Enzyme-linked immunosorbent assay (ELISA), the soluble fraction was diluted 4-fold and the insoluble fraction 30-fold. A solid-phase sandwich ELISA (KHB3441, Thermo Fisher Scientific, United States) was performed as per the manufacturer's instructions. Cytokine concentrations were interpolated from a standard curve based on optical density measurements and were normalized to the total protein concentration of each respective fraction.

For Insulin ELISA (Thermo Fisher Scientific, United States) mice were fasted for 6 h prior to terminal blood collection. Whole blood was collected into EDTA-coated tubes, centrifuged at $3,000 \times g$ for 10 min at 4°C to obtain serum, and stored at -80 °C until assay. Plasma was diluted 1:4 to 1:10 in assay diluent to ensure readings fell within the kit's dynamic range of 5-400 $\mu\text{IU/mL}$. This solid-phase sandwich ELISA kit employs a pre-coated 96-well microplate with target-specific capture antibody. Diluted plasma samples (100 μL /well, duplicates), standards, and controls were added to wells and incubated for 2 h at room temperature. Following three washes with wash buffer, biotinylated detection antibody was added (1 h incubation), then streptavidin-HRP conjugate (45 min). Plates were washed five times, TMB substrate added for color development (15-30 min in the dark), and reaction stopped with 1 M H_2SO_4 . Absorbance was measured at 450 nm. The assay recognizes both natural and recombinant mouse insulin, with sensitivity of 5 $\mu\text{IU/mL}$, intra-assay CV <10%, and inter-assay CV <12 %.

2.10.7. Liver histology

Liver tissues were harvested from female mice immediately following euthanasia by cervical dislocation. The liver was then rinsed in ice-cold PBS to remove excess blood. Excised liver lobes (left lateral and median) were trimmed of connective tissue, embedded in Optimal Cutting Temperature compound in peel-away embedding molds, and snap-frozen in 2-methylbutane (isopentane) cooled to -80 °C over dry ice or liquid nitrogen for 1 min. Frozen blocks were stored at -80 °C until sectioning. Sections (7 μm thick) were cut using a cryostat at -20 °C to -25 °C, thaw-mounted onto Superfrost Plus charged glass slides, and air-dried for 30 min at room temperature before fixation.

Slides were fixed in 10% neutral buffered formalin for 10 min or cold acetone (-20°C) for 5 min, then rehydrated in graded ethanol (95%, 70%, distilled water; 2 min each). Sections were stained in Harris-modified hematoxylin (Abcam, Vilnius, Lithuania) (5 min), rinsed under running tap water (5 min), counterstained with eosin Y (Abcam, Vilnius, Lithuania) (3min), dehydrated in graded ethanol (70%, 95%, 100%; 1 min each), cleared in xylene (2 × 2 min), and cover slipped with DPX (Abcam, Vilnius, Lithuania) or Permount mounting medium (Abcam, Vilnius, Lithuania). Slides were examined under light microscopy (Olympus BX53) at 20× and 40× magnification for assessment of hepatocyte morphology, steatosis, inflammation, and fibrosis.

2.10.8. Colon histology

For histological analysis, female mice colons fixed in 4% paraformaldehyde for 24 h, washed in PBS, and embedded in paraffin. The colon tissue was cut into 10 µm thick slices, placed on glass slides, and stored at +4°C. Tissue sections 10 µm were stained with hematoxylin. For glycan detection, Periodic acid-Schiff (PAS) (Sigma-Aldrich, Lithuania) and alcian blue (Thermo Fisher Scientific, United States) were used to stain general intestinal structure. Slides were examined under light microscopy (Olympus BX53) at 20× and 40× magnification. Analyses were performed using ImageJ software (version 1.53f51) and GraphPad software (version 9.3.1; USA). The images were analyzed, and different structural parameters were calculated.

2.10.9. Gene expression analysis

cDNA synthesis was performed using the Maxima H Minus First Strand cDNA Synthesis Kit, with dsDNase (Thermo Fisher Scientific, Lithuania). Real-time PCR was performed using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Lithuania), with Luminaris Color HiGreen Master Mix, Low ROX (Thermo Fisher Scientific, Lithuania). Primer concentrations in the reaction mix were equal to 0.4 µM, and 2 µl of cDNA sample was added per 20 µl reaction. Gene expression levels were standardized to alpha-tubulin (Tub1a) expression. The primer sequences can be found in Supplementary material Table S1.

2.10.10. Bioinformatics pipeline

2.10.10.1. 16S rRNA Data Processing and Taxonomic Profiling

For cecal microbiota profiling, the full-length bacterial 16S rRNA gene ($\approx$ 1,500 bp, spanning all nine hypervariable regions) was amplified and sequenced using insert sequencing platform and primer pair used, per the Novogene sequencing report — likely PacBio single-molecule real-time (SMRT) circular consensus sequencing (CCS) with the near-universal full-length primer pair 27F/1492R, rather than a single short hypervariable region. Raw full-length 16S rRNA gene amplicon sequencing reads (16S full-length amplicon analysis; Novogene, UK) were subsequently processed in-house using QIIME 2 (v. 2024.10)(Bolyen et al., 2019). Sequence data were organized into a manifest file and imported into a QIIME 2 artifact using the Sample Data [SequencesWithQuality] format. Quality profiles of demultiplexed reads were examined to determine trimming and truncation thresholds.

Denoising was performed with the DADA2 plugin using fixed parameters (trim-left = 13; trunc-len = 220). This step removed low-quality bases, corrected sequencing errors, filtered chimeras, and generated amplicon sequence variants (ASVs). Further, Taxonomic classification of ASVs was conducted using the QIIME 2 feature-classifier plugin with a pre-trained SILVA 138 Naïve Bayes classifier. All steps were executed on a High-Throughput Computing cluster using 64 CPUs and 272 GB RAM under job submission mode.

2.10.10.2 Microbial diversity analysis

For alpha diversity, microbial richness and diversity within each sample were estimated using observed richness, Shannon, and Simpson indices, calculated with the vegan package. For beta diversity analysis, genus-level abundance data were first transformed using the centered log-ratio method to account for compositionality. Pairwise differences between groups were then calculated using Euclidean distance. Permutation-based analysis of variance with 999 permutations was used to test the statistical significance of group differences, implemented via the pairwise Adonis package. Community patterns were visualized with principal coordinate analysis (PCoA) using ggplot2 for

graphical representation. All analyses were conducted in a reproducible R (version 4.3.3) workflow, ensuring consistent data processing and visualization of microbial diversity patterns.

2.10.11. Statistical analysis

All *in vitro* experiments were performed in triplicate. Results are expressed as mean $\pm$ standard deviation (SD) at a 95% confidence level. Group differences were evaluated using one-way and two-way ANOVA, followed by Tukey's multiple comparisons test to examine differences between means. A p-value of less than 0.05 was considered statistically significant.

For the *in vivo* experiments, data normality was first assessed using the Shapiro–Wilk normality test. Normally distributed data are expressed as mean $\pm$ standard error of the mean (SEM) and represented graphically as bar diagrams with superimposed scatter plots of individual data points. Non-normally distributed data are presented as median with interquartile range and displayed as boxplots indicating quartiles and whiskers reflecting the minimum and maximum values. For parametric data, one-way ANOVA followed by Dunnett's post hoc multiple comparisons test was applied in reference to the HFD-fed (HF) group. For non-parametric data, the Kruskal–Wallis test followed by Dunn's multiple comparisons test was used to compare treatment groups with the HF group. All tests were two-tailed, and a p-value of less than 0.05 was considered statistically significant throughout. All statistical analyses were performed using GraphPad Prism versions 8.4.3 and 9.3.1 (GraphPad Software Inc., Boston, USA).

3. RESULTS

During the *in vitro* investigation the morphologically different 150 LAB isolates were isolated from numerous FF of Lithuania. Later 43 isolates were selected and the isolation was divided into two different batches, batch I and batch II. These isolates were subsequently added to the probiotic library curated by the Department of Microbiology and Biotechnology, Institute of Biosciences, Life Sciences Center, Vilnius University, Lithuania. The isolated strains of batch I is listed in supplementary table 4, and batch II is listed in supplementary table 5. The LAB isolates underwent testing for probiotic properties, aiming at their resilience to acidic gastric juices they would encounter after consumption, and their ability to colonize the gut and produce metabolites essential for their probiotic functionality. These steps focused on assessing their suitability as probiotic candidates.

3.1 Batch I: survival of gastrointestinal tract

3.1.1 Survival at low pH

The ability of LAB strains to survive acidic gastric conditions is a crucial criterion for probiotic selection. During digestion, the stomach produces gastric acid with a pH between 1 and 3, which, while essential for protein digestion and pathogen defense, poses a significant challenge for ingested microorganisms. To assess this, all 23 batch I isolates were subjected to pH 2 in phosphate-saline buffer for *in vitro* acid tolerance evaluation. Of the 23 isolates tested, 18 demonstrated survival rates above the 50% threshold, confirming their ability to withstand highly acidic conditions. The four best-performing strains were 55w, 18B, 2T, and 9S, with survival rates of 97.58, 96.02, 95.37, and 90.03%, respectively, indicating strong acid resistance. The remaining strains showed varying degrees of tolerance, with the majority maintaining acceptable survival levels. However, three strains, 57B, 42T, and 29A, fell below the 50% survival threshold and were excluded from further analysis, as their markedly reduced viability suggested limited capacity to endure subsequent harsher GI conditions. The 18 surviving strains were advanced to pepsin resistance testing (Table 3.1).

3.1.2 Resistance to pepsin

Pepsin is a key proteolytic enzyme activated in the acidic environment of the stomach and represents another barrier that probiotic candidates must overcome. The 18 acid-tolerant isolates were subsequently tested for their ability to survive in the presence of pepsin at pH 2. Results showed considerable variation among the strains. Four isolates demonstrated pepsin resistance exceeding 90%: 18B (97.58%), 55w (94.99%), 33E (93.74%), and 3A (90.75%), suggesting these strains possess robust protective mechanisms against proteolytic degradation. In contrast, five strains, 35s (18.11%), 10w (45.83%), 30b (46.35%), 62W (46.63%), and 68B (46.77%), showed survival rates below 50% and were excluded from subsequent steps. The remaining 13 strains, demonstrating adequate pepsin tolerance, were advanced to bile salt and pancreatin resistance testing (Table 3.1).

3.1.3 Resistance to bile salts and pancreatin

Following successful gastric simulation, the 13 remaining isolates were subjected to simulated intestinal fluid containing 0.3% bile salts and 1 mg/mL pancreatin for 6 h. Six strains exhibited particularly high tolerance, surpassing the 90% survival threshold: 25E (95.40%), 9S (95.37%), 2T (94.20%), 3A (92.07%), 55w (90.90%), and 40C (90.83%). A total of 12 strains-maintained survival rates of at least 50%, while strain 11t showed the lowest survival of 32.60% and was excluded. The 12 strains demonstrating adequate GI resilience were advanced to functional characterization (Table 3.1).

3.2 Functional characterization of batch I probiotic candidates

3.2.1 Antimicrobial activity

The antimicrobial potential of the 12 selected LAB strains was evaluated by testing their CFS against five clinically relevant pathogenic bacteria: *S. aureus* ATCC 29213, *S. Typhimurium* ATCC 14028, *S. pyogenes* ATCC 12384, *S. pyogenes* ATCC 19615, and *K. pneumoniae* ATCC 13883. Probiotic bacteria can produce a range of bioactive metabolites, including bacteriocins and OA, which inhibit pathogenic microorganisms and contribute to host gut health. Results revealed varying degrees of antimicrobial activity across the strains. Notably, strains 18B, 25E, 48C, and 66W demonstrated the broadest inhibitory profiles, with CFS from these isolates inhibiting at least four of the

five tested pathogens. Strain 66W was the most potent, showing antagonistic activity against all five pathogens tested. Strains 2T, 11w, and 33E showed more limited activity, inhibiting only two pathogenic species. Strains 1A, 3A, 9S, and 40C showed moderate activity against two to three pathogens. All 12 strains were retained for further investigation given their varying but potentially beneficial antimicrobial profiles (Table 3.2).

3.2.2 Trolox equivalent antioxidant concentration

The antioxidant capacity of the CFS from all 13 LAB candidates was assessed using the TEAC assay. Probiotic-derived antioxidants play an important role in neutralizing reactive oxygen species, contributing to host protection against oxidative stress-related conditions including aging, inflammation, and chronic disease. TEAC values varied considerably across isolates. Strains 3A and 55w exhibited the highest antioxidant activity, with TEAC values of 26.37 $\mu\text{g/mL}$ and 26.06 $\mu\text{g/mL}$, respectively, suggesting strong free radical scavenging capability. In contrast, strain 40C showed the lowest TEAC value of 9.57 $\mu\text{g/mL}$ (Table 3.2). Despite the differences in antioxidant activity, all isolates were retained and subjected to further evaluation of their colonization potential, as antioxidant capacity alone was not used as an exclusion criterion at this stage.

3.2.3 Auto-aggregation

The auto-aggregation capacity of all 12 strains was measured after 24 h of incubation at 37°C. Strain 11w demonstrated the highest auto-aggregation of 79.96%, followed closely by 55w (76.76%) and 40C (76.63%). In contrast, strain 3A showed the lowest aggregation of 24.26%, falling well below the 50% threshold. Four strains 2T (26.63%), 3A (24.26%), 9S (25.18%), and 25E (36.37%) exhibited aggregation below 50% after 24 h and were excluded from further analysis (Table 3.2). The eight strains exceeding the 50 % aggregation threshold were advanced to adhesion testing on HCT116 cells.

3.2.4 Adhesion to HCT116 cells

Among the eight isolates tested, adhesion levels ranged from below 10% to above 25%. Strain 11w showed the highest adhesion ability at $25.67 \pm 0.43\%$, while isolate 1A showed the weakest adhesion at $9.26 \pm 0.97\%$. Strains 1A, 18B, and 55w all exhibited adhesion percentages below 12% and were

excluded from subsequent studies due to their limited colonization potential (Table 3.2). The remaining five strains, demonstrating stronger adhesion profiles, were retained as the most promising batch I probiotic candidates for further molecular characterization and genomic profiling.

3.3 Batch II investigation of survival in gastrointestinal tract

3.3.1 Survival at low pH

As with batch I, all 20 batch II isolates were subjected to pH 2.0 in phosphate-saline buffer to evaluate acid resistance. Of the 20 isolates tested, 15 achieved survival rates of 50% or above, demonstrating adequate acid tolerance. The highest survival was recorded in strains KA19 ($94.67 \pm 3.39\%$), KA13 ($93.76 \pm 3.71\%$), UV11 ($85.95 \pm 7.65\%$), and KA18 ($83.16 \pm 8.20\%$). Five strains KA3.2.2 ($30.78 \pm 8.81\%$), KA8.2 ($24.22 \pm 1.69\%$), KA9.2 ($23.81 \pm 8.24\%$), KA14 ($37.74 \pm 8.24\%$), and KA15.1 ($27.62 \pm 12.88\%$) fell below the 50% survival threshold and were excluded from further testing (Table 3.3). The 15 surviving strains were advanced to pepsin resistance testing.

3.3.2 Resistance to pepsin

The 15 acid-tolerant batch II isolates were subsequently tested for pepsin resistance at pH 2.0. Results revealed substantial variability among strains. Three isolates UV16 ($96.32 \pm 0.30\%$), KA9 ($94.45 \pm 2.06\%$), and KA18 ($82.80 \pm 1.57\%$) demonstrated pepsin resistance above 80%, suggesting that these strains may possess enhanced adaptive mechanisms enabling them to withstand proteolytic degradation in the gastric environment. In contrast, eight strains exhibited survival rates below 20%: KA2.1 ($19.01 \pm 5.22\%$), KA3.1 ($17.23 \pm 2.26\%$), KA9.2 ($13.10 \pm 1.93\%$), KA11.1 ($9.25 \pm 1.23\%$), KA14 ($19.52 \pm 3.35\%$), KA16 ($12.91 \pm 0.55\%$), KA17 ($19.69 \pm 1.31\%$), and UV11 ($17.46 \pm 2.13\%$). These eight strains were excluded due to their markedly reduced pepsin tolerance (Table 3.3). Six strains advancing beyond this stage were subjected to bile salt resistance testing.

Table 3.1. Batch I Gastrointestinal survival screening. N=23, ★ = highest value in column. ↓ = lowest value. ≥50% = passed threshold; exact value from figure not extracted. Not tested = excluded at prior step

Strain	Acid tolerance pH 2 (%)	Pepsin resistance (%)	Bile salts (%)	Pancreatin (%)	GI outcome	Reason excluded
Strains passing all GI stages — advanced to functional characterization						
55w	97.58 ★	94.99	90.90	≥50%	Passed	—
18B	96.02	97.58 ★	≥50%	≥50%	Passed	—
2T	95.37	≥50%	94.20	≥50%	Passed	—
9S	90.03	≥50%	95.37 ★	≥50%	Passed	—
3A	≥50%	90.75	92.07	≥50%	Passed	—
25E	≥50%	≥50%	95.40 ★	≥50%	Passed	—
11w	≥50%	≥50%	≥50%	≥50%	Passed	—
33E	≥50%	93.74	≥50%	≥50%	Passed	—
40C	≥50%	≥50%	90.83	≥50%	Passed	—
48C	≥50%	≥50%	≥50%	≥50%	Passed	—
66W	≥50%	≥50%	≥50%	≥50%	Passed	—
1A	≥50%	≥50%	≥50%	≥50%	Passed	—
Strains excluded during GI screening						
57B, 42T, 29A	<50%	Not tested	Not tested	Not tested	Excluded	Acid tolerance
35s, 10w, 30b, 62W, 68B, 60WB	≥50%	<50%	Not tested	Not tested	Excluded	Pepsin resistance
11t	≥50%	≥50%	32.60	≥50%	Excluded	Bile/pancreatin

Table 3.2. Batch I Functional characteristics of probiotic candidates. N=12 ★ = *highest value*. ↓ = *lowest value*. – = *not reported*.
TEAC = Trolox equivalent antioxidant concentration.

Strain	Antimicrobial activity (pathogens inhibited)	TEAC (µg/mL)	Auto-aggregation 24 h (%)	HCT116 adhesion (%)	Final status
Final retained candidates					
11w	<i>S. aureus</i> , <i>K. pneumoniae</i>	–	79.96 ★	25.67 ★	Retained
33E	<i>S. pyogenes</i> 19615, <i>K. pneumoniae</i>	–	≥50%	≥12%	Retained
40C	<i>S. aureus</i> , <i>S. Typhimurium</i>	9.57 ↓	76.63	≥12%	Retained
48C	<i>S. aureus</i> , <i>S. Typhimurium</i> , <i>S. pyogenes</i> 19615, <i>K. pneumoniae</i>	–	≥50%	≥12%	Retained
66W	All 5 pathogens ★	–	≥50%	≥12%	Retained
Excluded at functional characterization stage					
2T, 3A, 9S, 25E	2–5 pathogens each	26.37 (3A) ★	<50% after 24 h	Not tested	Excluded (aggregation)
1A, 18B, 55w	3–4 pathogens each	26.06 (55w)	≥50%	<12% ↓	Excluded (adhesion)

3.3.3 Resistance to bile salts

Six batch II isolates were tested for their ability to survive in the presence of 0.3% bile salts in peptone water (pH 7.2) over a 6 h incubation period. All six strains-maintained viability under these conditions, although survival rates differed considerably. Strain KA12 showed the highest resilience, with a survival rate of $92.37 \pm 2.04\%$, suggesting a well-developed capacity to tolerate bile salt-induced membrane stress. Strains KA3.1.1 and KA15 followed with survival rates of $77.77 \pm 0.69\%$ and $73.94 \pm 3.78\%$, respectively (Table 3.3). These three strains appeared to have developed adaptive mechanisms conferring enhanced tolerance to the harsh intestinal conditions represented by bile salts. All six strains were advanced to pancreatin resistance testing.

3.3.4 Resistance to pancreatin

The final stage of GIT survival testing for batch II involved assessing tolerance to 1 mg/mL pancreatin in 0.85% NaCl at pH 8.0, simulating the alkaline enzymatic environment of the small intestine. Strain KA3.1.1 showed the highest survival of $96.82 \pm 1.49\%$, demonstrating exceptional resilience across all simulated GIT conditions. Strain KA15 showed acceptable viability at 67.04%, while KA18 recorded the lowest survival of 45.89%, falling below the 50% threshold and was subsequently excluded (Table 3.3). The remaining strains demonstrating survival above 50% were identified as suitable candidates for functional characterization, as their combined tolerance to acid, pepsin, bile salts, and pancreatin strongly supports their capacity to survive GIT transit and exert probiotic effects in the gut.

These Batch II survival ranges are broadly consistent with those observed for Batch I isolates under the same low-pH, pepsin, bile-salt, and pancreatin challenges (Section 3.1), with both batches showing wide strain-to-strain variability and a similar overall proportion of isolates retained past the 50% survival threshold, supporting the reproducibility of the GIT-survival screening approach across the two independent isolation campaigns.

3.4 Functional characterization of batch II probiotic candidates

3.4.1 Antimicrobial activity

The antimicrobial activity of batch II LAB strains was assessed using the disc diffusion method, evaluating CFS against five enteropathogenic bacteria: *S. enteritidis* ATCC 25928, *E. coli* P42, *S. Typhimurium* ATCC 14028, *K. pneumoniae* ATCC 13883, and *P. aeruginosa* ATCC 27853. Inhibition zones were recorded after 24 h and 48 h of incubation. KA9 and UV16 exhibited the broadest antimicrobial profiles, with CFS from both strains inhibiting all five tested pathogens. Strain KA9 produced inhibition zones ranging from 16.32 ± 0.08 mm against *E. coli* P42 to 21.01 ± 0.46 mm against *P. aeruginosa*, while UV16 showed zones between 11.13 ± 0.19 mm and 22.05 ± 0.07 mm. Strain KA15 also showed strong activity, particularly against *S. Typhimurium* (21.55 ± 0.48 mm) and *P. aeruginosa* (22.57 ± 0.27 mm). In contrast, KA3.1.1 showed no inhibitory activity against *K. pneumoniae* or *P. aeruginosa*, indicating a narrower antimicrobial spectrum. Notably, none of the five strains produced an inhibition zone exceeding 10 mm against *S. enteritidis*, indicating uniformly low anti-*S. enteritidis* efficacy across the panel, suggesting this pathogen may possess inherent resistance mechanisms against the bioactive compounds produced by these LAB strains (Table 3.4).

This pattern mirrors Batch I, where antimicrobial activity against the shared indicator pathogens *S. Typhimurium* and *Klebsiella pneumoniae* (Section 3.2.1) was likewise strain-dependent rather than uniform across isolates, indicating that variable, strain-specific antimicrobial spectra are a consistent feature across both independent screening campaigns rather than a batch-specific artefact.

For clarity, all five strains were tested against all five pathogens listed above; the omission of a pathogen from a given strain's row indicates a tested but non-detectable inhibitory effect, not that the pathogen was untested. Specifically: KA9 and UV16 inhibited all five pathogens; KA12 showed no detectable inhibition against *S. Typhimurium*; KA15 showed no detectable inhibition against *E. coli* (KA15 did inhibit *S. enteritidis*, 12.05 ± 0.17 mm, which should also be included among its inhibited pathogens above); and KA3.1.1 showed no detectable inhibition against *S. enteritidis* (0 mm), *K. pneumoniae*, or *P. aeruginosa*.

3.4.2 Auto-aggregation

Auto-aggregation was measured at multiple time points (0.25 h, 15 h, 16 h, 18 h, and 24 h) to assess the kinetics of cell-to-cell adhesion among batch II candidates. UV16 demonstrated the highest auto-aggregation ability, reaching $71.09 \pm 0.81\%$ as early as 0.25 h and increasing to $93.50 \pm 1.01\%$ after 24 h, indicating rapid and robust self-adhesion capacity. *P. pentosaceus* KA12 recorded the second highest values, with $59.73 \pm 0.67\%$ at 0.25 h and $74.60 \pm 0.63\%$ at 24 h. The lowest auto-aggregation was observed in KA3.1.1, which reached $41.68 \pm 0.45\%$ at 0.25 h and $63.12 \pm 0.83\%$ after 24 h, still exceeding the 50% threshold by the end of the incubation period. The strong self-aggregation demonstrated by all strains, particularly UV16, supports their potential for stable colonization and persistence within the GIT (Table 3.4).

3.4.3 Adhesion to HCT116 cells

Colonization capacity was assessed for all five batch II strains using the HCT116 intestinal epithelial cell model after 48 h of incubation. Adhesion levels ranged from 16.63% to 53.33%, representing a considerably broader range than observed in batch I. UV16 demonstrated the highest adhesion of $53.33 \pm 0.45\%$, suggesting a particularly strong affinity for intestinal epithelial surfaces. KA3.1.1 showed the weakest adhesion at $16.63 \pm 0.95\%$. Notably, three strains KA9, KA15, and UV16 exhibited adhesion percentages exceeding 40%, indicating strong colonization potential. Results were reproducible across replicates, confirming the consistency and reliability of these adhesion measurements (Table 3.4).

Table 3.3. Batch II Gastrointestinal survival screening. N=20 ★ = *highest value in column*. ↓ = *lowest value*. ≥50% = *passed threshold*; exact value from figure not extracted.

Strain	Acid tolerance pH 2 (%)	Pepsin resistance (%)	Bile salts (%)	Pancreatin (%)	GI outcome	Reason excluded
Strains passing all GI stages — advanced to functional characterization						
UV16	≥50%	96.32 ★	≥50%	≥50%	Passed	—
KA9	≥50%	94.45	≥50%	≥50%	Passed	—
KA12	≥50%	≥50%	92.37 ★	≥50%	Passed	—
KA15	≥50%	≥50%	73.94	67.04	Passed	—
KA3.1.1	≥50%	≥50%	77.77	96.82 ★	Passed	—
Strains excluded during GI screening						
KA3.2.2, KA8.2, KA9.2, KA14, KA15.1	<50%	Not tested	Not tested	Not tested	Excluded	Acid tolerance
KA2.1, KA3.1, KA11.1, KA16, KA17, UV11 + others	≥50%	<20%	Not tested	Not tested	Excluded	Pepsin resistance
KA18	83.16	82.80	≥50%	45.89	Excluded	Pancreatin

Table 3.4. Batch II Functional characterization of probiotic candidates. N=5 ★ = *highest value*. ↓ = *lowest among retained strains*.
All 5 pathogens = S. enteritidis, E. coli, S. Typhimurium, K. pneumoniae, P. aeruginosa.

Strain	Antimicrobial activity (pathogens inhibited)	Auto-aggregation 24 h (%)	HCT116 adhesion (%)	Final status
UV16	<i>S. enteritidis, E. coli, S. Typhimurium, K. pneumoniae, P. aeruginosa</i>	93.50 ★	53.33 ★	Retained
KA9	<i>S. enteritidis, E. coli, S. Typhimurium, K. pneumoniae, P. aeruginosa</i>	≥50%	>40%	Retained
KA12	<i>S. enteritidis, E. coli, K. pneumoniae, P. aeruginosa</i>	74.60	≥16%	Retained
KA15	<i>S. Typhimurium, K. pneumoniae, P. aeruginosa</i>	≥50%	>40%	Retained
KA3.1.1	<i>E. coli, S. Typhimurium</i>	63.12 ↓	16.63 ↓	Retained

3.5 Synbiotic potential of LAB isolates

3.5.1 Influence of prebiotics on LAB growth

The synbiotic potential of the selected LAB strains was investigated by evaluating their ability to utilize FOS and GOS as carbon sources. Prebiotics selectively stimulate the growth of beneficial bacteria and, when paired with compatible probiotic strains, form synbiotic combinations could enhance the gut health benefits. In batch I, all tested strains showed significant growth enhancement in the presence of GOS in a concentration-dependent manner. Strains 11w, 33E, 40C, 48C, and 66W demonstrated progressively increased growth as GOS concentration was raised from 2% to 4%, though growth profiles differed between strains, indicating strain-specific prebiotic utilization. In batch II, strains KA3.1.1, KA9, KA15, and UV16 showed enhanced growth in the presence of both 5% FOS and 5% GOS compared to standard MRS broth supplementation. Strain KA12 preferentially utilized GOS over FOS, showing greater growth enhancement with 5% GOS (Table 3.5). Collectively, these findings suggest that both FOS and GOS serve as effective carbohydrate sources for the tested LAB strains, supporting their proliferation and potential to modulate the GM by suppressing competing pathogenic bacteria.

3.5.2 Tryptophan production

In batch I, strain 11w produced the highest tryptophan concentration of $16.63 \pm 2.25 \mu\text{M}$ when grown in media supplemented with 4% GOS, while strain 40C produced $2.64 \pm 0.5 \mu\text{M}$ under the same conditions. In batch II, strains KA15 and KA12 were identified as tryptophan producers. Strain KA15 showed the most notable production, reaching $10.48 \mu\text{M}$ in MRS media supplemented with 5% FOS, a concentration within the range typically reported for tryptophan-producing LAB strains (approximately 2–16 μM). For strain KA12, tryptophan production was recorded at $2.69 \mu\text{M}$ in standard MRS and $1.69 \mu\text{M}$ in GOS-supplemented MRS; notably, no tryptophan production was detected in FOS-supplemented conditions for this strain (Table 3.5). These results suggest that the type and concentration of prebiotic supplementation significantly influence tryptophan biosynthesis in specific LAB strains, with potential implications for the development of synbiotic formulations targeting psychobiotic and gut-brain axis applications.

3.6 Molecular identification of LAB candidates

The selected 10 probiotic candidates from both batches were subjected to whole genome sequencing for definitive molecular identification. Sequences were compared against the NCBI GenBank database to identify the closest homologs based on core genome coverage. The novel strains were registered to NCBI GeneBank database.

Batch I candidates were identified as follows: strain 11w as *Lacticaseibacillus paracasei* (99.71% core genome coverage to *L. paracasei* strain R094), 33E as *Lactiplantibacillus paraplantarum* (99.56% to DSM 10667), 40C as *Lactiplantibacillus plantarum* (99.56% to JCM 1149), 48C as *Lactiplantibacillus plantarum* (99.71% to CIP 103151), and 66W as *Lactiplantibacillus paraplantarum* (99.85% to DSM 10667). The high core genome coverage values across all batch I isolate indicate a very close genetic relationship to their respective reference strains, providing strong confidence in the species-level identifications (Table 3.5).

Batch II candidates were identified as *Lactiplantibacillus plantarum* KA3.1.1 (74.3% core genome coverage to WLP04), *Lactiplantibacillus plantarum* KA9, *Pediococcus pentosaceus* KA12, *Leuconostoc mesenteroides* KA15, and *Limosilactobacillus fermentum* UV16. Phylogenetic trees constructed from core genome SNP data confirmed the close evolutionary relationships among these isolates and their reference strains. The notably lower core genome coverage of KA3.1.1 (74.3%) compared to batch I strains may suggest greater genomic divergence from the closest reference sequence, which could reflect unique strain-specific genomic features worthy of further investigation (Table 3.5).

3.7. Probiotic genes investigation in the lactic acid bacteria genomes

The distribution of nine functionally significant genes across ten LAB strains (11W, 33E, 40C, 48C, 66W, KA3.1.1, KA9, KA12, KA15, and UV16) is presented in Table 3.6. Among the genes investigated, *clpE*, *nhaC*, and *tetA* were universally present in all ten strains, indicating that virulence factor modulation, pH and sodium ion homeostasis, and tetracycline efflux-based resistance are conserved traits across the entire collection. The stress response gene *brcA* was detected in all strains except KA3.1.1, which was the only isolate lacking this gene. Similarly, *trpS2*, responsible for tryptophan

biosynthesis, was present in eight of the ten strains, with KA3.1.1 and KA12 being the exceptions. The acid tolerance gene *gadC* demonstrated a more restricted distribution, testing positive only in strains 11W, KA3.1.1, and KA12, while remaining absent in the other seven isolates. The multidrug resistance gene *mdlL* showed the most variable pattern, being present in strains 33E, 40C, 48C, 66W, and KA9, but absent in 11W, KA3.1.1, KA12, KA15, and UV16. With respect to adhesion-related genes, *fbp*, which facilitates adhesion to the mucus layer, was detected in seven strains (11W, 33E, 40C, 48C, 66W, KA3.1.1, and KA9), whereas *pdeK*, which mediates adhesion to the intestinal epithelium, was found only in strains 11W and KA3.1.1, making it the least prevalent gene in this study (Table 3.7). Collectively, these findings highlight considerable genomic diversity among the selected LAB strains, particularly in genes associated with acid survival, multidrug resistance, and epithelial adhesion, while core functional genes related to stress tolerance and ion homeostasis appear to be well conserved across all isolates.

3.8. Selection of probiotic candidate strain for *in vivo* investigation

Following the comprehensive *in vitro* screening of all batch I and batch II candidates, *L. paracasei* 11W was identified as the most promising probiotic candidate and was selected for advancement *in vivo* investigation. The strain 11W demonstrated robust GIT resilience, successfully surviving acidic conditions at pH 2, pepsin challenge, and exposure to bile salts and pancreatin, confirming its capacity to remain viable throughout the entire GIT. In *in vitro* functional characterization, 11W exhibited the highest auto-aggregation rate and the strongest adhesion to HCT116 IEC, indicating excellent colonization potential. Genomic analysis further confirmed the presence of key probiotic-associated genes, including *gadC* for acid survival, *fbp* and *pdeK* for adhesion to the mucus layer and intestinal epithelium, and *trpS2* for tryptophan biosynthesis. Notably, *pdeK* was detected exclusively in strain 11W among all batch I candidates, representing a unique genomic asset. Synbiotic evaluation further distinguished 11W from all other strains, as it produced the highest tryptophan concentration of 16.63 μM when supplemented with 4% GOS, exceeding the output of all other tested strains across both batches. This elevated tryptophan production capacity, combined with its strong GI survival, colonization ability, and comprehensive probiotic gene profile, collectively justified the selection of *L. paracasei* 11W as the lead probiotic candidate to proceed with *in vivo* testing for its therapeutic potential in a diet-induced mouse model.

Table 3.5. Synbiotic potential and molecular identification of both the batches. ★ = *highest value or strongest response*. ↓ = *notably low*. — = *data not reported*. Tryptophan range for LAB strains typically 2–16 µM. Species names in *italics* were identified by whole genome sequencing

Strain	Batch	GOS response	FOS response	Tryptophan production	Identified species	Food source	Core genome coverage
Batch I							
11w	I	Enhanced (2–4%)	Not tested	16.63 µM ★ (4% GOS)	<i>Lacticaseibacillus paracasei</i> 11w	Fermented pear	99.71%
33E	I	Enhanced (2–4%)	Not tested	Not detected	<i>Lactiplantibacillus paraplantarum</i> 33E	Fermented cherry tomato	99.56%
40C	I	Enhanced (2–4%)	Not tested	2.64 µM (4% GOS)	<i>Lactiplantibacillus plantarum</i> 40C	Fermented cherry tomato	99.56%
48C	I	Enhanced (2–4%)	Not tested	Not detected	<i>Lactiplantibacillus plantarum</i> 48C	Fermented cherry tomato	99.71%

66W	I	Enhanced (2–4%)	Not tested	Not detected	<i>Lactiplantibacillus paraplantarum</i> 66W	Fermented cucumber	99.85%
Batch II							
UV16	II	Enhanced (5%)	Enhanced (5%)	Not detected	<i>Limosilactobacillus fermentum</i> UV16	Cucumber pickle	60.9%
KA9	II	Enhanced (5%)	Enhanced (5%)	Not detected	<i>Lactiplantibacillus plantarum</i> KA9	Sauerkraut	72.7%
KA12	II	Enhanced (5%) ★	Minimal response	2.69 μM (MRS); 1.69 μM (GOS); none in FOS	<i>Pediococcus pentosaceus</i> KA12	Homemade Wine	78.9%
KA15	II	Enhanced (5%)	Enhanced (5%) ★	10.48 μM ★ (5% FOS)	<i>Leuconostoc mesenteroides</i> KA15	Kombucha	68.4%
KA3.1.1	II	Enhanced (5%)	Enhanced (5%)	Not detected	<i>Lactiplantibacillus plantarum</i> KA3.1.1	Fermented green beans	97.3% ↓

Table 3.6. Probiotic genes predeicted using WGS of the selected LAB strains. (–) indicates no presence of genes. (+) indicates presence of genes

Genes and its function	11W	33E	40C	48C	66W	KA3.1.1	KA9	KA12	KA15	UV16
<i>hrcA</i> (Regulates stress response)	+	+	+	+	+	–	+	–	+	+
<i>clpE</i> (Modulates the gene expression of some VF)	+	+	+	+	+	+	+	+	+	+
<i>gadC</i> (Bacterial survival in acidic environment)	+	–	–	–	–	+	–	+	–	–
<i>nhaC</i> (Maintains proper pH and sodium levels within the bacterial cell)	+	+	+	+	+	+	+	+	+	+
<i>tetA</i> (Resist the effects of	+	+	+	+	+	+	+	+	+	+

tetracycline antibiotics by actively pumping the antibiotic out of the cell)										
<i>trpS2</i> (Tryptophan biosynthesis)	+	+	+	+	+	–	+	–	+	+
<i>mdtL</i> (Multidrug resistance)	-	+	+	+	+	–	+	–	–	–
<i>fbp</i> (Adhesion to the mucus layer)	+	+	+	+	+	+	+	–	–	–
<i>pdeK</i> (Facilitate adhesion to the intestinal epithelium)	+	–	–	–	–	+	–	–	–	–

3.9. Protective effects of *L. paracasei* 11w and GOS synergistic combination on HFD-induced obesity and glucose intolerance in female mice

We ensured equal starting points between the experimental groups and fed the mice HFD and CDs for 25 weeks with daily oral gavage of either *L. paracasei* 11W (1×10^7 CFU/mL in 200 microL) or provided with PBS. Two-way ANOVA revealed a significant effect of diet and time on the weight in the female mice ($F(21, 256) = 2.544$; $p = 0.0003$). HFD fed mice exhibited a noticeable weight gain compared to the control group (Figure 3.1.A) and Subsequent Dunnett's post hoc test revealed that mice fed an HFD (HF, HF+11w, and HF+11w+GOS) displayed a progressive increase in body weight when compared to controls, the HF group reached statistical significance from week 12 through week 25 (Figure 3.1.A). Analysing data at week 25, the HFD fed groups showed a marked increase in final body weight (Kruskal–Wallis, $H = 14.07$, $p = 0.0021$). The HF group showed a significant increase in body weight compared to control group ($p = 0.0059$, Figure 3.1.C). Mice receiving probiotic or synbiotic supplementation (HF+11w, HF+11w+GOS) showed numerically lower body weight gains compared to HF, although these reductions did not reach statistical significance (Figure 3.1.C). Fasting glucose levels increased with age across the experimental groups. Two-way ANOVA revealed significant main effects of age [$F(6,232) = 48.03$, $p < 0.0001$]. Subsequent Dunnett's multiple comparisons test showed that mice receiving the HF+11W+GOS intervention exhibited significantly lower fasting glucose levels at weeks 5 and 25 compared with the HF group ($p < 0.05$). Similarly, the HF+11W group showed a significant reduction in fasting glucose levels at week 25 ($p < 0.01$) relative to HF-fed mice (Figure 3.1.B). ($F(6, 232) = 48.03$, $p < 0.0001$; Figure 3.1.B). The HF+11w+GOS intervention resulted in a significant decrease in fasting glucose at 5 and 25 weeks compared with the HF group ($p < 0.05$), whereas the HF+11W had a significant reduction after 25 weeks ($p < 0.01$; Figure 3.1.B).

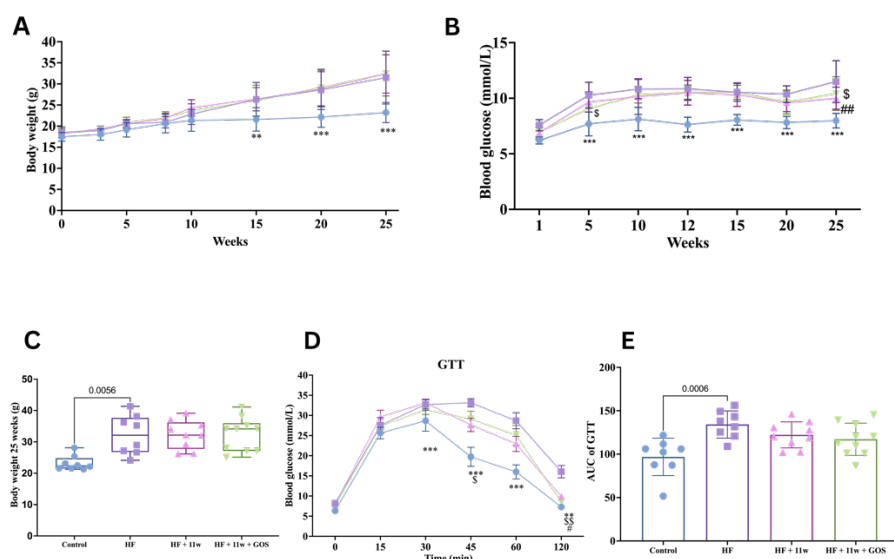


Figure 3.1. Effects of *L. paracasei* 11W and GOS supplementation on body weight and glucose homeostasis in HFD-fed female mice. (A) Body weight progression during the intervention period. (B) Fasting blood glucose levels are measured at different time points. (C) Final body weight at week 25. (D) Glucose tolerance test (GTT). (E) Area under the curve (AUC) derived from the GTT. Control mice received standard chow and PBS gavaging. HF mice received HFD and PBS gavaging. HF+11W mice received HFD and *L. paracasei* 11W gavaging. HF+11W+GOS mice received HFD, *L. paracasei* 11W gavaging, and 5% GOS in drinking water. Data are presented as mean $\pm$ SEM (A, B, D, E) or median with interquartile range (C), $n = 8$ – 10 per group. Two-way ANOVA followed by Dunnett's multiple comparisons test was used for panels A, B and D; Kruskal–Wallis followed by Dunn's multiple comparisons test for panel C; and one-way ANOVA followed by Dunnett's multiple comparisons test for panel E. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus HF; ## $p < 0.01$ versus HF+11W. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

GTT analysis at week 25 revealed substantially impaired glucose clearance in HF animals versus controls, with both HF+11w and HF+11w+GOS groups showing partial restoration of glucose tolerance, particularly at later time points post-glucose challenge (Figure 3.1.D). A decrease in blood glucose tolerance was observed in female mice ($F(15, 155) = 4.589$, $p < 0.0001$), particularly in HF animals ($p < 0.001$) at 30 min, 45 min, and 60 min compared to control animals, and HF+11W ($p < 0.05$) at 45 and 120 min compared to HF mice. Calculation of area under the curve for glucose during the GTT ($F(3, 31) = 6.115$, $p = 0.0022$) showed significantly increased glucose excursions

in HF mice ($p = 0.0006$ vs. control), with intervention groups displaying intermediate values between HF and control (Figure 3.1.E). Collectively, these results exhibit that HF feeding induces obesity and impaired glucose tolerance in female mice. At the same time, co-administration of *L. paracasei* 11W and GOS mitigates these adverse metabolic effects, but no difference observed in the weight gain.

3.10. Supplementation with *L. paracasei* 11W, either alone or in combination with GOS, attenuated anxiety-like behavior in female mice

To measure the anxiety levels in female mice, three different behavioral tests were performed. The OF, LD, and EPM provided an overview of general anxiety and exploratory behavior. In the center zone of OF, mice from all groups showed similar center visits, with no differences between groups ($F(3,31) = 1.771$; $p = 0.1731$; Figure 3.2.A). Whereas the percentage activity in the center zone, there was a significant difference between the groups ($F(3,33) = 3.496$; $p = 0.0263$). Moreover, the HF group exhibited a remarkable reduction in the percentage of total activity within the center zone compared to the HF+11W group ($p = 0.0168$; Figure 3.2.B). The intervention with *L. paracasei* 11W and GOS improved center-zone activity, which differed considerably from the HF group ($p = 0.0350$; Figure 3.2.B). In the LD test, the percentage time spent in the light compartment varied in all groups ($F(3, 31) = 2.825$; $p = 0.0549$). The HF group mice spent less time in the light compartment when compared to the control group ($p = 0.0266$). For the number of visits to the light compartment, the HF group notably reduced when compared to other groups (Kruskal–Wallis, $H = 13.03$, $p = 0.0046$). The HF group mice had fewer entries to the light component when compared to control ($p = 0.001$). Both supplementation groups trended toward more light box exploration, although statistical significance was not reached. In the EPM, firstly, the number of visits to the open arm was calculated. There were no notable differences in the number of open arm visits across groups ($F(3, 31) = 0.1595$; $p = 0.9227$; Figure 3.2.C). Secondly, the percentage time spent in open arm revealed a significant difference ($F(3, 32) = 5.574$, $p = 0.0034$). The percentage of duration spent on open arms was reduced in HF animals compared to controls ($p = 0.0102$; Figure 3.2.F). The HF+11W+GOS interventions increased open arm duration, with the marked recovery ($p = 0.0100$; Figure 3.10.F) when compared to HF group.

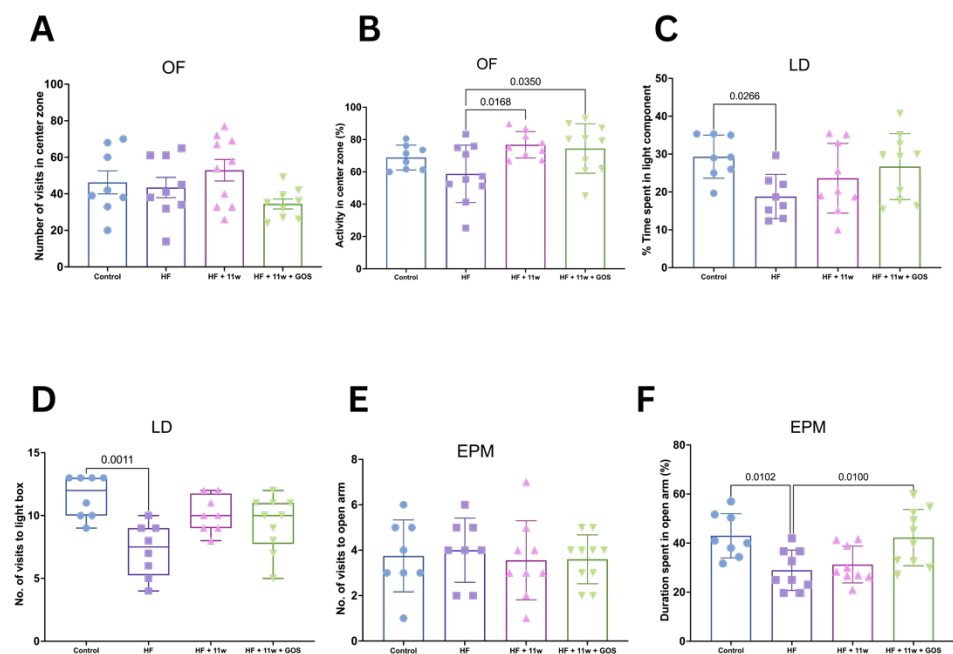


Figure 3.2. Effects of HFD, *L. paracasei* 11W supplementation, and GOS intervention on anxiety-like behaviors in female mice. (A) Number of visits to the center zone in the OF, (B) Percentage activity in the center zone in the OF, (C) The percentage time spent in the light compartment in the LD test. (D) Number of visits to the light box in the LD test. (E) Number of visits to the open arm in the EPM. (F) The percentage duration spent in the open arms in the EPM. Statistical analyses included Shapiro-Wilk tests for normality. One-way ANOVA (with F statistic, degrees of freedom, and Tukey's post hoc for normally distributed data), and Kruskal–Wallis plus Dunn's post hoc tests for non-normally distributed variables; significant p-values ($p < 0.05$) are indicated for pairwise comparisons. Data are shown as mean $\pm$ SEM ($n = 8$ – 10 per group). Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

3.11. Synbiotic and probiotic supplementation reverse HFD-induced deficits in memory, voluntary activity, and social interaction in female mice

Although a significant overall group effect was detected in the Y-maze alternation task (Kruskal–Wallis, $H = 10.54$, $p = 0.0145$), no individual group comparisons reached statistical significance. However, the HF+11w+GOS group showed higher variability and a greater median of alternations with synbiotic treatment (Figure 3.3.A). HF-fed mice exhibited a deficit in the NOR task (Kruskal–Wallis, $H = 18.91$, $p = 0.0003$), as revealed by a marked

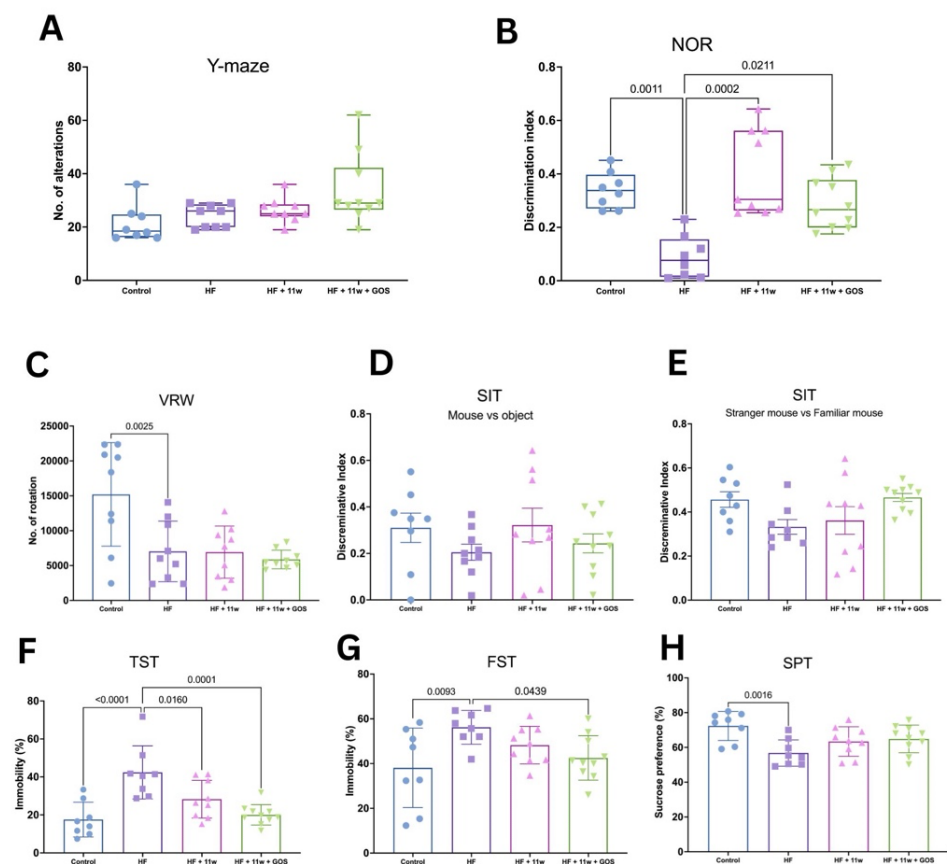


Figure 3.3. Assessment of Behavioral and cognitive profiling in female mice focusing on memory, exploratory activity, and sociability. (A) YM alternation task. (B) NOR: Discrimination index quantifies the ability to distinguish a novel object from a familiar object, a measure of recognition memory. (C) VRW to evaluate total physical activity: graphical representation of number of rotations. (D) SIT: mouse vs object: The discriminative index reflects preference for a mouse versus a novel object. (E) SIT: stranger mouse vs familiar mouse: Discrimination index for sociability and social novelty. Statistical analyses included Shapiro-Wilk tests for normality. One-way ANOVA (with F statistic, degrees of freedom, and Tukey's post hoc for normally distributed data), and Kruskal–Wallis plus Dunn's post hoc tests for non-normally distributed variables; significant p-values ($p < 0.05$) are indicated for pairwise comparisons. Data are shown as mean $\pm$ SEM ($n = 8$ – 10 per group). Control, blue; HF, purple; HF + 11w, pink; and HF + 11w + GOS, green.

reduction in the discrimination index compared to controls ($p = 0.0011$). Both HF+11w and HF+11w+GOS supplementation notably improved recognition memory performance versus HF-fed animals ($p = 0.0002$ and $p = 0.0211$,

respectively), restoring cognitive function to levels like controls (Figure 3.3.B). The total number of rotations measured for the interaction of diet and activity was prominent ($F(3,32) = 7.522, p = 0.006$). The VRW test revealed that HF mice exhibited significantly fewer wheel rotations than controls ($p = 0.0025$), indicating suppressed voluntary activity. The intervention groups displayed partial restoration of locomotor activity (Figure 3.3.C). In the SIT, both object interaction (mouse vs. object) and sociability (stranger mouse vs. familiar mouse) were conducted to observe the female mice's preference for a new environment. The outcomes did not differ prominently across groups. The female mice didn't reveal differences in mouse vs object ($F(3, 32) = 1.055, p = 0.3819$) (Figure 3.3.D) and for the stranger mouse vs. familiar mouse ($F(3, 31) = 2.720, p = 0.0614$) (Figure 3.3.E), with all cohorts showing a consistent preference for social or novel targets.

3.12. Effects of probiotic and synbiotic supplementation on HF-Induced depressive-like behaviors and anhedonia in female mice

HFD feeding induced pronounced depressive-like phenotypes and anhedonia in female mice, as evidenced by robust increased immobility in both the TST ($F(3, 31) = 10.68, p < 0.0001$; Figure 3.4.A) and FST ($F(3, 31) = 3.822, p = 0.0194$; Figure 3.4.B). Immobility in the TST rose to approximately 60% in HF-fed mice and was substantially greater than that of the control group (~25%; $p < 0.0001$; Figure 3.4.A). Supplementation with *L. paracasei* 11W (HF+11w) and combined synbiotic intervention (HF+11w+GOS) both significantly lowered immobility relative to HF ($p = 0.0160$ and $p = 0.0001$), approaching control values and indicating partial rescue of behavioral despair. In the FST, HF mice also exhibited considerably more immobility (~65%) versus controls (~45%; $p = 0.0093$; Figure 3.4.B), with both intervention groups showing reductions in immobility, reaching significance for HF+11w ($p = 0.0439$ vs. HF) and further improvement for HF+11w+GOS. The SPT revealed a substantial drop in sucrose preference among HF animals (~60%) compared to controls (~80%; $p = 0.0016$), consistent with anhedonia. Notably, both intervention cohorts showed sucrose preferences indistinguishable from those of control mice, supporting recovery of hedonic response. These results collectively demonstrate that HF triggers depressive-like states and reduced reward sensitivity, while synbiotic supplementation can alleviate these affective deficits in female mice.

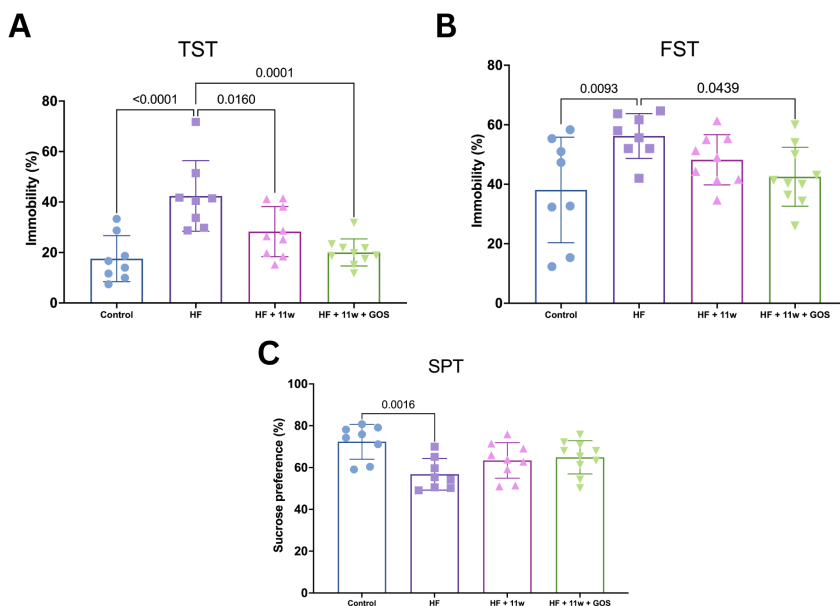


Figure 3.4. Effects of HFD and interventions on depressive-like behavior and anhedonia in female mice. (A) Tail Suspension Test (TST) immobility (%), (B) Forced Swim Test (FST) immobility (%), and (C) Sucrose Preference Test (SPT) sucrose preference (%). Data are presented as mean $\pm$ SEM ($n = 8-10$ per group). Statistical analyses included the Shapiro–Wilk test for normality followed by one-way ANOVA with appropriate post hoc multiple-comparison tests. Significant differences between groups are indicated as $p < 0.05$. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

3.13. *L. paracasei* 11W and GOS supplementation in HFD-fed mice modulate colonic pro-inflammatory cytokine expression

The HFD resulted in salient upregulation of pro-inflammatory cytokines in the mouse colon, compared to CD. For TNF- α non-normally distributed outcomes, a Kruskal-Wallis test was performed ($H = 9.324$, $p = 0.0253$) (Figure 3.5.A). Specifically, TNF- α levels were significantly increased in the HF group relative to the control group ($p = 0.0139$, Figure 3.5.A). Supplementation with *L. paracasei* 11W (HF+11W group) markedly reduced TNF- α levels compared to the HF group, approaching control values. Furthermore, for IFN- γ showed a prominent effect of diet with intervention, $F(3, 31) = 2.809$, $p = 0.0557$. Colonic IFN- γ concentration was also considerably elevated in the HF group compared to the HF+11W+GOS group ($p = 0.0325$, Figure 3.5.B). The addition of GOS to *L. paracasei* 11W further reduced IFN- γ expression, suggesting an additive anti-inflammatory effect.

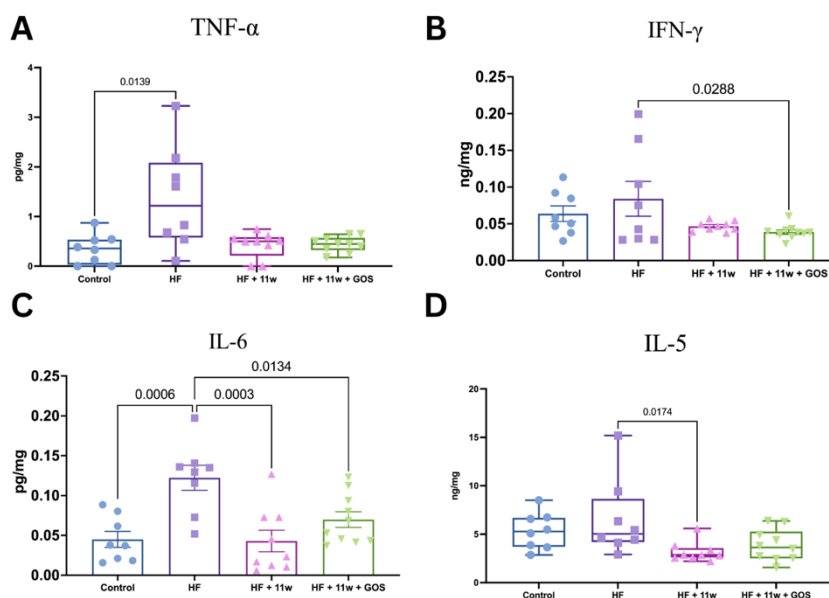


Figure 3.5. Effects of HFD and interventions on colonic levels of pro-inflammatory cytokines. Colonic cytokine concentrations of (A) TNF- α , (B) IFN- γ , (C) IL-6, and (D) IL-5 were quantified by ELISA in four experimental groups: Control, HF, HF+11W, and HF+11W+GOS. Data are shown as pg/mg or ng/mg tissue; individual values are overlaid. Statistical analyses included Shapiro-Wilk tests for normality. One-way ANOVA (with F statistic, degrees of freedom, and Tukey's post hoc for normally distributed data), and Kruskal–Wallis plus Dunn's post hoc tests for non-normally distributed variables; significant p-values are indicated for pairwise comparisons. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

For the IL-6 concentrations a substantial effect of diet with intervention was observed, $F(3, 31) = 8.325$, $p = 0.0003$. The IL-6 demonstrated the strongest pro-inflammatory response to HFD feeding, with the highest levels observed in the HF group. Both HF+11W and HF+11W+GOS groups showed significant decreases in IL-6 concentrations compared to the HF group ($p = 0.0003$ and 0.0134 , respectively). In contrast, for IL-5 concentrations, on-normally distributed outcomes, a Kruskal–Wallis test was performed ($H = 11.02$, $p = 0.0116$). The HF group values were elevated significantly relative to the HF+11W group ($p = 0.0174$, Figure 3.5.D), indicating that *L. paracasei* 11W supplementation also suppressed IL-5 overexpression induced by the HF diet. Whereas the HF+11W+GOS exhibits the potential in suppressing the HF-induced IL-5 expression, but no significance was observed. These data demonstrate that HF induces colonic inflammation, as shown by upregulated

pro-inflammatory cytokines, and that both *L. paracasei* 11W and its combination with GOS effectively ameliorate this inflammatory response (Figure 3.5).

3.14. Colon histology and morphometric analysis reveal HFD-induced changes reversed by *L. paracasei* 11W and GOS supplementation

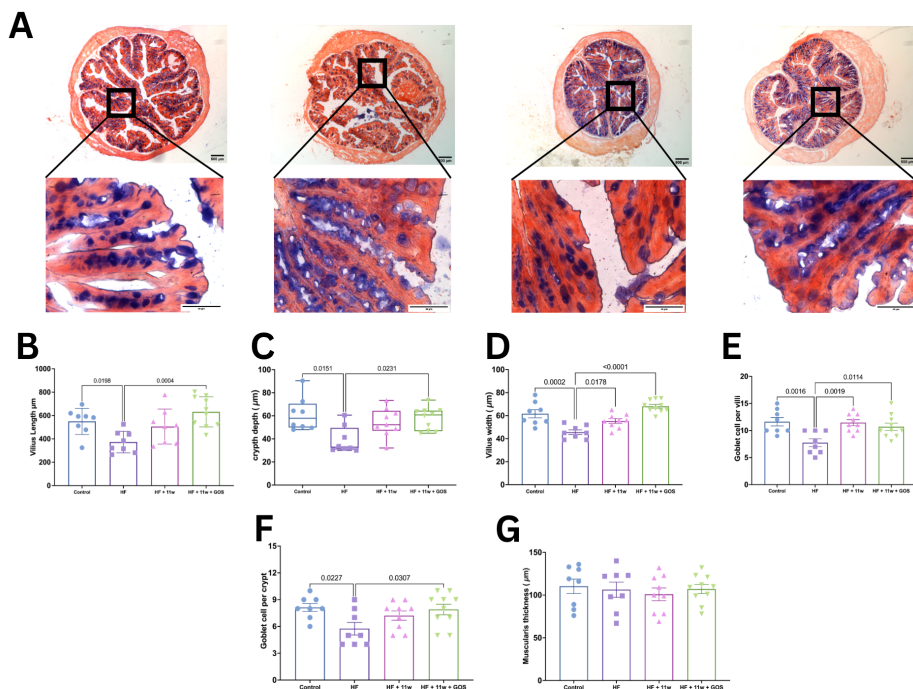


Figure 3.6. Quantitative analysis of colonic histology. (A) Representative AB PAS-stained cross-sections of colon tissue from each group, with magnified images highlighting mucosal architecture and goblet cell distribution (scale bars: upper, 500 µm; lower, 40 µm). (B–G) Quantitative morphometric parameters, shown as dot plots (mean ± SEM) or box plots based on data distribution: (B) Villus length, (C) Crypt depth, (D) Villus width, (E) Goblet cells per villus, (F) Goblet cells per crypt, (G) Mucous thickness. Statistical analyses included Shapiro-Wilk tests for normality. One-way ANOVA (with F statistic, degrees of freedom, and Tukey's post hoc for normally distributed data), and Kruskal–Wallis plus Dunn's post hoc tests for non-normally distributed variables; significant p-values are indicated for pairwise comparisons. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

Colon histological quantification revealed considerable morphometric alterations induced by HFD administration, with partial or complete

restoration by *L. paracasei* 11W and in combination with GOS supplementation. Villus length (Figure 3.6.B) was significantly decreased in HF mice compared to controls ($F(3,30) = 6.728$, $p = 0.0013$), and supplementation restored villus length in the HF+11W+GOS group ($p = 0.0004$). Crypt depth (Figure 3.6.C) also differed between the groups. The Kruskal–Wallis test revealed $H = 9.934$ and $p = 0.0191$, with improvement following synbiotic intervention (HF+11W+GOS: $p = 0.0231$). Villus width (Figure 3.6.D) was notably reduced by HF feeding ($F(3,31) = 17.05$, $p < 0.0001$), with both interventions restoring width with HF+11W ($p = 0.0002$) and HF+1W+GOS ($p = 0.0178$). Goblet cell counts per villus revealed HFD significantly reduced the count ($F(3,31) = 6.484$, $p = 0.0016$; Figure 3.6.E). The intervention groups HF+11W ($p = 0.0019$) and HF+11W+GOS ($p = 0.0114$) restored the goblet cells count like the control groups ($p = 0.0016$). Correspondingly, the goblet cell count per crypt was significantly reduced in HF groups ($F(3,31) = 3.293$, $p = 0.0334$), whereas the intervention group HF+11W+GOS ($p = 0.0307$) improved the goblet cell count as the control group ($p = 0.0227$). Mucous thickness (Figure 3.6.G) was also assessed, showing a trend towards an increase in intervention groups, though not reaching statistical significance in all comparisons. Overall, histological quantification demonstrates that HFD impairs colon morphology and goblet cell abundance, while interventions with *L. paracasei* 11W and GOS counteract many of these detrimental changes.

3.15. Impact of HFD and GOS supplementation on liver histology and metabolic parameters in female mice

Difference in the liver morphology was observed in the different administration groups, by visceral lipid accumulation in the HFD fed mice (Figure 3.7.A). HFD markedly induced hepatic morpho pathological changes, steatosis, and metabolic disturbance in female mice (Figure 3.7.A). Steatosis grade (Figure 3.7.B) was increased in HF compared to control group ($F(3,31) = 42.51$, $p < 0.0001$), with both intervention groups showing substantially reduced steatosis grade. The HF+11W+GOS exhibited a significant reduction ($p = 0.0343$) as compared to the HF group, whereas the HF+11W group showed a potential reduction the steatosis. Ballooning score (Figure 3.7.C) was notably higher in HFD mice (Kruskal–Wallis, $H = 21.08$, $p = 0.0001$). Both treatments reduced hepatocyte ballooning, but no significance was observed.

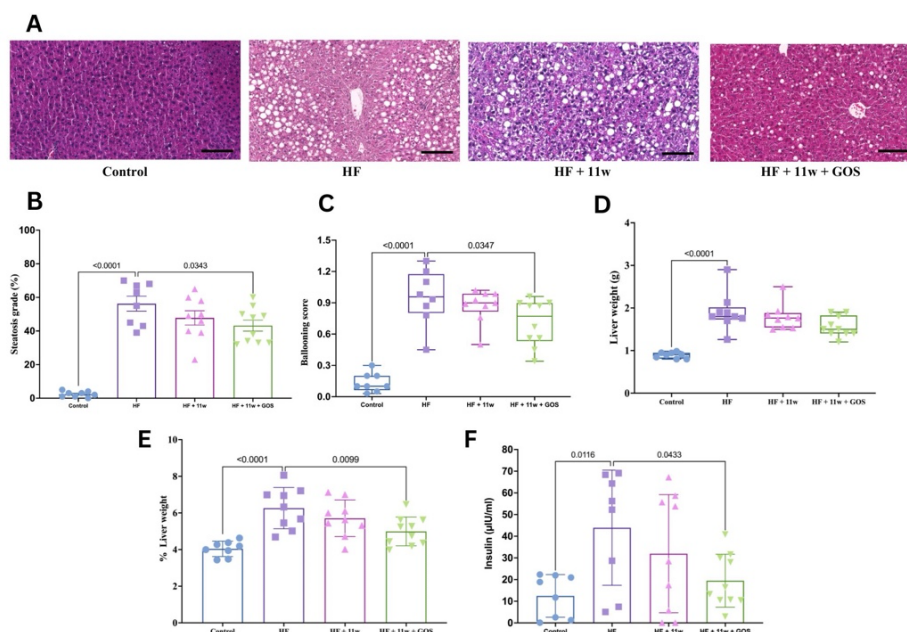


Figure 3.7. Quantitative analysis of liver histology and serum insulin parameters in experimental groups. (A) Representative H&E-stained liver sections showing hepatic morphology and steatosis (scale bar = 200 μ m; original magnification $\times 200$). (B–F) Quantitative assessment of hepatic and metabolic parameters presented as bar graphs (mean $\pm$ SEM) or box plots according to data distribution: (B) steatosis grade (%), (C) hepatic ballooning score, (D) liver weight (g), (E) liver weight (% body weight), and (F) serum insulin concentration (μ IU/mL). Histological quantification was performed on liver sections ($n = 3$ per group). Statistical analyses included the Shapiro–Wilk test for normality, followed by one-way ANOVA with Tukey’s post hoc test for normally distributed data or Kruskal–Wallis test with Dunn’s multiple-comparison test for non-normally distributed data. Significant differences are indicated as $p < 0.05$. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

3.16. HFD increases hippocampal pro-inflammatory cytokine expression with modulation by *L. paracasei* 11W and GOS

Hippocampal cytokine analysis revealed that an HFD significantly increased pro-inflammatory cytokine expression in the brain. Whereas, with only partial normalization upon *L. paracasei* 11W or synbiotic supplementation. *Il-6* mRNA expression (Figure 3.8. A) showed a difference between the diets ($F(3, 12) = 13.06$, $p = 0.0004$). *Il-6* expression was significantly elevated in the HFD group compared to controls ($p = 0.0001$), and between HF and HF+11W ($p = 0.0226$). The supplementation with *L. paracasei* 11W attenuated HF-

induced *Il-6* upregulation. The HF+11W+GOS group also showed lower levels of *Il-6* compared to HF, but no significance was observed.

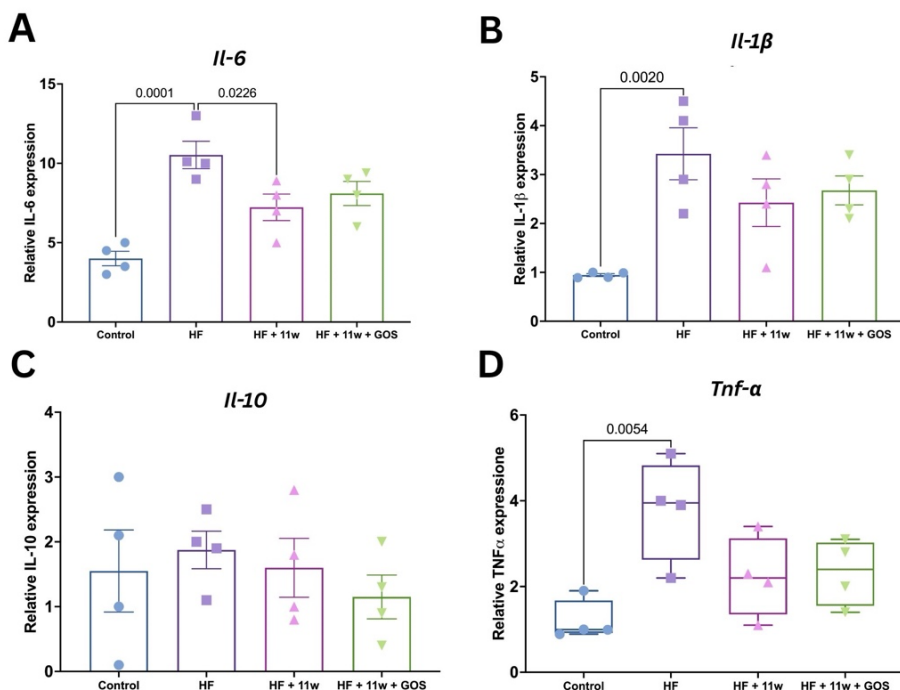


Figure 3.8. Cytokine gene expression in the hippocampus following dietary interventions. Relative mRNA expression levels of (A) *Il-6*, (B) *Il-1β*, (C) *Il-10*, and (D) *Tnf-α* were quantified in hippocampal tissue from four groups. Bar graphs (A–C) depict for normally distributed data (analyzed by one-way ANOVA with Dunnett's multiple comparisons test); box plot (D) displays non-normally distributed TNF- α expression. Statistical analyses included Shapiro-Wilk tests for normality. One-way ANOVA (with F statistic, degrees of freedom, and Tukey's post hoc for normally distributed data), and Kruskal–Wallis plus Dunn's post hoc tests for non-normally distributed variables; significant p-values are indicated for pairwise comparisons. Data are shown as mean $\pm$ SEM (n = 4 per group). Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

Il-1β expression (Figure 3.8.B) was also increased in the HF group. There was an observable difference between the control and HF groups ($p = 0.0020$). Intervention groups displayed a lower mean *Il-1β* compared to HF, though without significance in pairwise comparisons. No statistically significant differences were detected in anti-inflammatory *Il-10* expression (Figure 3.8.C) across groups, suggesting the interventions did not substantially alter IL-10 under these dietary conditions. For *Tnf-α* (Figure 3.8.D), significant group

differences (Kruskal–Wallis, $H = 9.772$, $p = 0.0061$). There was an identified markedly increased *Tnf- α* in HF mice compared to control group ($p = 0.0054$), with both intervention groups displaying reduced *Tnf- α* levels that did not differ significantly from HF. These results demonstrate that an HFD induces a pronounced hippocampal pro-inflammatory cytokine response, particularly for *Il-6*, *Il-1 β* , and *Tnf- α* . Supplementation with *L. paracasei* 11W and synbiotics partially reversed these changes, most notably for *Il-6*.

3.17. HFD induces cecal microbiota dysbiosis with partial restoration by extended feeding of *L. paracasei* 11W and GOS supplementation

Within the mice cecal samples, microbial diversity was assessed using Observed richness index, the Simpson index, and the Shannon index across the four experimental groups (control, HF+11w, and HF+11w+GOS). Across all three metrics, the HFD alone did not lead to a significant reduction in alpha diversity compared with the control group, as confirmed by Wilcoxon rank-sum tests. The observed richness index showed a modest decline of diversity in the HF+11w group relative to HF, although these differences were not statistically significant. Supplementation with GOS following long-term HFD feeding partially restored richness, with values approximating those of the HF group (Figure 3.9).

We noticed a comparable trend emerged for the Simpson index. Diversity in the HF group remained like controls, whereas the HF+11w group exhibited a slight increase in evenness. The HF+11w+GOS group showed diversity levels consistent with the HF+11w group, indicating that community evenness was broadly preserved across dietary interventions. For the Shannon index, both HF and HF+11w groups demonstrated slightly elevated values relative to controls. The HF+11W+GOS group showed alpha-diversity values comparable to those observed in the HF and HF+11W groups. Although numerical differences were observed among the groups, these differences were not statistically significant, indicating that the interventions had only limited effects on overall microbial alpha diversity. Some pairwise comparisons suggested moderate shifts (e.g., HF vs. HF+11w; HF vs. HF+11w+GOS), the overall pattern indicated that dietary interventions induced subtle rather than pronounced alterations in microbial diversity.

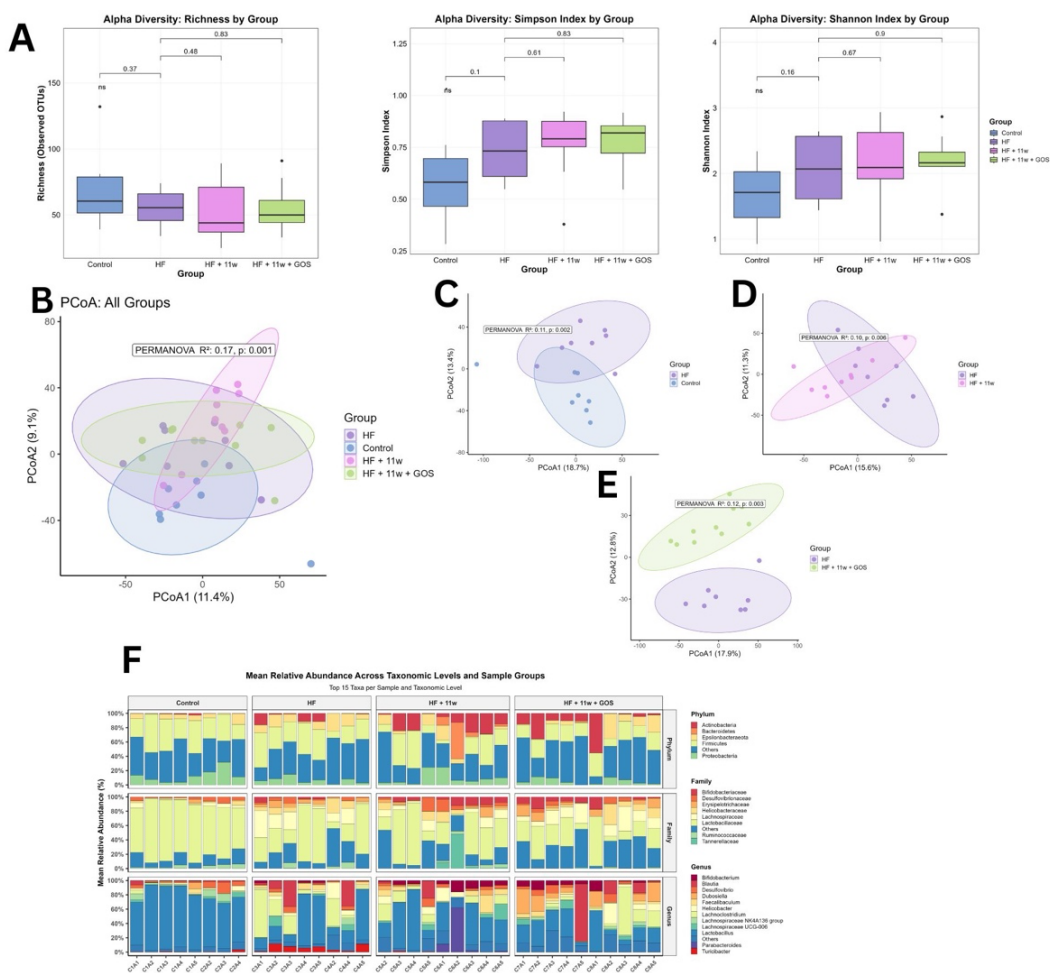


Figure 3.9. Alpha and beta diversity and Taxonomic composition of the GM across dietary groups. Alpha diversity (richness, Simpson, Shannon) did not differ significantly between HF and control mice, although a non-significant trend toward reduced diversity was apparent for some indices; the 11W intervention and GOS supplementation did not produce statistically significant restoration (A). PCoA plots (Aitchison distance (Euclidean distance on centred-log-ratio-transformed genus-level abundances)) reveal significant differences in community structure between groups. HF mice clustered separately from controls, the 11w intervention shifted the microbiota toward recovery, and the addition of GOS produced the most distinct improvement (PERMANOVA significant for all comparisons) (B–E). (F) presents stacked bar plots showing the mean relative abundance of the top 15 taxa at the phylum, family, and genus levels for individual samples.

Furthermore, differences in microbial community composition among the experimental groups were assessed by PCoA based on Aitchison distance

(Euclidean distance on centred-log-ratio-transformed genus-level abundances). Permutational multivariate analysis of variance (PERMANOVA) was applied to quantify group-level differences in overall community composition. Overall comparison across all four groups (control, HF, HF+11w, and HF+11w+GOS), we observed a significant separation of microbial communities ($R^2 = 0.17$, $p = 0.001$), indicating that dietary interventions exerted measurable effects on the GM structure (Figure 3.9.B). Although the groups showed partial overlap, each diet condition formed a distinguishable cluster, demonstrating that the four treatments contributed to detectable multivariate variation.

Pairwise comparisons further clarified these shifts. The HF diet produced a significant compositional difference relative to the control group ($R^2 = 0.11$, $p = 0.003$; Figure 3.9.C), suggesting that HFD feeding altered the overall microbial configuration despite minimal changes in alpha diversity. Long-term HFD feeding with *L. paracasei* 11W (HF+11w) also remained distinct from controls ($R^2 = 0.13$, $p = 0.006$; Figure 3.9.D), indicating a sustained restructuring of the microbial community over the intervention period.

Importantly, supplementation with synbiotic intervention after long-term HFD feeding (HF+11w+GOS) resulted in a community structure that diverged significantly from HF+11w alone ($R^2 = 0.12$, $p = 0.003$; Figure 3.9.E). This separation suggests that GOS introduced an additional shift in multivariate space, potentially reflecting a modulatory effect on taxa affected by long-term HFD feeding. Overall, beta-diversity analyses indicate that the HFD induces significant community-level alterations and that both the duration of HFD exposure and the addition of synbiotic intervention further reshape the GM landscape.

Across all taxonomic levels, HFD feeding induced a pronounced shift in GM composition (Figure 3.9.F). In control of animals, the community was dominated by *Firmicutes* and *Bacteroidetes* with stable distributions across samples. HFD feeding distinctly increased the proportion of *Firmicutes* and reduced *Bacteroidetes*, alongside a higher contribution from *Actinobacteria* and *Proteobacteria*. These alterations were only partially corrected by the 11w intervention. In contrast, the combined intervention with synbiotics produced a clearer recovery, restoring the phylum-level structure toward that of controls, as also reflected by the group-level mean abundances. At the family and genus levels, HFD feeding led to enrichment of *Lachnospiraceae*, *Ruminococcaceae*, *Blautia*, and *Turicibacter*, accompanied by a marked

reduction in *Bacteroidaceae*, *Lactobacillaceae*, *Bifidobacterium*, and *Lactobacillus* (Figure 3.9.F). The *L. paracasei* 11w intervention attenuated these shifts, whereas synbiotic supplementation produced the strongest correction, with notable enrichment of *Bifidobacteriaceae* and related saccharolytic genera. Overall, the combined intervention most effectively counteracted HF-induced dysbiosis, aligning both individual-sample profiles and group-level mean phylum abundances with the control microbiota.

Analysis of cecum samples by 16S rRNA sequencing revealed that HFD feeding induced marked shifts in several dominant genera. These shifts were differentially modulated by *L. paracasei* 11W and its combination with GOS. The nine genera presented in detail (*Lactobacillus*, *Blautia*, *Lachnoclostridium*, *Helicobacter*, *Turicibacter*, *Bifidobacterium*, *Dubosiella*, *Faecalibaculum*, and *Lachnospiraceae* NK4A136) were selected because they satisfied two conditions jointly: (i) a statistically significant Kruskal–Wallis group effect ($p < 0.05$) in this dataset, and (ii) prior literature evidence linking the genus to either HFD-induced dysbiosis/metabolic disturbance (e.g., *Blautia*, *Lachnoclostridium*, *Turicibacter*, *Helicobacter*) or to a beneficial, fibre/SCFA-associated profile (e.g., *Lactobacillus*, *Bifidobacterium*, *Dubosiella*, *Faecalibaculum*, *Lachnospiraceae* NK4A136), so that the selection reflects neither a purely data-driven ‘top hits’ list nor a purely literature-based a priori list, but the intersection of the two. *Lactobacillus* abundance (Figure 3.10.A) was remarkably reduced in HFD mice (Kruskal–Wallis, $H = 13.21$, $p = 0.0042$). When compared to control and HF group the *Lactobacillus* genera showed a significant reduction ($p = 0.0025$). HF+11W and HF+11W+GOS groups displayed partial restoration of *Lactobacillus* relative abundance towards control levels, although there was no significance when compared to HF group.

HFD promoted the abundance of several genera which are associated with metabolic disturbance. The genus *Blautia* and *Lachnoclostridium* (Kruskal–Wallis, $H = 14.15$, $p = 0.0027$) were both enriched in HF mice. *Blautia* revealed significantly higher in HF group when compared to HF+11W+GOS ($p = 0.0488$) (Figure 3.10.B). A higher *Lachnoclostridium* genus was observed in HF compared to control ($p = 0.0013$), as well as when compared to HF+11W+GOS ($p = 0.0494$) (Figure 3.10.C). *Helicobacter* genera (Figure 3.10.D) abundance was similarly increased in HF when compared with control ($p = 0.0353$). The HF+11W+GOS showed a marked reduction in *Helicobacter* genera, indicating suppression under synbiotic treatment. Moreover,

Turicibacter (Figure 3.10.E) exhibited strongest diet-associated shifts (Kruskal–Wallis, $H = 24.20$, $p < 0.0001$). It had a pronounced increase in HF group and notable depletion in HF+11W ($p = 0.004$) and HF+11W+GOS ($p < 0.0001$) groups. In contrast, beneficial genera, *Bifidobacterium*, were significantly promoted by probiotics and synbiotics treatment (Kruskal–Wallis, $H = 20.30$, $p = 0.0001$). *Bifidobacterium* genera were low in control and HF group (Figure 3.10.F). There was a significant increase in HF+11W ($p = 0.0291$) and HF+11W+GOS ($p = 0.0242$) when compared to the HF group (Figure 3.10.F).

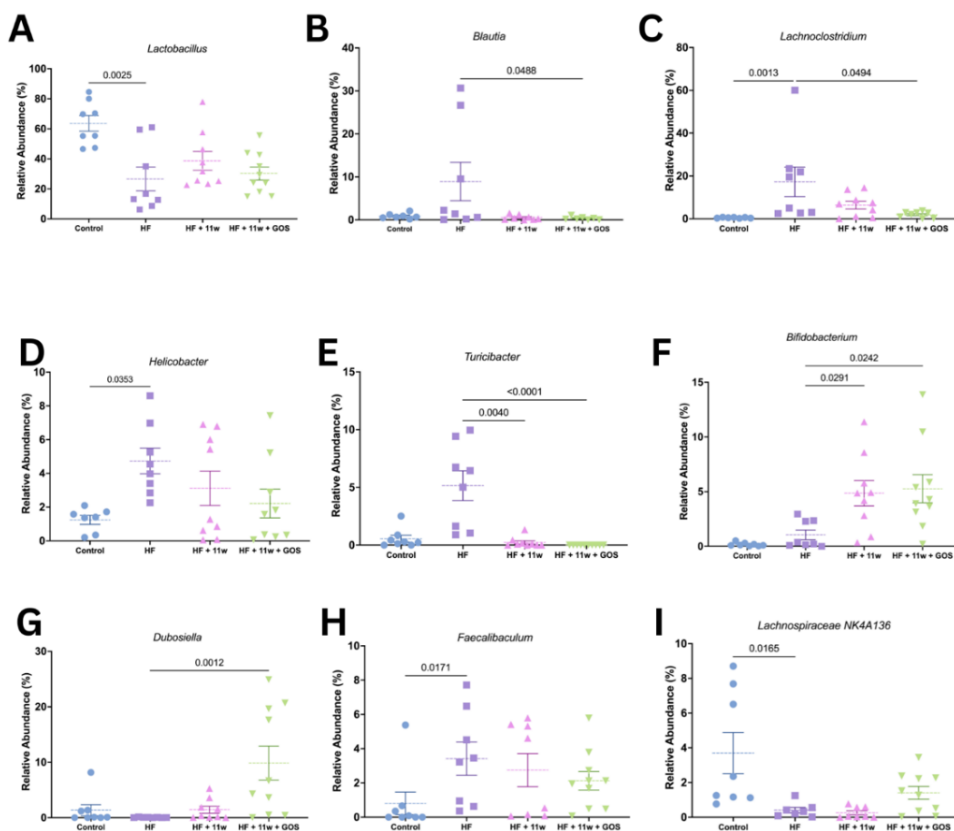


Figure 3.10. Relative abundance percentage of selected bacterial genera in fecal microbiota. Scatter plots show individual mice with mean $\pm$ SEM of the relative abundance (%) for (A) *Lactobacillus*, (B) *Blautia*, (C) *Lachnospiraceae*, (D) *Helicobacter*, (E) *Turicibacter*, (F) *Bifidobacterium*, (G) *Dubosiella*, (H) *Faecalibaculum*, and (I) *Lachnospiraceae* NK4A136. Relative abundances were derived from 16S rRNA gene amplicon sequencing. Group differences were assessed using Kruskal–Wallis tests followed by Dunn’s multiple-comparison test; exact p-values ($p < 0.05$) for significant pairwise comparisons are indicated above the

corresponding brackets. Control, blue; HF, purple; HF + 11W, pink; and HF + 11W + GOS, green.

Microbial genera that are typically found in lean or fiber-rich diets were also affected by HFD. *Dubosiella* was significantly increased in intervention group (Kruskal–Wallis, $H = 13.65$, $p = 0.0034$). When compared to HF and HF+11W+GOS, the synbiotic supplementation significantly improved *Dubosiella* genera ($p = 0.0012$). Moreover, the *Faecalibaculum* (Figure 3.18.H) was reduced in HFD fed mice (Kruskal–Wallis, $H = 8.329$, $p = 0.0397$). When compared to control versus HF group there was a significant reduction in *Faecalibaculum* genera ($p = 0.0171$), while there was a partial recovery in both intervention groups. Finally, *Lachnospiraceae* NK4A136 was significantly depleted in HFD fed groups (Kruskal–Wallis, $H = 14.97$, $p = 0.0018$). When compared to HF versus control a significant reduction was observed ($p = 0.0165$), whereas an intermediate improvement was observed in HF+11W and HF+11W+GOS. HFD shifts the GM towards pro-inflammatory or dysbiosis genera. It also depletes the beneficial bacteria *Lactobacillus* and *Bifidobacterium* which produced SCFA. The intervention with *L. paracasei* 11W and the synbiotics (*L. paracasei* 11W + GOS) restores more favorable community structures.

3.18. Correlations between gut microbiota phylum populations, biological parameters, metabolic measurements, behavioral tests, colon and hippocampus inflammation.

In HF+11W + GOS group, the phylum the *Lactobacillus* showed a negative correlation with the carmine test ($R = -0.653$, $p < 0.05$). the phylum *Blautia* showed positive correlation to the body weight at 25 weeks ($R = 0.634$, $p < 0.05$). Moreover, *Lachnospiraceae* UGC-006 negatively correlated with iAUC GGT ($R = -0.634$, $p < 0.05$), liver weight ($R = -0.643$, $p < 0.05$) and moisture content in pellet ($R = -0.857$, $p < 0.01$), and *Helicobacter* negative correlation to cecum weight ($R = -0.681$, $p < 0.05$) and positive correlation to serum insulin levels ($R = 0.700$, $p < 0.05$). Moreover, the phylum *Dubosiella* was negatively correlated to Body weight at 25 weeks ($R = -0.750$, $p < 0.05$) and positively correlated to % liver weight ($R = 0.689$, $p < 0.05$). The *Faecalibaculum* negatively correlated with the serum insulin levels ($R = -0.673$, $p < 0.05$) (Figure 3.11. A).

In the correlation between the GM population and the behavioral tests conducted on mice, the phylum *Blautia* showed positive correlation to visits to center zone in OF (R= 0.817, $p<0.01$). Whereas *Lachnospiraceae* UGC006 showed negative correlation to the NOR DI (R= -0.700 , $p<0.05$) and *Bifidobacterium* showing negative correlation to FST % immobility (R= -0.763 , $p<0.05$). The *Parabacteroides* showed positive correlation to visits to center zone of OF (R= 0.862, $p<0.001$) (Figure 3.11.B).

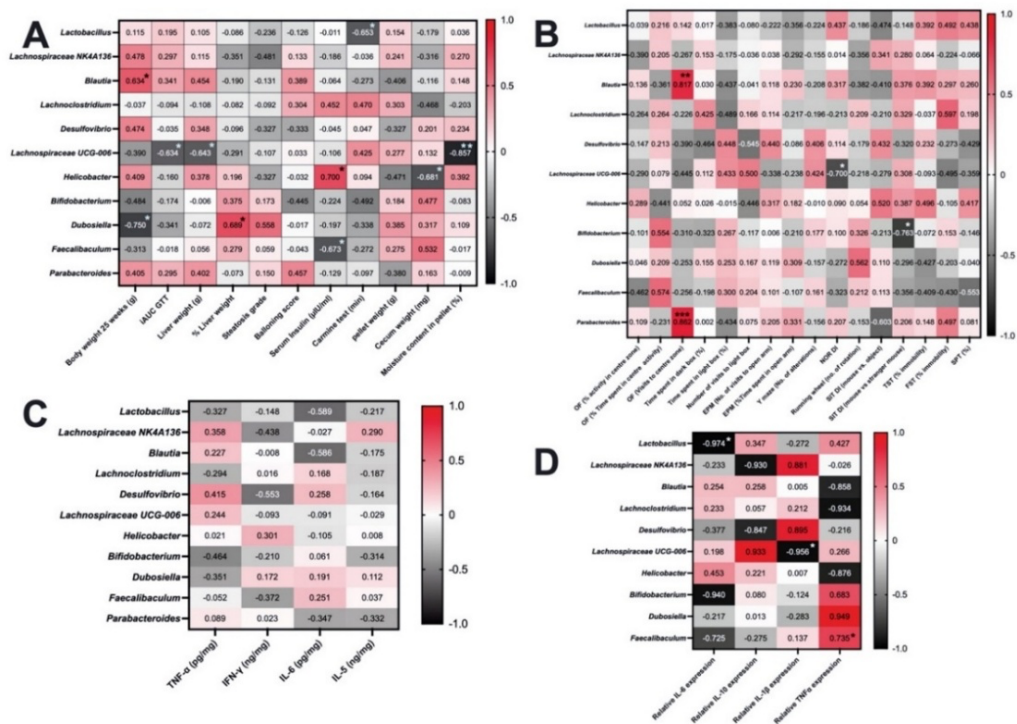


Figure 3.11. Spearman correlation heatmaps showing associations between GM genera and (A) biological and metabolic parameters, (B) behavioral outcomes, (C) colonic inflammatory cytokine expression, and (D) hippocampal inflammatory cytokine expression in female mice receiving HF+11W+GOS (synbiotic supplementation). Correlation coefficients (Spearman's r) are represented by the color scale ranging from -1 to $+1$. Positive correlations are indicated in red and negative correlations in black. Statistical significance is denoted by asterisks ($p < 0.05$, $p < 0.01$, $p < 0.001$).

For the cytokines expressed in hippocampal region, *Lactobacillus* negatively correlated with the IL-6 expression (R= -0.974 , $p<0.05$). The phylum *Lachnospiraceae* UGC-006 negatively correlated to IL-1 β (R= -0.956 ,

p<0.05) and *Facalibaculum* positively correlated to the TNF α expression (R=0.735, p<0.05) (Figure 3.11.D).

4. DISCUSSION

This thesis identified several LAB strains isolated from traditional Lithuanian FF with promising probiotic characteristics, including high GIT stress tolerance, adhesion capacity, antimicrobial activity, and favorable safety profiles. Among the strains evaluated, *Lacticaseibacillus paracasei* 11W emerged as the most promising candidate due to its ability to utilize prebiotic substrates, produce L-tryptophan, and satisfy key probiotic selection criteria. In an HFD-induced mouse model, supplementation with *L. paracasei* 11W, particularly in combination with GOS, improved glucose homeostasis, reduced hepatic steatosis and systemic metabolic disturbances, attenuated colonic and hippocampal inflammation, ameliorated anxiety-like, depressive-like, and cognitive impairments. These findings support the potential of food-derived LAB as probiotic and psychobiotic candidates and suggest that synbiotic supplementation may modulate the MGB axis to improve both metabolic and neurobehavioral outcomes.

4.1. Survival under gastrointestinal conditions: a critical determinant of probiotic efficacy

A fundamental requirement for a probiotic candidate selection is its ability to survive the harsh conditions of the GIT, including low gastric pH, digestive enzymes, and bile salts. In this study, LAB strains isolated from Lithuanian fermented vegetables and fruits were tested through a four-step *in vitro* simulated stress criteria's including pH 2.0, pepsin, 0.3% bile salts, and 1 mg/mL pancreatin. Several isolates demonstrated high survival under simulated gastrointestinal conditions, indicating strong tolerance to gastric acidity and digestive enzymes. The strong performance of *L. fermentum* UV16 suggests that this species may have effective stress-response systems that help it survive multiple GIT stress conditions (Bisson et al., 2023; Sionek et al., 2023b, 2024). Moreover, this observation is in line with other studies reporting that *L. fermentum* and related LAB species can retain high viability under acidic and enzymatic stress (Namshir et al., 2025). A low pH in gastric juices significantly influences the membrane pathway and cellular mechanisms of LAB by affecting their survival and functionality. LAB relies on the F1Fo-ATPase enzyme to regulate their intracellular pH and generate proton motive force (Corcoran et al., 2005). The differences among strains show that acid resistance is strain specific and depends on mechanisms such

as pH control inside the cell and bile salt hydrolase activity (Matsui & Cvitkovitch, 2010).

Several isolates also exhibited strong bile salt tolerance, supporting their potential to persist in the small intestine. The strain KA12 showed more than 90% survival at 0.3% bile salts, which indicates strong bile resistance and may be related to bile salt hydrolase activity. Such resistance may be associated with bile salt hydrolase activity, which is frequently linked to probiotic persistence in the small intestine (Kusada et al., 2021). High survival under pancreatin exposure further supports the ability of selected isolates to withstand intestinal digestive conditions. These results obtained show that Lithuanian fermented vegetables and fruits are promising source of robust LAB strains with probiotic potential (Ruiz-Ramírez et al., 2023). The stepwise screening method used here is more rigorous than testing only acid or bile tolerance separately, and it gives a better estimate of how well the strains may survive in the human GIT (Hassanzadazar et al., 2012). By exposing the isolates sequentially to low pH, pepsin, bile salts, and pancreatin, this approach better reflects the conditions that probiotic bacteria encounter during passage through GIT. This allows for the identification of strains that can remain viable across multiple digestive challenges rather than under a single stress condition alone. As a result, the multi-step screening strategy is likely to provide a more reliable indication of a strain's potential to survive GIT and subsequently colonize the host intestine.

4.2. Colonization potential of LAB isolates

Besides survival in the GIT, a probiotic must also be able to attach to IEC for a short time. This adhesion helps the strain compete with pathogens, interact with the host immune system, and persist longer in the gut. The ability of probiotic candidates to adhere to IEC surfaces is considered an important functional characteristic because it may facilitate transient colonization, enhance host–microbe interactions, and promote competitive exclusion of pathogenic microorganisms. Although permanent colonization is not required for probiotic efficacy, adhesion may increase the duration of probiotic–host interactions and thereby enhance biological activity within the gastrointestinal tract. In this study, adhesion was measured using HCT116 IECs, which are commonly used for probiotic adhesion tests. HCT116 cells are derived from human colorectal carcinoma and may exhibit differences in surface glycoprotein composition, receptor expression, and mucus production

compared to normal IEC (T. M. Yeung et al., 2010). Consequently, adhesion results obtained using this model should be interpreted as an initial screening of colonization potential rather than a direct representation of *in vivo* intestinal adhesion (Rosidi et al., 2023). In our study, several isolates demonstrated strong adhesion to IECs, exceeding commonly accepted criteria for probiotic selection. This variation may be due to differences in surface proteins such as EF-Tu and GAPDH, as well as differences in exopolysaccharide production (J. Wang et al., 2023). A similar adhesion range was observed in the study of numerous LAB strains on HCT116 cells (Emmawati et al., 2016).

For auto-aggregation testing, UV16 also showed the strongest auto-aggregation. High auto-aggregation suggests better ability to form stable communities and to compete with harmful bacteria in the intestine (Zawistowska-Rojek et al., 2022). Strong auto-aggregation can also be related to the formation of biofilms, creating stable communities that enhance probiotic survival and activity within the host's gastrointestinal tract (Chantanawilas et al., 2024). In addition to auto-aggregation, probiotic bacteria utilize exopolysaccharides, surface proteins, and various other adhesion molecules to bind to both host epithelial cells and pathogenic bacteria (Kaur & Dey, 2023; S. Singh et al., 2021). Although adhesion assays using epithelial cell lines provide a useful screening tool, successful colonization *in vivo* is influenced by additional factors, including host diet, mucus composition, microbial competition, and immune responses, which cannot be fully replicated *in vitro*.

L. fermentum UV16 and KA9 exhibited broad antimicrobial activity against both Gram-positive and Gram-negative pathogens. Similar findings have been reported for other LAB strains, where inhibition is attributed to the production of OA, hydrogen peroxide, and bacteriocin-like compounds (Anumudu, Miri, and Onyeaka 2024b; Cirat et al. 2024). The lower effect against *Salmonella enteritidis* may be related to its stronger resistance to acidic stress (Kostoglou & Giaouris, 2025).

4.3. Safety assessment and genomic characterization: alignment with EFSA standards

Safety assessment is critical component of probiotic strain selection. In this study, safety was evaluated using complementary phenotypic and WGS approaches. The phenotypic tests included hemolytic activity, mucin

degradation, gelatin hydrolysis, and antibiotic susceptibility. WGS was used to detect transferable AMR genes and VF at the genome level. This combined approach follows the EFSA recommendation for QPS evaluation (Rychen et al., 2018). Most of the tested strains showed no hemolytic activity and did not break down mucin or gelatin. This pattern is typical of many well-known probiotic *Lactobacillus* and *Pediococcus* strains (Baek et al., 2025). WGS also confirmed that most candidates did not carry transferable AMR genes or known VF. This included the main strains *L. paracasei* 11W, *L. fermentum* UV16, and *L. mesenteroides* KA15. However, *P. pentosaceus* KA12, and *L. plantarum* 33C showed plasmids and had broader antibiotic resistance profiles. This finding highlights the importance of strain-level safety assessment, as probiotic suitability cannot be inferred solely from species designation (Bothoulath et al., 2025). Similar findings have been reported in other food-associated LAB, where antimicrobial resistance genes may potentially be transferred through horizontal gene transfer (Rychen et al., 2018). Genome analysis of *L. plantarum* 33C also identified genes linked to stress tolerance, adhesion, and bile salt hydrolase activity, which matches its good survival in GIT stress tests. In addition, the genes *trpA* and *trpB* were found in *L. paracasei* 11W, *P. pentosaceus* KA12, and *L. mesenteroides* KA15. Enzymes encoded by the *trp* operon, particularly *trpA* and *trpB*, catalyze the final steps of converting indole-3-glycerol phosphate to L-tryptophan. The presence of these genes in the genomes of several isolates indicates their genetic capacity to synthesize tryptophan (Ferrer et al., 2022; Michalska et al., 2021).

4.4. Synbiotic Interactions: prebiotic utilization and tryptophan biosynthesis

Synbiotics are combinations of probiotics and a prebiotic that work better together than either one alone. In this study, the synbiotic potential of the LAB strains was tested by checking whether they could grow well on FOS and GOS, and whether these conditions increased tryptophan production, which is important for 5-HT synthesis. In batch I, all five selected strains grew better when GOS was added at 2–4%, showing that GOS is a suitable prebiotic for these isolates. In batch II, both FOS and GOS improved growth in four out of five strains, while KA12 preferred GOS more strongly. Many LAB strains differ in their ability to utilize prebiotics. The probiotic strain *L. plantarum* has consistently shown improved growth with both GOS and FOS (Cao et al., 2019). This shows that carbohydrate use differs from strain to strain, depending on the enzymes they have for breaking down these sugars (Cao et al., 2019).

Recent research on FOS and GOS supplementation indicates an increase in LAB populations while reducing *E. coli* and *Clostridium perfringens* in animal and human models (Chang et al., 2022; Davani-Davari et al., 2019). These LAB isolates possess specific enzymes that pathogenic bacteria lack, such as β -galactosidase and β -fructosidase, enabling them to metabolize FOS and GOS (Maske et al., 2021).

Tryptophan levels were evaluated by HPLC-MS in this study. Strain 11W produced the highest amount, $16.63 \pm 2.25 \mu\text{M}$, when grown with 4% GOS, which was higher than the other tested strains. *L. mesenteroides* KA15 also produced a notable amount of tryptophan, $10.48 \mu\text{M}$, with 5% FOS. Tryptophan is important because it is the starting compound used to make 5-HT (H. Gao & Zhou, 2020). Since tryptophan is a precursor for serotonin biosynthesis, increased tryptophan availability may influence host serotonergic signaling. However, serotonin concentrations, serotonergic signaling pathways, and tryptophan metabolism within the host were not directly assessed in the present study. Therefore, any potential effects of bacterial tryptophan production on host serotonin synthesis remain speculative and require experimental confirmation. Tryptophan biosynthesis in bacteria occurs through the shikimate pathway, which generates aromatic amino acids from central metabolic intermediates (Shende et al., 2024). In this pathway, phosphoenolpyruvate and erythrose-4-phosphate serve as key precursors leading to the formation of chorismate, the common precursor of aromatic amino acids including tryptophan (Tzin & Galili, 2010). The agreement between genomic identification of *trpA/trpB* genes and the observed tryptophan production strengthens the biological plausibility of the proposed psychobiotic properties of the selected strains. Importantly, the current findings demonstrate the capacity of selected strains to synthesize tryptophan under *in vitro* conditions but do not establish whether microbially produced tryptophan reaches sufficient concentrations *in vivo* to influence host neurotransmitter production or MGB axis signaling. Taken together, the ability of *L. paracasei* 11W to utilize GOS and produce tryptophan supports its potential as a psychobiotic candidate. However, the extent to which these properties contribute to host neurochemical, behavioral, or MGB axis outcomes remains to be determined. Future studies should investigate whether bacterial tryptophan production influences host tryptophan availability, serotonin metabolism, microbial tryptophan-derived metabolites, and MGB axis signaling *in vivo*. Such studies would help clarify whether the behavioral improvements observed following probiotic or synbiotic supplementation are

directly linked to tryptophan metabolism or are mediated through alternative mechanisms, including modulation of GM composition, SCFA production, or immune signaling pathways.

4.5. Metabolic effects: body weight, glucose homeostasis, and hepatic pathology

The 25-week dietary intervention in female C57BL/6J mice established a robust HFD-induced metabolic syndrome model, characterized by progressive obesity, fasting hyperglycemia, impaired glucose tolerance, hepatic steatosis, and elevated serum insulin. The selection of female mice is particularly relevant given the documented sexual dimorphism in HFD metabolic responses and increasing recognition that female-specific hormonal influences on the MGB axis produce distinct behavioral phenotypes compared to male rodents (Lefebvre et al. 2024).

Although supplementation with *L. paracasei* 11W alone or in combination with GOS did not significantly attenuate total weight gain relative to the HFD group at week 25, this finding is consistent with several other single-strain probiotic studies in severe obesity models (Álvarez-Arraño and Martín-Peláez 2021). Consistent with our findings, previous studies have reported that *L. paracasei* strains improve metabolic parameters in HFD-fed mice through modulation of the GM composition and inflammatory pathways, even without marked effects on body weight (Y. Sun et al., 2023). Moreover, the intervention significantly modulated glucose homeostasis. The synbiotic group (HF+11W+GOS) significantly reduced fasting blood glucose at weeks 5 and 25 compared with HP group, and GTT analysis revealed partial restoration of glucose clearance, particularly at 45 and 120 min post-challenge. The AUC for glucose excursion was significantly elevated in HF mice and showed intermediate reduction in intervention groups. These findings closely parallel outcomes reported for *L. paracasei* supplementation in murine T2D models (Dang et al., 2018) and support previous reports suggesting that certain *Lactobacillus* species may improve insulin sensitivity through modulation of GLP-1 secretion, SCFA signaling, and attenuation of intestinal LPS translocation (Dang et al., 2018). However, these pathways were not directly assessed in the present study and therefore should be considered potential mechanisms that warrant further investigation.

Hepatic pathology was markedly induced by HFD, manifesting as elevated steatosis grade, hepatocyte ballooning, increased liver weight, and hyperinsulinemia. Synbiotic supplementation significantly reduced steatosis grade, liver weight, and serum insulin concentrations compared with HF group animals. Recent studies adhere to these findings, where the *L. paracasei* strains have the potential to ameliorate HF-induced NAFLD by modulating GM composition and suppressing hepatic lipid accumulation (Ji et al., 2022; Teofilović et al., 2025). The reduction in hepatic steatosis observed following *L. paracasei* 11W and GOS supplementation is consistent with previous reports demonstrating beneficial effects of *Lactobacillus* species on hepatic lipid metabolism. Previous studies demonstrated that hepatic steatosis was attenuated by *Lactobacillus* species in reducing hepatic lipid accumulation through downregulation of lipogenic gene expression (SREBP-1c, FAS) and upregulation of fatty acid beta-oxidation pathways (Arellano-García et al., 2025; C. Tang et al., 2020). Because these molecular pathways were not evaluated in the present study, their contribution to the observed improvements in hepatic pathology remains speculative. The additive protective effect of GOS over probiotic alone may reflect GOS-driven enrichment of butyrate-producing colonic microbiota (Kalkan et al., 2025). However, SCFA concentrations and functional microbial metabolites were not measured in the present study, and therefore the mechanisms underlying the enhanced effects of the synbiotic formulation remain to be elucidated. Although several biological mechanisms described in the literature may explain the observed metabolic improvements, including modulation of microbial metabolites, host inflammatory pathways, and lipid metabolism, the present study was not designed to directly investigate these processes. Future studies incorporating metabolomic, transcriptomic, and mechanistic analyses will be necessary to determine the pathways through which *L. paracasei* 11W and GOS exert their effects.

4.6. Behavioral and cognitive outcomes associated with HFD-induced metabolic dysfunction

In this study, it was demonstrated that HFD-induced metabolic dysfunction in female mice is accompanied by behavioral and cognitive alterations, including anxiety-like behavior, increased immobility in behavioral despair paradigms, anhedonia-like responses, and impaired cognitive performance and that these behavioral impairments are substantially ameliorated by *L. paracasei* 11W supplementation, with additional benefit conferred by GOS supplementation.

We next assessed anxiety-like behaviors in female mice. In the OFT, HFD-fed mice showed reduced percentage activity in the center zone, consistent with anxiety-like avoidance behavior. Both probiotic and synbiotic interventions restored center-zone activity. The LD test further confirmed HFD-induced anxiety, with HF mice showing reduced time and entries into the illuminated compartment. In the elevated plus maze, HFD animals showed significantly reduced open arm duration, which was substantially reversed by synbiotic supplementation. The consistency of these findings across three independent anxiety paradigms employing distinct neurological underpinnings (neophobia in OFT, light aversion in LD, and height aversion in EPM) strengthens the conclusion that HFD induces genuine anxiogenic states rather than confounding changes in locomotion or arousal. These findings are consistent with previous work from our laboratory demonstrating that HFD-induced metabolic dysfunction in male mice is associated with altered microglial function and increased neuroinflammation (Vijaya et al., 2025). The present study extends these observations to female mice, showing that HFD-induced metabolic disturbances are also accompanied by behavioral impairments and that probiotic and synbiotic interventions can ameliorate these effects. Although the underlying mechanisms were not directly investigated, the behavioral improvements observed alongside changes in inflammatory and GM-related parameters support a potential role for MGB interactions in mediating these responses. Similarly, another study demonstrated that *L. paracasei* strains reduce anxiety-like behavior in ciliary trials (Y. Guan et al. 2025) exposed to chronic stress or diet-induced dysbiosis, consistent with your findings of improved exploration and reduced anxiety in probiotic/synbiotic-treated female mice (Y. Guan et al., 2025; Wu et al., 2025). The synbiotic improvement observed in our study mirrors findings where combining probiotics with prebiotics such as GOS potentiated anxiolytic effects versus probiotics alone, particularly in female rodents, possibly due to sex-dependent MGB axis interactions (Saadati et al., 2024). Additionally, reduced anxiety-like behavior and neuroinflammation in HFD-fed mice supplemented with probiotics, demonstrating that probiotic modulation decreases inflammatory markers like IL-6 and is linked to behavioral improvements in anxiety paradigms akin to LD (N. P. Schmidt et al., 2024; Sempach et al., 2024).

Depressive-like behavior, assessed through the TST and FST, was robustly elevated in HFD mice (TST immobility ~60%; FST ~65%), with both

interventions producing statistically significant reductions. Recent study by murine models supports these findings, demonstrating that probiotics and synbiotics are effective in alleviating depressive-like behaviors in preclinical models (J. Zhang et al., 2025). The SPT revealed pronounced anhedonia in HFD animals (~60% preference vs. ~80% in controls), fully reversed by both probiotic and synbiotic supplementation. Although the present study did not directly assess dopaminergic signaling, anhedonia is mechanistically linked to mesolimbic dopaminergic hypofunction and is among the most treatment-resistant symptoms in clinical depression (Ricci et al., 2025). Its reversal by *L. paracasei* 11W is therefore of translational relevance, though the specific molecular mechanisms whether mediated through 5-HT precursor availability, SCFA signaling, or vagal afferent modulation remain to be fully elucidated (L. Wang et al., 2023).

Cognitive outcomes assessed by NOR and YM demonstrated HFD-induced deficits in recognition memory and spatial working memory. Both probiotic alone and synbiotic supplementation restored NOR performance to control levels. This is consistent with evidence implicating hippocampal neuroinflammation driven by elevated IL-6 and IL-1 β as found in this study as a central mechanism in diet-induced cognitive decline (H. Wang et al., 2026)(Vijaya et al. 2024b). The VRW activity was suppressed by HFD and partially restored by interventions, suggesting systemic effects on motivational and energy homeostatic pathways beyond the strictly neuropsychiatric domain. These psychobiotic effects of *L. paracasei* 11W are mechanistically plausible given the strain's capacity for tryptophan biosynthesis, which can augment serotonergic neurotransmission, combined with its demonstrated anti-inflammatory properties that reduce neuroinflammatory cytokine expression (Del Toro-Barbosa et al., 2020). A meta-analysis analyzing 14 studies with depressive symptoms in both animals and humans revealed that probiotics and synbiotics significantly reduce depressive symptom scores, while prebiotics alone showed no significant effect (L.-Y. Dai et al., 2025).

4.7 Colonic and hippocampal inflammation: cytokine expression and histopathology

HFD feeding elevated pro-inflammatory colonic cytokine expression, with significant upregulation of TNF- α , IFN- γ , IL-6, and IL-5 compared to controls. *L. paracasei* 11W supplementation significantly reduced colonic

TNF- α , approaching control values, while the synbiotic combination further reduced IFN- γ and IL-6. The strongest effect was observed for IL-6, which showed the most pronounced HFD-induced upregulation and was significantly attenuated by both interventions. These data are consistent with previous reports demonstrating immunomodulatory capacity of *L. paracasei* species, which have been reported to reduce colonic TNF- α and IL-6 through Toll-like receptor 2 engagement, regulatory T cell induction, and enhanced production of anti-inflammatory IL-10 by colonic macrophages (Bosco et al. 2011; Y. Guo et al. 2023). However, these pathways were not directly evaluated in the present study and should therefore be considered potential mechanisms rather than confirmed explanations for the observed effects.

Colon histomorphometry revealed HFD-induced reductions in villus length, crypt depth, villus width, and goblet cell density. Goblet cell depletion is particularly significant, as these cells are the primary producers of the protective mucus layer that forms the first line of defense against luminal pathogens and bacterial endotoxin translocation (Raya Tonetti et al., 2024). Both interventions significantly restored goblet cell counts per villus in HF+11W, and HF+11W+GOS groups compared with HF group. The synbiotic combination further restoring crypt depth and goblet cell counts per crypt. Previous studies have reported that GOS fermentation can increase butyrate production, which has been associated with improved epithelial integrity and goblet cell function (Godínez-Méndez et al., 2025), because SCFA concentrations and microbial metabolites were not measured in the present study, the mechanisms underlying the improved histological outcomes remain speculative.

In the hippocampus, HFD significantly elevated *Il-6* and *Il-1 β* , with *Tnf- α* expression also markedly increased. *L. paracasei* 11W supplementation significantly attenuated hippocampal *Il-6*, while the synbiotic trend showed further *Il-6* reduction without reaching significance. The more modest effects observed in the hippocampus compared with the colon may reflect the complexity of gut-brain communication and the indirect nature of GM-mediated modulation of neuroinflammatory processes. (Gryka-Martón et al., 2025). Interestingly, similar findings were observed in a recent study from our laboratory using male APP/PS1 mice, a transgenic AD model, where supplementation with the synbiotic combination of *Akkermansia muciniphila* and GOS improved metabolic parameters, behavior, and hippocampal microglial cell abundance (Kunevičius et al., 2025). Despite differences in

disease model and probiotic strain, the beneficial effects observed in both studies suggest that GOS-containing synbiotic interventions may represent a promising strategy for modulating MGB axis. However, the indirect routes of MGB communication including vagal afferent signaling, circulating SCFA, tryptophan metabolites, and systemic LPS reduction remain active pathways through which the GM modulates neuroinflammation (Burokas et al., 2017b; Golubeva et al., 2017b).

4.8 Gut Microbiota remodeling: dysbiosis, diversity, and restoration

The microbiota analyses indicate that prolonged HFD exposure induced substantial alterations in community composition, despite having relatively limited effects on overall microbial richness and diversity. This suggests that HFD-associated dysbiosis in the present model was driven primarily by changes in microbial community structure rather than by a loss of microbial diversity. Similar observations have been reported in other murine HFD studies, where compositional shifts rather than reductions in alpha diversity were associated with metabolic dysfunction (Mamun et al., 2025)(Du et al., 2025). In contrast to alpha diversity, beta-diversity analyses demonstrated clear separation between dietary groups, indicating that HFD profoundly altered microbial community composition. Phylum-level changes featured HF-driven Bacteroidetes reductions, plus Proteobacteria rises, partially mitigated by HF+11W but strongly corrected by GOS toward controls. This is similar to a study of HF mouse models with PERMANOVA-detected beta separations and Firmicutes dominance despite alpha stability, linking to metabolic dysbiosis (Perez-Munoz et al., 2019). Cecum-specific HF effects, like Bacteroidetes loss, match rodent studies emphasizing site-specific resilience versus fecal changes (W. Du et al. 2025; Mamun et al. 2025).

Prolonged HF exposure sustained these compositional shifts, whereas GOS supplementation induced an additional, distinct modulation of the GM genera. A major finding of this study was that synbiotic supplementation produced a more pronounced restoration of HFD-induced dysbiosis than probiotic administration alone. Although *L. paracasei* 11W partially attenuated microbial alterations, the addition of GOS promoted a broader restructuring of the GM toward a community profile more closely resembling that of control animals. This observation supports the concept that prebiotics provide ecological support for beneficial microorganisms and may enhance the efficacy of probiotic interventions. A similar pattern was observed in a recent

study from our laboratory using a synbiotic intervention consisting of *Akkermansia muciniphila* and GOS in APP/PS1 mice, where GOS supplementation contributed also to microbiota remodeling (Kunevičius et al., 2025). Comparable to a HF and western diet rodent study where GOS restored cecum profiles, enriched *Lachnospiraceae* and *Bifidobacterium*, and boosted SCFA versus standalone diets (Choi et al. 2021; Sulistyowati et al. 2022). Probiotic interventions with *Lactobacillus* spp. partially restored beta diversity; however, recovery of the GM at the phylum level was less pronounced in the absence of GOS supplementation (Huang et al. 2023; Ponpichai et al. 2025; Zhuang et al. 2025). Synbiotic supplementation in this study promotes *Bifidobacterium*, resembles studying on human and mouse prebiotic trials shifting communities post-HF-like stressors (Johnstone et al. 2021; Looijesteijn et al. 2024). Collectively, these findings suggest that prolonged HFD exposure induces persistent microbiota alterations, whereas probiotic and particularly synbiotic supplementation may partially restore microbial community structure.

At the genus level, HFD was associated with depletion of several taxa commonly linked to intestinal homeostasis and enrichment of taxa frequently associated with dysbiosis and inflammation. Synbiotic supplementation partially reversed these alterations by increasing the abundance of beneficial microorganisms while reducing taxa associated with inflammatory and metabolic disturbances. Such shifts are consistent with previous reports indicating that restoration of a more balanced microbial community may contribute to improvements in metabolic health and immune regulation (J.-K. Jo et al. 2021; Yi et al. 2025). The synbiotic administration enhanced *Bifidobacterium* similarly to previous study alleviating HF dysbiosis via SCFA-producers (Yi et al., 2025). A notable feature of the synbiotic intervention was the enrichment of taxa commonly associated with saccharolytic metabolism and short-chain fatty acid production, together with a reduction in microorganisms frequently linked to inflammation and HFD-induced dysbiosis. Similar microbiota remodeling has been reported in previous studies of prebiotic and synbiotic supplementation, where enrichment of beneficial microbial populations was associated with improved gut barrier function, reduced inflammatory responses, and enhanced metabolic outcomes (He et al. 2022; J.-K. Jo et al. 2021; Mamun et al. 2025). Importantly, these microbial shifts occurred in parallel with improvements in glucose homeostasis, inflammatory markers, and behavioral outcomes in the present study, suggesting a potential association between restoration of

microbial community structure and improved host physiology. However, causal relationships between specific microbial changes and host responses cannot be established from the present findings.

Overall, microbiota findings indicate that synbiotic supplementation was more effective than probiotic administration alone in attenuating HFD-induced dysbiosis and promoting restoration of microbial community structure. The observed shifts were characterized by enrichment of taxa generally associated with intestinal homeostasis and metabolic health, together with reductions in taxa commonly linked to inflammation and metabolic dysfunction. Importantly, these changes occurred in parallel with improvements in glucose homeostasis, hepatic pathology, inflammatory markers, and behavioral outcomes. Collectively, these findings support a potential association between microbiota remodeling and the beneficial physiological effects observed following synbiotic supplementation (Song et al. 2023; Yi et al. 2025). However, as the present study was not designed to establish causality, the specific contributions of individual microbial taxa to these host responses remain to be determined.

Correlation analyses were performed within the synbiotic group to explore associations between gut microbial taxa and metabolic, behavioral, and inflammatory outcomes. These analyses were intended to generate hypotheses regarding microbiota–host relationships rather than to establish causal mechanisms. Overall, the observed correlations suggest that taxa enriched following synbiotic supplementation were generally associated with more favorable metabolic profiles, whereas taxa linked to HFD-induced dysbiosis tended to associate with adverse metabolic parameters. These associations are consistent with previous literature linking GM composition with intestinal transit, glucose regulation, adiposity, and inflammatory status (Calvete-Torre et al. 2024a; L. Jia et al. 2024; Y. Li et al. 2025; Maleki et al. 2025).

Correlation analyses identified associations between specific microbial taxa and behavioral outcomes in the synbiotic group. However, these findings should be interpreted as exploratory and hypothesis-generating rather than evidence of causal microbiota-behavior relationships. The positive correlation observed between *Lactobacillus* and percentage immobility in the FST parallels studies in chronic stress models where *Lactobacillus* abundance has been associated with altered depression-like behaviors (A. Tang et al., 2025). *Blautia*'s positive association with OF visits to the center zone and negative

association with LD time spent in the light box may indicate an association between this genus and anxiety-related exploratory behavior. Previous studies have also linked *Blautia* to anxiety symptom trajectories, suggesting potential relevance for affective regulation (Hieta et al., 2025). The negative correlation between *Lachnospiraceae* UCG-006 and NOR DI aligns with emerging evidence that specific Lachnospiraceae genera have been associated with cognitive-related outcomes. This may involve SCFA-related modulation of neuroinflammation and synaptic plasticity; however, SCFA were not measured in the present study, and therefore this mechanism remains speculative (Calvete-Torre et al., 2024b). *Parabacteroides* positive association with OF outcomes is consistent with dietary and obesity studies associating this genus with behavioral and metabolic regulation (K. Wang et al., 2019).

Correlations between microbial taxa and colonic or hippocampal cytokine expression suggest potential links between GM composition and inflammatory status. Several taxa enriched following synbiotic supplementation were associated with lower expression of pro-inflammatory cytokines, whereas taxa associated with HFD-induced dysbiosis tended to show associations with inflammatory markers. These findings are consistent with previous studies linking GM composition to both intestinal and neuroinflammatory processes (L. Jia et al. 2024; Paiva et al. 2024). Importantly, these microbiota-cytokine associations occurred alongside improvements in glucose homeostasis, colonic inflammation, hippocampal cytokine expression, and behavioral outcomes. This observation supports the possibility that microbiota remodeling contributed to the overall physiological benefits observed following probiotic and synbiotic supplementation. However, the present analyses cannot determine whether specific microbial taxa directly regulate inflammatory pathways or whether both microbial and inflammatory changes reflect broader responses to dietary intervention.

Therefore, these findings should be regarded as hypothesis-generating and provide a basis for future mechanistic investigations examining the interactions between GM composition, inflammatory signaling, and MGB axis function. Further studies incorporating microbial metabolite profiling, immune-cell characterization, and causal intervention approaches will be required to determine the specific mechanisms underlying these associations.

In conclusion, this thesis identified and characterized novel LAB strains from traditional Lithuanian FF with probiotic and synbiotic potential. Several

isolates demonstrated key probiotic properties, including survival under simulated gastrointestinal conditions, adhesion to IEC, antimicrobial activity, and compliance with EFSA QPS safety requirements. Genomic and metabolite analyses further revealed functional traits associated with probiotic activity, including prebiotic utilization and tryptophan biosynthesis. Among the strains evaluated, *Lacticaseibacillus paracasei* 11W emerged as the most promising candidate. In a female mouse model of HFD-induced metabolic dysfunction, *L. paracasei* 11W, particularly in combination with GOS, improved glucose homeostasis, reduced hepatic steatosis and inflammation, ameliorated behavioral impairments, and partially restored GM dysbiosis. The synbiotic intervention was more effective than the probiotic alone at reversing HFD-induced microbiota alterations, and these changes were associated with improved metabolic, inflammatory, and behavioral outcomes. Overall, this work provides a scientific basis for the development of novel probiotic and synbiotic interventions targeting MGB axis dysfunction and highlights traditional Lithuanian FF as a valuable source of functionally relevant microbial strains.

LIMITATIONS AND FUTURE DIRECTION

Despite the scientific investigation and multidimensional scope of this study, several limitations should be acknowledged. First, all *in vivo* experiments were performed exclusively in female mice. While this was intentional and allowed sex-specific biological responses to be examined, the findings cannot be directly extended to male subjects. Since males and females are known to respond differently to HFD and gut-brain signaling, future studies should include male mice for comparison and to determine whether the observed effects are sex-dependent.

Second, the behavioral tests used in this study are indirect measures of conditions such as depression and anxiety in humans. The gap between animal behavior models and human psychiatric disorders remains a recognized challenge in psychobiotic research, and caution is warranted when interpreting these results in a clinical context.

Third, the incomplete recovery of hippocampal neuroinflammatory markers may be due to a suboptimal probiotic dose, a treatment period that was too short, or the inherent limitations of microbiota-based interventions in resolving brain inflammation. Future work should assess BBB integrity, markers of neurogenesis, microglial cell function, and vagal signaling pathways to better characterize MGB axis mechanisms. In addition, future studies should directly quantify microbiota-derived metabolites implicated in MGB axis signaling, including SCFA, tryptophan-derived metabolites, and circulating serotonin precursors. Integrating metabolomic analyses with neuroinflammatory assessments would provide greater mechanistic insight into how probiotic and synbiotic interventions influence gut–brain communication.

Fourth, 16S rRNA sequencing was used for microbial community profiling, which provides only genus-level identification and does not allow strain-level distinction or functional assessment of microbial activity. Shotgun metagenomics, metatranscriptomics, and metabolomics would provide greater functional and mechanistic resolution in future investigations. Such approaches could identify microbial metabolic pathways involved in SCFA production, tryptophan metabolism, neurotransmitter precursor synthesis, and inflammatory regulation, thereby improving understanding of the mechanisms underlying the observed physiological and behavioral effects.

Fifth, only *L. paracasei* 11W was evaluated *in vivo*, meaning the potential of other candidate strains remain untested in animal models. Notably, *L. fermentum* UV16 showed the highest intestinal adhesion and auto-aggregation, and *L. mesenteroides* KA15 produced the most tryptophan among batch II isolates. These candidates warrant individual and combination testing in future preclinical studies.

Sixth, although multiple associations were identified between microbial taxa and metabolic, inflammatory, and behavioral outcomes, correlation analyses do not establish causality. Future studies employing fecal microbiota transplantation, gnotobiotic approaches, or mechanistic validation experiments will be required to determine causal relationships between specific microbial taxa and host phenotypes.

Future studies should also focus on establishing causal relationships between microbiota alterations and host responses through integrated mechanistic approaches. Combining microbial profiling with metabolomic, immunological, and neurobiological analyses would help determine whether specific microbial metabolites, including SCFA and tryptophan-derived compounds, mediate the improvements in metabolic function, inflammation, and behavior observed in this study. Such investigations would provide a more comprehensive understanding of the pathways linking probiotic and synbiotic interventions to MGB axis regulation.

Finally, translation to human use will require well-designed randomized controlled trials in individuals with metabolic syndrome. The optimal dose, treatment duration, and safety profile of *L. paracasei* 11W in humans cannot be assumed from mouse data alone.

CONCLUSIONS

1. Among 150 LAB isolates recovered from traditional Lithuanian FF, screening identified ten promising probiotic candidates representing several LAB genera, demonstrating clear strain-specific differences in gastrointestinal survival, epithelial adhesion, and antimicrobial activity.
2. The selected LAB isolates differed in their utilization of FOS and GOS and in L-tryptophan production, with *L. paracasei* 11W and *L. mesenteroides* KA15 showing the strongest synbiotic potential. *L. paracasei* 11W showed the highest L-tryptophan production in the presence of 4% GOS, whereas *L. mesenteroides* KA15 showed the strongest response with 5% FOS, identifying these strains as particularly promising candidates for targeted synbiotic combinations.
3. WGS confirmed the presence of genes associated with probiotic traits, including stress tolerance, adhesion, and tryptophan biosynthesis across selected strains.
4. Safety profiling identified five LAB strains that fulfilled EFSA QPS criteria through the absence of transferable antimicrobial resistance determinants, virulence factors, and hemolytic activity. These strains were the best probiotic candidates selected.
5. *L. paracasei* 11W was selected as the lead probiotic candidate based on its overall *in vitro*, genomic, and safety characteristics. *L. paracasei* 11W with GOS synbiotic supplementation reduced HFD-induced metabolic dysfunction, neuroinflammation, behavioral impairment, and GM dysbiosis in part of the analysed traits in female mice, supporting its potential as a synbiotic intervention for metabolic and neuroinflammatory disorders.
6. Probiotic and synbiotic supplementation induced selective modulation of GM composition, particularly increasing *Bifidobacterium* abundance and altering HFD-associated bacterial taxa, despite no significant changes in overall alpha diversity.

RECOMMENDATIONS

The findings of this thesis demonstrate the potential of LAB isolated from traditional Lithuanian FF as probiotic and synbiotic candidates for modulating metabolic, inflammatory, and MGB axis-related outcomes. Among the strains evaluated, *Lacticaseibacillus paracasei* 11W exhibited favorable probiotic characteristics, including gastrointestinal stress tolerance, adhesion to IEC, tryptophan production, and a suitable safety profile. In a high-fat diet mouse model, supplementation with *L. paracasei* 11W, particularly in combination with GOS, improved glucose homeostasis, reduced hepatic pathology and inflammation, modulated GM composition, and ameliorated anxiety-like, depressive-like, and cognitive impairments.

The enhanced effects observed with the synbiotic formulation suggest that combining *L. paracasei* 11W with GOS may provide additional benefits compared with probiotic administration alone. These findings support the potential role of synbiotic approaches in addressing metabolic dysfunction and associated neurobehavioral disturbances.

This work also highlights traditional Lithuanian FF as a valuable source of LAB with desirable functional properties. The integrated screening and validation approach used in this study, encompassing phenotypic characterization, WGS, metabolite analysis, and preclinical evaluation, provides a framework for the identification and assessment of novel probiotic and psychobiotic candidates.

Future studies should focus on confirming the efficacy and safety of *L. paracasei* 11W and its synbiotic combination with GOS in human clinical trials. Further investigation of the underlying mechanisms, particularly those involving GM modulation, microbial metabolites, and microbiota–gut–brain axis signaling, will improve understanding of their potential applications in metabolic and neuropsychiatric disorders.

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SUPPLEMENTARY MATERIALS

Supplementary table 1. List of reagents used in this study

Reagent / Material	Supplier
MRS agar (de Man, Rogosa & Sharpe)	Oxoid, Wesel, Germany
MRS broth	Oxoid, Wesel, Germany / BD Life Science, USA
Sterile peptone water (0.1%)	Sigma-Aldrich, Poznań, Poland
Phosphate-buffered saline (PBS)	Sigma-Aldrich, Poznań, Poland / Corning, USA
Nutrient agar	
Blood agar (5% sheep blood)	Sigma-Aldrich, Poznań, Poland
Tryptic/Tryptone soy broth	Carl Roth, Karlsruhe, Germany
Pepsin (3 mg/ml)	Sigma-Aldrich, Poznań, Poland
Bile salts (0.3% w/v)	Sigma-Aldrich, Poznań, Poland
Pancreatin (1 mg/ml)	Sigma-Aldrich, Poznań, Poland
Hydrochloric acid (1 M HCl)	Carl Roth, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe, Germany
DNeasy PowerSoil Pro Kit	QIAGEN
QuantiFluor® dsDNA System	Promega Italia / Promega France
Nextera XT DNA Library Preparation Kit	Illumina, Warszawa, Poland
IDT Unique Dual Indexes	IDT (Integrated DNA Technologies)
AMPure magnetic beads	Beckman Coulter
QIAGEN EB elution buffer	QIAGEN
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific
Galacto-oligosaccharides (GOS), 5%	KingPrebiotics® / New Francisco (Yunfu City) Biotechnology Corp. Ltd., China

Fructo-oligosaccharides (FOS), 5%	KingPrebiotics® / New Francisco (Yunfu City) Biotechnology Corp. Ltd., China
Acetonitrile (HPLC grade, mobile phase B)	Sigma-Aldrich, Poznań, Poland
Formic acid (0.1% aq., mobile phase A)	Sigma-Aldrich, Poznań, Poland
Tyrosine standard compound	Sigma-Aldrich, Poznań, Poland
GABA standard compound	Sigma-Aldrich, Poznań, Poland
Rifampicin antibiotic disc	Carl Roth, Karlsruhe, Germany
Erythromycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Penicillin G antibiotic disc	Carl Roth, Karlsruhe, Germany
Ampicillin antibiotic disc	Carl Roth, Karlsruhe, Germany
Lincomycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Kanamycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Gentamycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Streptomycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Vancomycin antibiotic disc	Carl Roth, Karlsruhe, Germany
Tetracyclin antibiotic disc	Carl Roth, Karlsruhe, Germany
Mucin from porcine stomach (0.5% w/v)	Sigma-Aldrich, St. Louis, MO, USA
Gelatin (3% w/v)	Sigma-Aldrich, Poznań, Poland
PES membrane filter, 0.22 µm	Guangzhou Jet Bio-Filtration Co. Ltd., Guangzhou, China
Control diet , 3.87 kcal/g	Altromin, Germany
High-fat diet (HFD), 5.21 kcal/g (60% fat)	Altromin, Germany

D-Glucose (i.p. injection, 2 g/kg)	Sigma-Aldrich, Poznań, Poland
Sucrose solution (2%, sucrose preference test)	Sigma-Aldrich, Poznań, Poland
4% Paraformaldehyde (PFA)	Sigma-Aldrich, Poznań, Poland
Optimal Cutting Temperature (OCT) compound	Sigma-Aldrich, Poznań, Poland
2-Methylbutane (isopentane)	Sigma-Aldrich, Poznań, Poland
10% neutral buffered formalin	Sigma-Aldrich, Poznań, Poland
Harris-modified hematoxylin	Sigma-Aldrich, Poznań, Poland
Eosin Y	Sigma-Aldrich, Poznań, Poland
Xylene	Sigma-Aldrich, Poznań, Poland
DPX / Permount mounting medium	Sigma-Aldrich, Poznań, Poland
Periodic acid-Schiff (PAS) stain	Sigma-Aldrich, Lithuania
Alcian blue stain	Thermo Fisher Scientific, USA
Graded ethanol series (70%, 95%, 100%)	Sigma-Aldrich, Poznań, Poland
Cold acetone (−20 °C, fixation)	Sigma-Aldrich, Poznań, Poland
Complete protease inhibitor cocktail	Roche
PMSF (phenylmethylsulfonyl fluoride), 1 mM	Sigma-Aldrich
Sodium orthovanadate, 2 mM	Sigma-Aldrich
Formic acid (70%, insoluble fraction)	Thermo Fisher Scientific, USA
Tris-base buffer (1 M, pH 9, neutralisation)	Thermo Fisher Scientific, USA
Bradford Protein Assay reagent	Thermo Fisher Scientific, USA
Pro-inflammatory cytokine ELISA kit (IL-6, TNF- α , IFN- γ , IL-5)	Thermo Fisher Scientific, USA
Insulin ELISA kit	Thermo Fisher Scientific, USA

Potassium EDTA blood collection tubes	Thermo Fisher Scientific, USA
Maxima H Minus First Strand cDNA Synthesis Kit (with dsDNase)	Thermo Fisher Scientific, Lithuania
Luminaris Color HiGreen Master Mix, Low ROX	Thermo Fisher Scientific, Lithuania

Supplementary table 2. List of equipment and devices used in this study

Equipment / Device	Supplier
Incubator (35–37 °C, aerobic)	Thermo Fisher Scientific, Binder, Memmert
Deep freezer (–80 °C)	Froilabo, Livingston, UK
High-speed centrifuge (10,000 × g / rpm)	Eppendorf, Beckman Coulter, Thermo Fisher Scientific
Denville Micro 260D Microcentrifuge (5,000 × g)	Denville Scientific Inc., Metuchen, NJ, USA
Refrigerated centrifuge (3,000–4,000 × g, 4 °C)	Eppendorf, Beckman Coulter
High-speed refrigerated centrifuge (14,000 × g, 4 °C)	Beckman Coulter, Thermo Fisher Scientific
Dounce homogeniser	Wheaton, Kimble Chase
GloMax Plate Reader System (DNA quantification)	Promega Italia / Promega France
Qubit 4 Fluorometer	Thermo Fisher Scientific
Eppendorf BioSpectrometer® (OD600)	Eppendorf, Hamburg, Germany
CONTOUR® NEXT GEN blood glucose monitor	Ascensia Diabetes Care, USA
Illumina NovaSeq (2 × 150 bp; WGS)	Illumina, Warszawa, Poland
Illumina MiSeq (16S rRNA amplicons)	Novogene, UK
Shimadzu Prominence HPLC system	Shimadzu, Kyoto, Japan
Photodiode array (PDA) detector	Shimadzu, Kyoto, Japan
LCMS-2020 mass spectrometer (ESI)	Shimadzu, Kyoto, Japan
YMC Pack Pro C18 column (3 × 150 mm)	YMC, Kyoto, Japan
QuantStudio 3 Real-Time PCR System	Thermo Fisher Scientific, Lithuania
Olympus BX53 light microscope (20× and 40×)	Olympus
PowerLab hardware & LabChart-8 software	ADInstruments Ltd, UK

Supplementary table 3. Biological material and reference strains

Strain / Biological material	Source / Supplier	ATCC / ID no.
<i>Staphylococcus aureus</i> (antimicrobial activity, haemolysis)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 29213
<i>Salmonella Typhimurium</i> (antimicrobial activity)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 14028
<i>Streptococcus pyogenes</i> (antimicrobial activity)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 12384
<i>Streptococcus pyogenes</i> (antimicrobial activity)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 19615
<i>Klebsiella pneumoniae</i> (antimicrobial activity)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 13883
<i>Staphylococcus aureus</i> (gelatin degradation QC)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 6538
<i>Escherichia coli</i> (mucin degradation positive control)	Dept. Microbiology, Vilnius University, Lithuania	ATCC 25922
<i>L. paracasei</i> 11w (novel probiotic strain; isolated from fermented pear, Halės Turgus)	Dept. Microbiology, Faculty of Life Sciences Centre, Vilnius University, Lithuania	In-house isolate from fermented pear
C57BL/6J mice, female, 7 weeks old (<i>in vivo</i> model)	Dept. of Biological Models, Vilnius University, Lithuania	<i>First generation bred mice</i>

Supplementary table 4. Bacterial strains and their sources.

Isolates	Sources
1A	Fermented orange
2T	Fermented orange
3A	Fermented orange
9S	Fermented orange
10w	Fermented pear
11w	Fermented pear
11t	Fermented pear
18B	Fermented pear
25E	Fermented pear
29A	Fermented pear

30b	Fermented cherry tomato
32T	Fermented cherry tomato
33C	Fermented cherry tomato
35s	Fermented orange
40C	Fermented cherry tomato
42T	Fermented orange
48C	Fermented cherry tomato
55w	Fermented cucumber
57B	Fermented cucumber
60wb	Fermented cucumber
62W	Fermented cucumber
66W	Fermented cucumber
68B	Fermented cucumber

Supplementary table 5. Sources of samples and isolates names.

Isolates	Source
KA2.1	Homemade cheese
KA3.1	Fermented green beans
KA3.1.1	Fermented green beans
KA3.2.2	Fermented green beans
KA11	Canned tomatoes
KA11.1	Canned tomatoes
KA12	Homemade Wine (from strawberries)
KA13	Sauerkraut
KA14	Sauerkraut
KA15	Kombucha
KA15.1	Kombucha
KA16	yogurt
KA17	Sauerkraut
KA18	Sauerkraut
KA19	Cucumber pickle
KA8.2	Fermented cucumbers
KA9	Sauerkraut
KA9.2	Sauerkraut
UV11	Yogurt
UV16	Cucumber pickle

SANTRAUKA

IVADAS

Fermentuoti maisto produktai (FF) jau daugelį amžių yra svarbi žmogaus mitybos dalis ir vertinami ne tik dėl skonio savybių, aromato bei ilgesnio išsilaikymo, bet ir dėl galimo teigiamo poveikio sveikatai (Leech ir kt., 2020; Castellone ir kt., 2021). Fermentacijos metu mikroorganizmai sintetina įvairius biologiškai aktyvius junginius, įskaitant organines rūgštis, vitaminus, bioaktyvius peptidus, egzopolisacharidus ir polifenolius (Praveen ir Brogi, 2025). Vieną svarbiausių vaidmenų fermentacijos procesuose atlieka pieno rūgšties bakterijos (LAB), plačiai naudojamos fermentuotų pieno produktų, daržovių, vaisių ir grūdinių produktų gamyboje bei pasižymintys ilga saugaus vartojimo istorija (Santa ir kt., 2025).

Pastaraisiais metais daugėja įrodymų, kad tam tikri probiotiniai LAB kamienai gali veikti ne tik žarnyno mikrobiotą ir virškinamojo trakto funkcijas, bet ir metabolizmą, imuninę sistemą bei centrinę nervų sistemą per mikrobiotos-žarnyno-smegenų (MGB) sąveiką (Leeuwendaal ir kt., 2022; Abouelela ir Helmy, 2024; Szota ir kt., 2026). Tačiau šis poveikis yra labai kamienui specifinis, todėl kiekvienas potencialus probiotinis kamienas turi būti išsamiai įvertintas pagal fenotipines, genomines ir metabolines savybes, prieš jį pripažįstant saugiu ir veiksmingu probiotiku (Churin ir kt., 2026).

Tradiciniai lietuviški FF gali būti vertingas naujų probiotinių LAB kamienų šaltinis. Natūralios fermentacijos metu mikroorganizmai susiduria su įvairiais stresiniais veiksniais, tokiais kaip žemas pH, padidėjusi druskų koncentracija ir ribotas deguonies kiekis, todėl gali būti atrenkami kamienai, pasižymintys geresniu išgyvenamumu virškinamajame trakte bei didesniu biologiškai aktyvių metabolitų sintezės potencialu (Dimidi ir kt., 2019; Leeuwendaal ir kt., 2022). Nepaisant to, lietuviški FF vis dar yra menkai ištirti kaip naujų probiotinių mikroorganizmų šaltinis (Megur ir kt., 2026).

Kad LAB galėtų būti laikomi probiotikais, jie turi išgyventi virškinamojo trakto sąlygas, būti atsparūs skrandžio rūgščiai, pepsinui, tulžies druskoms ir pankreatinui bei gebėti adhezuoti prie žarnyno epitelio ląstelių, kas svarbu kolonizacijai ir sąveikai su šeimininko organizmu (Megur ir kt., 2023). Taip pat svarbios jų antimikrobinės savybės bei gebėjimas veikti sinergiškai su prebiotikais, tokiais kaip fruktooligosacharidai (FOS) ir

galaktooligosacharidai (GOS), sudarant sinbiotikus (De Jesus Raposo ir kt., 2016; Mitmesser ir Combs, 2017; Sako ir Tanaka, 2011).

Vienas iš šio darbo tikslų buvo įvertinti atrinktų LAB kamienų gebėjimą sintetinti L-triptofaną – svarbų serotoniną (5-HT) ir kitų biologiškai aktyvių metabolitų pirmtaką (Friedman, 2018). Triptofano biosintezės genų identifikavimas kartu su metabolitų analize leido įvertinti šių bakterijų potencialą dalyvauti MGB sąveikos procesuose ir pasižymėti psichobiotinėmis savybėmis.

Saugumo vertinimas yra neatsiejama probiotikų tyrimų dalis. Pagal Europos maisto saugos tarnybos (EFSA) kvalifikuotos saugumo prielaidos (QPS) procedūrą būtina įvertinti galimus antimikrobinio atsparumo ir virulentiškumo veiksnius, taikant tiek fenotipinius metodus, tiek viso genomo sekoskaitos (WGS) analizę (Kwon ir kt., 2021; Roe ir kt., 2022). Tik saugūs LAB kamienai gali būti atrenkami tolesniems tyrimams.

Baigiamojoje šios disertacijos dalyje buvo tiriamas iš tradicinių lietuviškų FF išskirto *Lactocaseibacillus paracasei* 11W kamieno poveikis riebalais praturtintų pašarų (HFD) sukeltame metabolinio sindromo modelyje. Vertintas šio kamieno, skiriamo vieno arba kartu su GOS, poveikis metaboliniams rodikliams, žarnyno mikrobiotai, uždegiminiams procesams ir elgsenos parametrams. Šis tyrimas prisideda prie naujų probiotikų ir sinbiotikų paieškos bei kūrimo strategijų ir praplečia žinias apie tradicinių lietuviškų FF mikroorganizmų potencialą moduluoti metabolinius bei su MGB sąveika susijusius procesus.

Tyrimo tikslas ir uždaviniai

Šios daktaro disertacijos tikslas buvo identifikuoti ir charakterizuoti naujus probiotinius LAB kamienus, išskirtus iš tradicinių lietuviškų FF, bei įvertinti atrinkto probiotinio kandidato poveikį metabolizmui, elgsenai, uždegiminiams procesams ir žarnyno mikrobiotos sudėčiai pelių HFD sukulto metabolinio sutrikimo modelyje.

Šiam tikslui pasiekti buvo iškelti šie uždaviniai:

1. Išskirti morfologiškai skirtingus LAB kamienus iš tradicinių lietuviškų FF ir įvertinti jų probiotinį potencialą, tiriant

- išgyvenamumą virškinamojo trakto sąlygomis, adheziją prie epitelio ląstelių ir antimikrobinį aktyvumą *in vitro*.
2. Įvertinti atrinktų LAB kamienų sinbiotinį potencialą, tiriant FOS ir GOS panaudojimą bei biologiškai aktyvių metabolitų gamybą.
 3. Atlikti perspektyviausių LAB kamienų WGS analizę ir identifikuoti su probiotinėmis savybėmis susijusius genus, dalyvaujančius atsake į stresą, adhezijoje, tulžies druskų hidrolizėje ir triptofano biosintezėje.
 4. Įvertinti išskirtų LAB kamienų saugumą, taikant fenotipinius ir genominius metodus virulentiškumo veiksniams bei pernešamiems antimikrobinio atsparumo genams nustatyti pagal EFSA QPS procedūros reikalavimus.
 5. Ištirti *Lacticaseibacillus paracasei* 11W kamieno, skiriamo vieno arba sinbiotiniame derinyje su GOS, poveikį metaboliniams elgsenos, uždegiminiams ir žarnyno mikrobiotos rodikliams HFD sukeltame metabolinio sutrikimo modelyje pelių patelėms.

Mokslinis naujumas

Šio darbo mokslinį naujumą sudaro naujų probiotinių pieno rūgšties bakterijų (LAB) kamienų, išskirtų iš tradicinių lietuviškų fermentuotų maisto produktų, identifikavimas ir išsamus charakterizavimas. Šie produktai iki šiol buvo mažai tirti kaip potencialus probiotinių mikroorganizmų šaltinis. Darbe pirmą kartą kompleksiskai įvertintas atrinktų kamienų probiotinis, sinbiotinis ir psichobiotinis potencialas, derinant *in vitro* funkcinius tyrimus, viso genomo sekoskaitą (WGS), metabolitų profiliavimą ir *in vivo* metabolinio sutrikimo modelį. **Toks integruotas metodologinis požiūris leido nuosekliai įvertinti perspektyvius LAB kamienus visais etapais – nuo jų išskyrimo ir funkcinio charakterizavimo iki saugumo vertinimo ir biologinio veiksmingumo patvirtinimo *in vivo*.** Iš daugiau kaip 150 išskirtų bakterijų izoliatų, atlikus daugiapakopę probiotinių savybių, saugumo ir genominę atranką, buvo pasirinktas perspektyviausias kandidatas - *Lacticaseibacillus paracasei* 11W. Šis kamienas pasižymėjo palankiu saugumo profiliu, gebėjimu sintetinti triptofaną ir, kartu su GOS, mažino HFD sukeltus metabolinius, uždegiminius, elgsenos bei žarnyno mikrobiotos pokyčius. Gauti rezultatai praplečia žinias apie tradicinių lietuviškų FF mikroorganizmų biologinį potencialą ir sudaro prielaidas naujų sinbiotinių produktų kūrimui metabolinių bei su MGB sąveika susijusių sutrikimų prevencijai ir korekcijai.

Ginamieji disertacijos teiginiai

1. Atrinkti LAB kamienai, išskirti iš tradicinių lietuviškų FF, pasižymi probiotinėms savybėms būdingomis charakteristikomis, įskaitant išgyvenamumą virškinamojo trakto sąlygomis, antimikrobinį aktyvumą ir adheziją prie žarnyno epitelio ląstelių.
2. Atrinkti LAB kamienai pasižymi sinbiotinėmis sąveikomis su prebiotikais, kurios skatina jų augimą ir moduliuoja L-triptofano gamybą.
3. WGS analizė atskleidė, kad atrinktų LAB kamienų genomuose nustatyti įvairūs genai, susiję su probiotinėmis savybėmis, įskaitant atsaką į stresą, adheziją, tulžies druskų metabolizmą ir triptofano biosintezę.
4. Atrinkti LAB kamienai atitinka pagrindinius EFSA QPS procedūros reikalavimus probiotikų taikymui, įskaitant hemolizinio aktyvumo, virulentiškumo veiksnių ir pernešamų antimikrobinio atsparumo genų nebuvimą.
5. *Lacticaseibacillus paracasei* 11W ir GOS moduliuoja HFD sukeltus metabolinius, elgsenos ir uždegiminius pokyčius bei ir žarnyno mikrobiotos disbiozę.

MEDŽIAGOS IR METODAI

Tyrimo eiga

Tyrimą sudarė *in vitro* ir *in vivo* eksperimentai. *In vitro* dalyje buvo išskirti ir charakterizuoti LAB kamienai iš tradicinių lietuviškų fermentuotų maisto produktų, įvertintos jų probiotinės savybės, metabolinis aktyvumas, saugumas ir genomo charakteristikos. Remiantis *in vitro* atrankos rezultatais, tolesniems *in vivo* tyrimams buvo pasirinktas *Lacticaseibacillus paracasei* 11W kamienas. Jo poveikis vertintas HFD sukeltame metabolinio sindromo modelyje naudojant C57BL/6J pelių linijos pateles.

Mėginių rinkimas ir LAB kamienų išskyrimas

LAB kamienai buvo išskirti iš tradicinių lietuviškų fermentuotų maisto produktų, įskaitant raugintas daržoves, fermentuotus pieno produktus ir kombučą. Išskirti mikroorganizmai buvo gryninami, kultivuojami ir saugomi tolimesniems tyrimams.

Atsparumas simuliuojamoms virškinamojo trakto sąlygoms

Atsparumas rūgštinei terpei buvo vertinamas suspendavus kultūras 1 M HCl iki pH 2,0 parūgštintame PBS tirpale ir inkubuojant 37 °C temperatūroje 4 val. Atsparumas pepsinui buvo tiriamas simuliuotose skrandžio sultyse (3 mg/mL pepsinas, pH 2,0, 37°C, 4 val.). Tulžies druskų tolerancija buvo nustatoma 0,3% (m/t) tulžies druskų tirpale (pH 8,0, 37°C, 6 val.), o atsparumas pankreatinui – 1 mg/mL pankreatino tirpale (pH 7,2, 37°C, 6 val.). Gyvybingumas prieš ir po inkubacijos buvo nustatomas plokštelių skaičiavimo metodu ir išreikštas kaip išgyvenamumas procentais.

Žarnyno kolonizacijos savybių vertinimas

Antimikrobinis aktyvumas buvo nustatytas agaro difuzijos į šulinėlius metodu, naudojant pH neutralizuotus kultūrų supernatantus (CFS), patikrinant prieš penkis referencinius patogenus (*S. aureus* ATCC 29213, *Salmonella Typhimurium* ATCC 14028, *S. pyogenes* ATCC 12384/19615 ir *K. pneumoniae* ATCC 13883). Autoagregacija ir koagregacija su patogenais buvo matuojamos spektrofotometriškai prie 600 nm per 4, 12 ir 24 val. Adhezija prie HCT116 žmogaus storosios žarnos epitelio linijos ląstelių buvo kiekybiškai įvertinta po 3 val. inkubacijos 37°C, prie 5% CO₂; neprilipusios bakterijos buvo pašalintos PBS plovimais, o prisitvirtinusios prie ląstelių bakterijos suskaičiuotos MRS agaro plokštelėse po tripsinizacijos.

Prebiotikų įtaka augimui ir metabolitų analizė

LAB kamienų augimas MRS terpėje, papildytoje GOS arba FOS, buvo vertinamas matuojant optinį tankį prie 600 nm kas 4 val. 37°C temperatūroje. Bakterijų metabolitų profiliavimas atliktas HPLC-MS metodu, nustatant triptofano, tirozino ir gama-amino sviesto rūgšties (GABA) koncentracijas (Shimadzu Prominence sistema, YMC Pro C18 kolonėlė, ESI jonizacija); triptofanas, tirozinas ir GABA buvo kiekybiškai įvertinti pagal kalibravimo kreives ir patikrinti adityvumo metodu.

Genomo analizė ir saugumo vertinimas

DNR buvo išskirta naudojant QIAGEN DNeasy PowerSoil Pro rinkinį, o viso genomo sekoskaita atlikta Illumina NovaSeq platformoje

(2×150 bp). Genomų funkcinė anotacija buvo atlikta naudojant Prokka programinę įrangą. Antimikrobinio atsparumo genų paieška atlikta naudojant Abricate ir ResFinder duomenų bases, o virulentiškumo veiksnių analizė – VFDB duomenų bazę (≥90% sekų tapatumas ir ≥60% padengimas). MLST analizė atlikta naudojant PubMLST duomenų bazę ir BLAST algoritmą. Fenotipinių lygmeniu buvo vertinamos hemolizinės savybės 5% avių kraujo agare, jautrumas antibiotikams disko difuzijos metodu (10 antibiotikų), taip pat želatinos ir mucino skaidymo gebos.

Gyvūnų modelis ir eksperimentinė intervencija

36 C57BL/6J linijos pelių patelės (7 savaičių amžiaus) buvo suskirstytos į keturias grupes (n = 8–10): standartinių pašarų kontrolinė grupė (Control), HFD grupė, HFD+*L. paracasei* 11W grupė ir HFD+*L. paracasei* 11W + 5 % GOS grupė. HFD pašarai sudarė 60 % energinės vertės iš riebalų (5,21 kcal/g; Altromin, Vokietija). *L. paracasei* 11W kamienas (1×10^7 CFU/mL PBS) buvo sugirdomas naudojant skrandžio zondą. GOS (5%) buvo tiekiami su geriamuoju vandeniu ir keičiami 2 kartus savaitėje. Intervencija truko 25 savaites. Visi eksperimentai su gyvūnais buvo patvirtinti Lietuvos Valstybinės maisto ir veterinarijos tarnybos (leidimo Nr. G2–174) ir atlikti laikantis Direktyvos 2010/63/ES reikalavimų.

Elgesio ir metaboliniai tyrimai

Nerimo pobūdžio elgesys buvo vertinamas atviro lauko, šviesos-tamsos dėžutės ir pakylėto kryžminio labirinto testais. Pažintinės funkcijos buvo vertinamos naujojo objekto atpažinimo ir Y-labirinto testais. Depresijai būdingas elgesys buvo vertinamas uodegos pakabinimo ir priverstinio plaukimo testais. Taip pat buvo vertinamas socialinis elgesys, anhedonija ir valingas fizinis aktyvumas. Gliukozės tolerancija buvo vertinama atliekant intraperitoninį gliukozės tolerancijos testą (2 g/kg kūno masės, po 14 val. badavimo), gliukozės koncentraciją kraujyje nustatant po 0, 15, 30, 45, 60 ir 120 min.

Audinių, mikrobiotos ir statistinė analizė

Po gyvūnams atliktos cervikalinės dislokacijos storojoje žarnoje buvo nustatytos uždegiminių citokinų (IL-6, TNF- α , IFN- γ ir IL-5) koncentracijos, o kraujo plazmoje – insulino koncentracija ELISA metodu. Kepenų

histologiniai pokyčiai buvo vertinami kriostatiniuose pjūviuose, dažytuose hematoksilino ir eozino (H&E) dažais. Storosios žarnos morfologiniai pokyčiai buvo vertinami pjūvius dažant H&E, PAS ir Alcian Blue dažais. Genų raiška storioje žarnoje ir hipokampe buvo analizuojama RT-kPGR metodu, normalizuojant pagal referencinį geną *Tubala*. Žarnyno mikrobiotos sudėtis buvo nustatyta 16S rRNR geno amplikonų sekoskaitos metodu, naudojant QIIME 2 programinę įrangą ir SILVA 138 duomenų bazę.

Duomenų normalumas buvo vertintas Shapiro–Wilk testu. Normalųjį pasiskirstymą atitinkantys duomenys analizuoti vienfaktore arba dvifaktore dispersine analize (ANOVA), taikant Dunnett'o post hoc testą. Neparametriniams duomenims naudotas Kruskal–Wallis testas su Dunn'o post hoc testu. Statistiškai reikšmingais laikyti skirtumai, kai $p < 0,05$.

REZULTATAI

Atliekant *in vitro* tyrimus, iš įvairių Lietuvoje gaminamų FF buvo išskirti LAB izoliatai, atrinkti pagal morfologiškai skirtingas kolonijas. Mikroorganizmų išskyrimas iš FF buvo vykdoma dviem etapais – pirmosios ir antrosios atrankos metu. Išskirti izoliatai buvo įtraukti į Vilniaus universiteto Gyvybės mokslų centro Molekulinės mikrobiologijos ir biotechnologijos katedros probiotinių mikroorganizmų kolekciją. Pirmosios atrankos kamienų sąrašas pateiktas 4 priede, o antrosios atrankos kamienų sąrašas – 5 priede.

LAB izoliatai buvo vertinami pagal jų probiotines savybes, įskaitant atsparumą virškinamojo trakto sąlygoms, gebėjimą kolonizuoti žarnyną, antimikrobinį aktyvumą ir metabolinį potencialą. Tyrimų tikslas buvo atrinkti perspektyviausius probiotinius kandidatus tolesnei geno, funkciniai ir *in vivo* analizei.

3.1. Pirmosios atrankos LAB kamienų atsparumas virškinamojo trakto sąlygoms

3.1.1. Išgyvenimas esant žemam pH

LAB kamienų gebėjimas išgyventi rūgščioje skrandžio aplinkoje yra vienas svarbiausių probiotinių savybių vertinimo kriterijų. Siekiant įvertinti atsparumą rūgščiai, visi 23 pirmosios atrankos izoliatai buvo veikiami pH 2,0 sąlygomis. Iš 23 tirtų izoliatų 18 pasižymėjo didesniu nei 50 %

išgyvenamumu. Didžiausias išgyvenamumas nustatytas 55W (97,58 %), 18B (96,02 %), 2T (95,37 %) ir 9S (90,03 %) kamienuose. Trys kamieniai – 57B, 42T ir 29A – nepasiekė 50 % išgyvenamumo ribos ir buvo pašalinti iš tolesnių tyrimų. Likę 18 kamienų buvo atrinkti pepsino tolerancijos vertinimui (3.1 lentelė).

3.1.2. Atsparumas pepsinui

Aštuoniolikos rūgščiai atsparių izoliatų gebėjimas išgyventi pepsino aplinkoje buvo vertinamas simuliuotose skrandžio sultyse esant pH 2,0. Didžiausiu atsparumu pasižymėjo 18B (97,58 %), 55W (94,99 %), 33E (93,74 %) ir 3A (90,75 %) kamieniai. Penkių kamienų išgyvenamumas nesiekė 50 %, todėl jie buvo pašalinti iš tolesnių tyrimų. Likę 13 kamienų buvo atrinkti tulžies druskų ir pankreatino tolerancijos vertinimui (3.1 lentelė).

3.1.3. Atsparumas tulžies druskoms ir pankreatinui

Trylikos atrinktų kamienų atsparumas simuliuotoms plonosios žarnos sąlygoms buvo vertinamas naudojant tulžies druskas ir pankreatiną. Šeši kamieniai pasižymėjo didesniu nei 90 % išgyvenamumu: 25E (95,40 %), 9S (95,37 %), 2T (94,20 %), 3A (92,07 %), 55W (90,90 %) ir 40C (90,83 %). Iš viso 12 kamienų išlaikė didesnę nei 50 % gyvybingumą, o 11T kamienas pasižymėjo mažiausiu išgyvenamumu (32,60 %) ir buvo pašalintas iš tolesnių tyrimų. Dvylika atspariausių kamienų buvo atrinkti funkcinių savybių vertinimui (3.1 lentelė).

3.2. Pirmosios atrankos LAB kamienų funkcinių savybių vertinimas

3.2.1. Antimikrobinis aktyvumas

Dvylikos atrinktų LAB kamienų antimikrobinis aktyvumas buvo vertinamas naudojant kultūrų supernatantus (CFS) prieš penkis referencinius patogenus: *Staphylococcus aureus* ATCC 29213, *Salmonella Typhimurium* ATCC 14028, *Streptococcus pyogenes* ATCC 12384, *Streptococcus pyogenes* ATCC 19615 ir *Klebsiella pneumoniae* ATCC 13883. Keli kamieniai pasižymėjo plačiu antimikrobinio aktyvumo spektru. Didžiausias aktyvumas nustatytas 66W kamienne, kurio CFS slopino visų penkių tirtų patogenų augimą. Taip pat stipriu antimikrobinio aktyvumu pasižymėjo 18B, 25E ir 48C kamieniai. Visi 12 kamienų buvo atrinkti tolesniems tyrimams (3.2 lentelė).

3.2.2. Antioksidacinis aktyvumas (TEAC)

Dvylikos atrinktų LAB kamienų CFS antioksidacinis aktyvumas buvo vertinamas TEAC metodu. Didžiausias aktyvumas nustatytas 3A (26,37 $\mu\text{g/mL}$) ir 55W (26,06 $\mu\text{g/mL}$) kamienuose, o mažiausias – 40C kamiene (9,57 $\mu\text{g/mL}$). Nepaisant nustatytų skirtumų, visi kamienai buvo toliau vertinami pagal jų kolonizacijos potencialą (3.2 lentelė).

3.2.3. Autoagregacijos vertinimas

Dvylikos atrinktų kamienų autoagregacijos geba buvo vertinama po 24 val. inkubacijos. Didžiausia autoagregacija nustatyta 11W (79,96 %), 55W (76,76 %) ir 40C (76,63 %) kamienuose. Mažiausia autoagregacija buvo nustatyta 3A kamiene (24,26 %). Keturių kamienų (2T, 3A, 9S ir 25E) autoagregacija nesiekė 50 %, todėl jie buvo pašalinti iš tolesnių tyrimų. Aštuoni kamienai buvo atrinkti adhezijos prie HCT116 ląstelių tyrimui (3.2 lentelė).

3.2.4. Adhezija prie HCT116 ląstelių

Aštuonių atrinktų kamienų gebėjimas prisitvirtinti prie HCT116 žmogaus storosios žarnos epitelio ląstelių svyravo nuo $9,26 \pm 0,97$ % iki $25,67 \pm 0,43$ %. Didžiausia adhezija nustatyta 11W kamiene, o mažiausia – 1A kamiene. Kamienų 1A, 18B ir 55W adhezija buvo mažesnė nei 12 %, todėl jie nebuvo atrinkti tolesniems tyrimams dėl riboto kolonizacijos potencialo. Likę penki kamienai buvo pasirinkti detalesnei molekulinei ir funkicinei analizei.

3.3. Antrosios atrankos LAB kamienų atsparumas virškinamojo trakto sąlygoms

3.3.1. Išgyvenimas esant žemam pH

Kaip ir pirmosios atrankos atveju, visi 20 antrosios atrankos izoliatų buvo veikiami pH 2,0 fosfato-fiziologiniu buferiu, siekiant įvertinti jų atsparumą rūgščiai. Iš 20 tirtų izoliatų 15 pasižymėjo didesniu nei 50 % išgyvenamumu. Didžiausias išgyvenamumas nustatytas KA19 ($94,67 \pm 3,39$ %), KA13 ($93,76 \pm 3,71$ %), UV11 ($85,95 \pm 7,65$ %) ir KA18 ($83,16 \pm 8,20$ %) kamienuose. Penki kamienai – KA3.2.2 ($30,78 \pm 8,81$ %), KA8.2 ($24,22 \pm 1,69$ %), KA9.2 ($23,81 \pm 8,24$ %), KA14 ($37,74 \pm 8,24$ %) ir KA15.1 ($27,62 \pm 12,88$ %) –

nepasiekė 50 % išgyvenamumo ribos ir buvo pašalinti iš tolesnių tyrimų (3.3 lentelė). Likę 15 kamienų buvo atrinkti pepsino tolerancijos vertinimui.

3.3.2. Atsparumas pepsinui

Penkiolikos rūgščiai atsparių antrosios atrankos izoliatų gebėjimas išgyventi pepsino aplinkoje buvo vertinamas esant pH 2,0. Didžiausiu atsparumu pasižymėjo UV16 ($96,32 \pm 0,30$ %), KA9 ($94,45 \pm 2,06$ %) ir KA18 ($82,80 \pm 1,57$ %) kamieniai. Aštuonių kamienų išgyvenamumas buvo mažesnis nei 20 %: KA2.1 ($19,01 \pm 5,22$ %), KA3.1 ($17,23 \pm 2,26$ %), KA9.2 ($13,10 \pm 1,93$ %), KA11.1 ($9,25 \pm 1,23$ %), KA14 ($19,52 \pm 3,35$ %), KA16 ($12,91 \pm 0,55$ %), KA17 ($19,69 \pm 1,31$ %) ir UV11 ($17,46 \pm 2,13$ %). Šie kamieniai buvo pašalinti iš tolesnių tyrimų dėl nepakankamos pepsino tolerancijos (3.3 lentelė). Likę šeši kamieniai buvo atrinkti tulžies druskų tolerancijos vertinimui.

3.3.3. Atsparumas tulžies druskoms

Šešių antrosios atrankos izoliatų gebėjimas išgyventi 0,3 % tulžies druskų tirpale buvo vertinamas po 6 val. inkubacijos. Visi šeši kamieniai išlaikė gyvybingumą šiomis sąlygomis. Didžiausiu atsparumu pasižymėjo KA12 kamienas, kurio išgyvenamumas siekė $92,37 \pm 2,04$ %. KA3.1.1 ir KA15 kamienų išgyvenamumas buvo atitinkamai $77,77 \pm 0,69$ % ir $73,94 \pm 3,78$ % (3.3 lentelė). Visi šeši kamieniai buvo atrinkti pankreatino tolerancijos vertinimui.

3.3.4. Atsparumas pankreatinui

Galutiniame virškinamojo trakto sąlygų modeliavimo etape buvo vertinamas kamienų atsparumas 1 mg/mL pankreatinui esant pH 8,0, modeliuojant plonosios žarnos fermentinę aplinką. Didžiausią išgyvenamumą parodė KA3.1.1 kamienas ($96,82 \pm 1,49$ %), pasižymėjęs aukštu atsparumu visų modeliutų virškinamojo trakto sąlygų metu. KA15 kamieno išgyvenamumas siekė 67,04 %, o KA18 kamieno – 45,89 %. Kadangi KA18 kamieno išgyvenamumas nesiekė 50 %, jis buvo pašalintas iš tolesnių tyrimų (3.3 lentelė). Likę kamieniai, kurių išgyvenamumas viršijo 50 %, buvo atrinkti funkcinių savybių vertinimui.

3.4. Antrosios atrankos LAB kamienų funkcinių savybių vertinimas

3.4.1. Antimikrobinis aktyvumas

Antrosios atrankos LAB kamienų antimikrobinis aktyvumas buvo vertinamas naudojant kultūrų supernatantus (CFS) prieš penkis patogeninius mikroorganizmus: *Salmonella enteritidis* ATCC 25928, *Escherichia coli* P42, *Salmonella Typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883 ir *Pseudomonas aeruginosa* ATCC 27853. Slopavimo zonos buvo vertinamos po 24 ir 48 val. inkubacijos. Plačiausiu antimikrobinio aktyvumo spektru pasižymėjo KA9 ir UV16 kamienai, kurių CFS slopino visų penkių tirtų patogenų augimą. KA15 kamienas taip pat pasižymėjo stipriu antimikrobinio aktyvumu, ypač prieš *S. Typhimurium* ($21,55 \pm 0,48$ mm) ir *P. aeruginosa* ($22,57 \pm 0,27$ mm). Visi kamienai pasižymėjo palyginti nedideliu aktyvumu prieš *S. enteritidis*, kas gali rodyti didesnę šio mikroorganizmo atsparumą LAB gaminamiems antimikrobiniais junginiams (3.4 lentelė).

3.4.2. Autoagregacijos vertinimas

Autoagregacija buvo vertinama po 0,25, 15, 16, 18 ir 24 val. inkubacijos, siekiant įvertinti ląstelių tarpusavio sąveikos dinamiką. Didžiausia autoagregacijos geba nustatyta UV16 kamienne – po 0,25 val. ji siekė $71,09 \pm 0,81$ %, o po 24 val. padidėjo iki $93,50 \pm 1,01$ % (3.4 lentelė). Visų tirtų kamienų autoagregacijos rodikliai buvo aukšti, o UV16 pasižymėjo geriausiais rezultatais. Šie duomenys rodo gerą kolonizacijos ir išlikimo virškinamajame trakte potencialą.

3.4.3. Adhezija prie HCT116 ląstelių

Penkių antrosios atrankos kamienų gebėjimas prisitvirtinti prie HCT116 žmogaus storosios žarnos epitelio ląstelių buvo vertinamas po 48 val. inkubacijos. Adhezijos rodikliai svyravo nuo 16,63 % iki 53,53 %, todėl buvo nustatyti didesni skirtumai nei pirmosios atrankos kamienų grupėje. Didžiausia adhezija nustatyta UV16 kamienne ($53,53 \pm 0,45$ %), o mažiausia – KA3.1.1 kamienne ($16,63 \pm 0,95$ %). Trys kamienai – KA9, KA15 ir UV16 – pasižymėjo didesne nei 40 % adhezija, rodydami stiprų kolonizacijos potencialą (3.4 lentelė).

3.5. Prebiotikų įtaka augimui ir metabolitų analizė

3.5.1. Prebiotikų įtaka LAB kamienų augimui

Atrinktų LAB kamienų sinbiotinis potencialas buvo vertinamas tiriant jų gebėjimą panaudoti fruktooligosacharidus (FOS) ir galaktooligosacharidus (GOS) kaip anglies šaltinius. Pirmosios atrankos kamienai pasižymėjo reikšmingai pagerėjusiu augimu terpėse, papildytose GOS. 11W, 33E, 40C, 48C ir 66W kamienų augimas nuosekliai didėjo didinant GOS koncentraciją nuo 2 % iki 4 %. Antrosios atrankos kamienai KA3.1.1, KA9, KA15 ir UV16 taip pat pasižymėjo geresniu augimu terpėse su 5 % FOS arba 5 % GOS, palyginti su standartine MRS terpe. KA12 kamienas pasižymėjo geresniu augimu terpėje su GOS nei su FOS (3.5 lentelė). Šie rezultatai rodo, kad tiek FOS, tiek GOS gali būti efektyvūs anglies šaltiniai tirtiems LAB kamienams.

3.5.2. Triptofano gamybos vertinimas

Pirmosios atrankos *Lacticaseibacillus paracasei* 11W kamieno kultūros supernatante nustatyta didžiausia triptofano koncentracija – $16,63 \pm 2,25 \mu\text{M}$, kai kamienas buvo augintas terpėje su 4 % GOS. Tuo tarpu *Lactiplantibacillus plantarum* 40C kamieno kultūros supernatante tomis pačiomis sąlygomis nustatyta $2,64 \pm 0,50 \mu\text{M}$ triptofano koncentracija. Antrosios atrankos metu triptofaną gaminančiais kamienais buvo identifikuoti KA15 ir KA12. Didžiausia triptofano koncentracija nustatyta *Leuconostoc mesenteroides* KA15 kultūros supernatante – $10,48 \mu\text{M}$ terpėje su 5 % FOS. *Pediococcus pentosaceus* KA12 kamieno kultūros supernatante triptofano koncentracija siekė $2,69 \mu\text{M}$ standartinėje MRS terpėje ir $1,69 \mu\text{M}$ GOS papildytoje MRS terpėje (3.5 lentelė). Šie rezultatai rodo, kad prebiotiko tipas ir koncentracija gali reikšmingai veikti triptofano biosintezę skirtinguose LAB kamienuose.

3.6. Molekulinė LAB kandidatų identifikacija

Galutiniai probiotiniai kandidatai iš pirmosios ir antrosios atrankos buvo identifikuoti taikant viso genomo sekoskaitą. Gautos sekos buvo lyginamos su NCBI GenBank duomenų baze. Pirmosios atrankos kandidatai identifikuoti kaip *Lacticaseibacillus paracasei* 11W, *Lactiplantibacillus paraplantarum* 33E, *Lactiplantibacillus plantarum* 40C, *Lactiplantibacillus plantarum* 48C ir *Lactiplantibacillus paraplantarum* 66W. Antrosios atrankos kandidatai identifikuoti kaip *Lactiplantibacillus plantarum* KA3.1.1, *Lactiplantibacillus*

plantarum KA9, *Pediococcus pentosaceus* KA12, *Leuconostoc mesenteroides* KA15 ir *Limosilactobacillus fermentum* UV16 (3.5 lentelė).

3.7. Su probiotinėmis savybėmis siejamų genų analizė

Su probiotinėmis savybėmis siejamų genų pasiskirstymas dešimtyje atrinktų LAB kamienų (11W, 33E, 40C, 48C, 66W, KA3.1.1, KA9, KA12, KA15 ir UV16) pateiktas 3.7 lentelėje. Genai *clpB*, *nhaC* ir *tetA* buvo aptikti visuose dešimtyje tirtų kamienų. Rūgščių tolerancijai svarbus genas *gadC* buvo nustatytas tik 11W, KA3.1.1 ir KA12 kamienuose. Daugiavaistį atsparumą lemiantis genas *mdlL* buvo aptiktas 33E, 40C, 48C, 66W ir KA9 kamienuose, tačiau nebuvo nustatytas 11W, KA3.1.1, KA12, KA15 ir UV16 kamienuose. Su adhezija prie žarnyno epitelio siejamas genas *pdeK* buvo aptiktas tik 11W ir KA3.1.1 kamienuose, todėl buvo rečiausiai nustatytas genas tarp tirtų kandidatų (3.7 lentelė).

3.8. Probiotinio kandidato atranka *in vivo* tyrimui

Atlikus išsamų pirmosios ir antrosios atrankos LAB kamienų vertinimą *in vitro*, *L. paracasei* 11W buvo atrinktas kaip perspektyviausias probiotinis kandidatas tolesniems *in vivo* tyrimams. Šis kamienas pasižymėjo geru atsparumu virškinamojo trakto sąlygoms – sėkmingai išgyveno esant pH 2, veikiant pepsinui, tulžies druskoms ir pankreatinui. Vertinant funkcinės savybės, *L. paracasei* 11W pasižymėjo didžiausia autoagregacijos geba ir stipriausia adhezija prie HCT116 žarnyno epitelio ląstelių, rodančia gerą kolonizacijos potencialą. Genomo analizė patvirtino svarbių su probiotinėmis savybėmis siejamų genų buvimą, įskaitant *gadC*, susijusį su išgyvenimu rūgščioje aplinkoje, *fbp* ir *pdeK*, siejamus su adhezija prie gleivių sluoksnio ir žarnyno epitelio, bei *trpS2*, dalyvaujantį triptofano biosintezėje. Pažymėtina, kad genas *pdeK* buvo aptiktas tik 11W kamiene tarp visų pirmosios atrankos kandidatų. Sinbiotinio potencialo vertinimas taip pat išskyrė 11W kamieną iš kitų kandidatų – jo kultūros supernatante nustatyta didžiausia triptofano koncentracija (16,63 μM), kai terpė buvo papildyta 4 % GOS. Šis rodiklis viršijo visų kitų abiejų atrankų metu tirtų kamienų rezultatus. Didelė triptofano gamyba, geras išgyvenamumas virškinamojo trakto sąlygose, stiprus kolonizacijos potencialas ir palankus su probiotinėmis savybėmis siejamų genų profilis pagrindė *L. paracasei* 11W kamieno atranką tolesniems *in vivo* tyrimams taikant HFD sukeltą pelių modelį.

3.9. Kūno masės ir gliukozės tolerancijos vertinimas

Pelės 25 savaites buvo šeriamos HFD arba standartiniais pašarais ir kasdien gavusios *L. paracasei* 11W (1×10^7 KSV/ml). Dvifaktoriškos dispersinės analizės (ANOVA) parodė reikšmingą pašarų tipo ir laiko poveikį kūno masei ($F(21,256) = 2,544$; $p = 0,0003$). HFD grupių pelių kūno masė buvo reikšmingai didesnė nei Control grupės nuo 12 iki 25 tyrimo savaitės (3.9 pav. A). 25 tyrimo savaitę HFD grupėje nustatytas reikšmingas kūno masės padidėjimas (Kruskal–Wallis testas, $H = 14,07$; $p = 0,0021$). Control grupės pelių kūno masė buvo reikšmingai mažesnė nei HFD grupės ($p = 0,0056$) (3.9 pav. C). HFD+11W ir HFD+11W+GOS grupėse nustatytas mažesnis kūno masės prieaugis nei HFD grupėje, tačiau šie skirtumai statistiškai reikšmingi nebuvo (3.9 pav. C). Gliukozės koncentracija kraujyje visose HFD grupėse buvo reikšmingai didesnė nei Control grupėje viso tyrimo metu (3.9 pav. B). Gliukozės tolerancijos testo (GTT) rezultatai 25 savaitę parodė ryškiai sutrikusį gliukozės pasisavinimą HFD grupėje, o HFD+11W ir HFD+11W+GOS grupėse buvo stebimos dalinio gliukozės tolerancijos atsistatymo tendencijos (3.9 pav. D, E).

3.10. Su nerimu susijusio elgesio vertinimas

Nerimo pobūdžio elgesiui įvertinti buvo atlikti atviro lauko testas (OFT), šviesos–tamsos dėžutės testas (LDB) ir pakylėto kryžminio labirinto testas (EPM). OFT metu reikšmingų skirtumų tarp grupių pagal apsilankymų centro zonoje skaičių nenustatyta ($F(3,31) = 1,771$; $p = 0,1731$; 3.10 pav. A). Tačiau HFD grupės pelės centro zonoje praleido reikšmingai mažiau laiko nei HFD+11W grupės pelės ($p = 0,0168$; 3.10 pav. B). LDB teste HFD grupės pelės reikšmingai mažiau laiko praleido šviesioje dalyje ($p = 0,0266$) ir rečiau į ją įėjo ($p = 0,001$), palyginti su Control grupės pelėmis. EPM teste nustatytas reikšmingas skirtumas pagal laiką, praleistą atvirose labirinto dalyse ($F(3,32) = 5,574$; $p = 0,0034$). HFD grupės pelės atvirose dalyse praleido mažiau laiko nei Control grupės pelės ($p = 0,0102$; 3.10 pav. F), o HFD+11W+GOS grupėje šis rodiklis buvo reikšmingai didesnis nei HFD grupėje ($p = 0,0100$; 3.10 pav. F). Šie rezultatai rodo, kad HFD skatina su nerimu susijusį elgesį, o *L. paracasei* 11W ir GOS skyrimas gali šį poveikį sušvelninti.

3.11. Pažintinių funkcijų, fizinio aktyvumo ir socialinio elgesio vertinimas

Y labirinto teste (Y-maze) nustatytas bendras grupės poveikis (Kruskalio–Valio testas, $H = 10,54$; $p = 0,0145$), tačiau poriniai grupių palyginimai statistiškai reikšmingų skirtumų neparodė (3.11 pav. A). HFD grupės pelės pasižymėjo sutrikusia naujo objekto atpažinimo testo (NOR) veikla (Kruskalio–Valio testas, $H = 18,91$; $p = 0,0003$). Tiek HFD+11W, tiek HFD+11W+GOS grupėse nustatytas reikšmingas atpažinimo atminties pagerėjimas, palyginti su HFD grupe (atitinkamai $p = 0,0002$ ir $p = 0,0211$), rodantis pažintinių funkcijų atsistatymą iki Control grupės lygio (3.11 pav. B). Savanoriško rato bėgimo teste nustatyta, kad HFD grupės pelės atliko reikšmingai mažiau rato apsisukimų nei Control grupės pelės ($p = 0,0025$), rodydamos sumažėjusį fizinį aktyvumą (3.11 pav. C). Socialinės sąveikos testo (SIT) metu reikšmingų skirtumų tarp grupių nenustatyta nei vertinant pasirinkimą tarp pelės ir objekto, nei tarp pažįstamos ir nepažįstamos pelės (3.11 pav. D, E).

3.12. Depresinio pobūdžio elgesio ir anhedonijos vertinimas

HFD sukėlė ryškius depresinio pobūdžio elgesio ir anhedonijos požymius. Pakabintos uodegos teste (TST) HFD grupės pelių nejudrumas siekė apie 60 % ir buvo reikšmingai didesnis nei Control grupės pelių (~25 %; $p < 0,0001$; 3.12 pav. A). Tiek HFD+11W, tiek HFD+11W+GOS grupėse nejudrumas buvo reikšmingai sumažėjęs, palyginti su HFD grupe (atitinkamai $p = 0,0160$ ir $p = 0,0001$). Priverstinio plaukimo teste (FST) HFD grupės pelės taip pat pasižymėjo didesniu nejudrumu (~65 %) nei Control grupės pelės (~45 %; $p = 0,0093$; 3.12 pav. B), o abiejose intervencinėse grupėse šis rodiklis sumažėjo. Sacharozės pirmenybės testas (SPT) parodė reikšmingai sumažėjusią sacharozės pirmenybę HFD grupėje (~60 %), palyginti su Control grupe (~80 %; $p = 0,0016$), kas rodo anhedonijos išsivystymą. Abiejų intervencinių grupių sacharozės pasirinkimo rodikliai nesiskyrė nuo Control grupės pelių (3.12 pav. C).

3.13. Storosios žarnos uždegiminių citokinų analizė

HFD sukėlė ryškų uždegiminių citokinų koncentracijų padidėjimą storajoje žarnoje. TNF- α koncentracija buvo reikšmingai padidėjusi HFD grupėje ($p = 0,0139$; 3.13 pav. A), o *L. paracasei* 11W skyrimas reikšmingai sumažino

TNF- α koncentraciją. IFN- γ koncentracija taip pat buvo reikšmingai didesnė HFD grupėje nei HFD+11W+GOS grupėje ($p = 0,0325$; 3.13 pav. B). Ryškiausias uždegiminis atsakas nustatytas vertinant IL-6 – didžiausios šio citokino koncentracijos buvo nustatytos HFD grupėje. Tiek probiotinis (HFD+11W), tiek sinbiotinis (HFD+11W+GOS) skyrimas reikšmingai sumažino IL-6 koncentraciją (atitinkamai $p = 0,0003$ ir $p = 0,0134$). IL-5 koncentracija buvo reikšmingai didesnė HFD grupėje nei HFD+11W grupėje ($p = 0,0174$; 3.13 pav. D). Šie rezultatai rodo, kad HFD skatina storosios žarnos uždegimą, o *L. paracasei* 11W ir GOS derinys veiksmingai slopina šį uždegiminį atsaką.

3.14. Storosios žarnos histologinė ir morfometrinė analizė

Storosios žarnos histologinė ir morfometrinė analizė parodė reikšmingus HFD sukeltus pokyčius, kurie buvo iš dalies arba visiškai atkurti taikant probiotinę ir sinbiotinę intervencijas. Gaurelių ilgis (3.14 pav. B) reikšmingai sumažėjo HFD grupėje ($F(3,30) = 6,728$; $p = 0,0013$), o sinbiotinė intervencija reikšmingai padidino šį rodiklį ($p = 0,0004$). Kriptų gylis (3.14 pav. C) taip pat reikšmingai skyrėsi tarp grupių ($H = 9,934$; $p = 0,0191$), o pagerėjimas buvo stebimas HFD+11W+GOS grupėje ($p = 0,0231$). Gaurelių plotis (3.14 pav. D) buvo reikšmingai sumažėjęs HFD grupėje ($F(3,31) = 17,05$; $p < 0,0001$), o abi intervencijos šį rodiklį atkūrė. Taurinių ląstelių skaičius gaureliuose ir kriptose taip pat buvo sumažėjęs HFD grupėje, tačiau probiotinis ir sinbiotinis skyrimas atkūrė šiuos rodiklius iki Control grupės lygio (3.14 pav. E, F). Šie rezultatai rodo, kad HFD neigiamai veikia storosios žarnos morfologiją ir taurinių ląstelių gausą.

3.15. Kepenų histologinė ir metabolinių rodiklių analizė

HFD sukėlė ryškius kepenų morfologinius pokyčius, steatozę ir metabolinius sutrikimus. Steatozės laipsnis (3.15 pav. B) buvo reikšmingai didesnis HFD grupėje nei Control grupėje ($F(3,31) = 42,51$; $p < 0,0001$), o abi intervencijos sumažino kepenų steatozę. Reikšmingas sumažėjimas nustatytas HFD+11W+GOS grupėje ($p = 0,0343$). Kepenų masė (3.15 pav. D) buvo reikšmingai padidėjusi HFD grupėje ($H = 21$; $p = 0,0001$), o sinbiotinė intervencija ją sumažino ($p = 0,0108$). Insulino koncentracija kraujo plazmoje (3.15 pav. F) taip pat buvo reikšmingai padidėjusi HFD grupėje ($F(3,31) = 3,815$; $p = 0,0195$), o HFD+11W+GOS grupėje nustatytas reikšmingas pagerėjimas ($p = 0,0433$). Šie rezultatai rodo, kad HFD sukelia kepenų

pažeidimus ir metabolinius sutrikimus, o *L. paracasei* 11W ir GOS derinys pasižymi apsauginiu poveikiu.

3.16. Hipokampo uždegiminių citokinų raiškos analizė

Hipokampo citokinų analizė parodė, kad HFD reikšmingai sustiprino uždegiminių citokinų raišką smegenyse. *Il6* mRNA raiška (3.16 pav. A) buvo reikšmingai padidėjusi HFD grupėje, palyginti su Control grupe ($p = 0,0001$), o *L. paracasei* 11W skyrimas reikšmingai sumažino šį padidėjimą ($p = 0,0226$). *I-1β* raiška (3.16 pav. B) taip pat buvo reikšmingai padidėjusi HFD grupėje ($p = 0,0020$). Statistiškai reikšmingų skirtumų *Il10* raiškoje tarp grupių nenustatyta (3.16 pav. C). *Tnf* raiška (3.16 pav. D) reikšmingai skyrėsi tarp grupių ($H = 9,772$; $p = 0,0061$), o didžiausia buvo HFD grupėje ($p = 0,0054$). Šie rezultatai rodo, kad HFD sukelia ryškų uždegiminį atsaką hipokampe, ypač didindama *Il6*, *Il1β* ir *Tnfα* raišką.

3.17. Aklosios žarnos mikrobiotos analizė

Aklosios žarnos mėginiuose mikrobiotos įvairovė buvo vertinama naudojant stebėta rūšių gausą, Simpsono ir Šanono indeksus keturiose eksperimentinėse grupėse. Apskritai HFD nesukėlė reikšmingo alfa įvairovės sumažėjimo, palyginti su Control grupe, kaip parodė atlikta statistinė analizė. Beta įvairovės analizė, atlikta naudojant pagrindinių koordinačių analizę (PCoA) ir PERMANOVA testą, parodė reikšmingą mikrobinių bendrijų atsiskyrimą tarp keturių eksperimentinių grupių ($R^2 = 0,17$; $p = 0,001$) (3.17 pav. B). HFD sukėlė reikšmingus mikrobiotos sudėties pokyčius, palyginti su Control grupe ($R^2 = 0,11$; $p = 0,003$; 3.17 pav. C). Sinbiotinė intervencija (HFD+11W+GOS) lėmė mikrobiotos struktūrą, kuri reikšmingai skyrėsi nuo HFD+11W grupės ($R^2 = 0,12$; $p = 0,003$; 3.17 pav. E).

Taksonominiu lygmeniu HFD reikšmingai pakeitė aklosios žarnos mikrobiotos sudėtį (3.17 pav. F). Control grupėje dominavo Firmicutes ir Bacteroidetes tipai. HFD padidino Firmicutes ir sumažino Bacteroidetes santykinę gausą. *L. paracasei* 11W skyrimas iš dalies atkūrė šiuos pokyčius, o sinbiotinė intervencija, taikant GOS, pasižymėjo ryškiausiu poveikiu, ypač didindamas *Bifidobacteriaceae* šeimos bakterijų gausą.

16S rRNA geno amplikonų sekoskaita atskleidė, kad HFD reikšmingai pakeitė daugelio dominuojančių bakterijų genčių gausą. *Lactobacillus* gausa (3.18

pav. A) buvo reikšmingai sumažėjusi HFD grupėje ($H = 13,21$; $p = 0,0042$). HFD taip pat didino su metaboliniais sutrikimais siejamų genčių, tokių kaip *Blautia* ir *Lachnoclostridium*, gausą. Ryškiausi pokyčiai nustatyti *Turicibacter* gentyje ($H = 24,20$; $p < 0,0001$), kurios gausa buvo reikšmingai sumažėjusi tiek HFD+11W ($p = 0,004$), tiek HFD+11W+GOS ($p < 0,0001$) grupėse (3.18 pav. E). Tuo tarpu *Bifidobacterium* genties bakterijų gausa reikšmingai padidėjo tiek probiotinėje (HFD+11W; $p = 0,0291$), tiek sinbiotinėje (HFD+11W+GOS; $p = 0,0242$) grupėse (3.18 pav. F).

3.18. Koreliacinė analizė tarp mikrobiotos, metabolinių, elgsenos ir uždegimo rodiklių

Koreliacinė analizė atskleidė reikšmingus ryšius tarp mikrobiotos sudėties ir fiziologinių rodiklių. HFD+11W+GOS grupėje *Lactobacillus* gausa koreliavo su karmino testo rodikliais ($R = 0,653$; $p < 0,05$). *Blautia* gausa teigiamai koreliavo su kūno mase 25-ąją tyrimo savaitę ($R = 0,634$; $p < 0,05$). *Lachnospiraceae UCG-006* gausa buvo susijusi su GTT rezultatų integruotu plotu po kreive (iAUC GTT) ($R = 0,634$; $p < 0,05$), kepenų mase ($R = 0,643$; $p < 0,05$) ir išmatų drėgme ($R = 0,857$; $p < 0,01$).

Vertinant elgsenos rodiklius, *Blautia* gausa teigiamai koreliavo su apsilankymų OFT centro zonoje skaičiumi ($R = 0,817$; $p < 0,01$). *Bifidobacterium* gausa buvo susijusi su mažesniu nejudrumu FST ($R = 0,763$; $p < 0,05$). Hipokampo uždegimo rodiklių analizė parodė, kad *Lactobacillus* gausa buvo susijusi su mažesne *Il6* raiška ($R = 0,974$; $p < 0,05$). Šie rezultatai rodo glaudžius ryšius tarp žarnyno mikrobiotos sudėties, metabolinių rodiklių, elgsenos parametrų ir uždegiminių procesų.

REZULTATŲ APTARIMAS

LAB yra vieni svarbiausių fermentuotų maisto produktų mikroorganizmų, prisidedantys prie fermentacijos procesų, maistinės vertės gerinimo ir įvairių bioaktyvių junginių susidarymo. Šiame darbe buvo įvertintas tradiciniuose lietuviškuose FF aptinkamų LAB kamienų probiotinis, sinbiotinis ir psichobiotinis potencialas. Tyrime taikyta nuosekli *in vitro* atranka, WGS analizė, metabolitų profiliavimas ir ilgalaikė *in vivo* intervencija HFD sukeltame metabolinio sindromo modelyje. Gauti rezultatai leido identifikuoti perspektyvius probiotinius kandidatus bei įvertinti *Lactocaseibacillus*

paracasei 11W ir jo derinio su GOS poveikį metaboliniams, uždegiminiams, elgsenos ir mikrobiotos pokyčiams.

4.1. Išgyvenimas virškinamajame trakte – svarbi probiotinių kamienų savybė

Vienas svarbiausių probiotinių kamienų atrankos kriterijų yra gebėjimas išgyventi virškinamojo trakto sąlygomis, įskaitant žemą skrandžio pH, virškinimo fermentų ir tulžies druskų poveikį. Šiame tyrime iš tradicinių lietuviškų fermentuotų maisto produktų išskirti LAB kamienai buvo vertinami taikant nuoseklų keturių etapų *in vitro* modelį. Dalis tirtų kamienų pasižymėjo dideliu atsparumu visoms modeliuotoms sąlygoms, o ypač gerus rezultatus parodė *Limosilactobacillus fermentum* UV16. Ankstesni tyrimai taip pat rodo, kad *L. fermentum* gali išlaikyti aukštą gyvybingumą rūgštinėmis ir fermentinėmis sąlygomis (Bisson et al., 2023; Sionek et al., 2023b; Sionek et al., 2024; Namshir et al., 2025). LAB atsparumas rūgštinei terpei siejamas su viduląstelinio pH reguliacijos mechanizmais, įskaitant F₁F₀-ATPazės aktyvumą, o atsparumas tulžies druskoms dažnai siejamas su tulžies druskų hidrolazių aktyvumu (Corcoran et al., 2005; Matsui ir Cvitkovitch, 2010; Kusada et al., 2021). Gauti rezultatai patvirtina, kad tradiciniai lietuviški FF gali būti vertingas perspektyvių probiotinių LAB kamienų šaltinis (Ruiz-Ramírez et al., 2023).

4.2. LAB kamienų kolonizacijos potencialas

Gebėjimas kolonizuoti žarnyną yra viena svarbiausių probiotinių mikroorganizmų savybių. Šiame tyrime atrinkti LAB kamienai pasižymėjo skirtingu autoagregacijos, adhezijos prie HCT116 žarnyno epitelio ląstelių ir antimikrobinio aktyvumo lygiu. Didžiausias adhezijos gebėjimas nustatytas *L. paracasei* 11W ir *L. fermentum* UV16 kamienams, o tai rodo jų potencialą efektyviai sąveikauti su šeimininko žarnyno epitelium. Ankstesni tyrimai parodė, kad stipri adhezija ir autoagregacija yra susijusios su geresniu kolonizacijos potencialu, konkurencija su patogenais bei ilgesniu išlikimu žarnyne (Collado et al., 2008; Monteagudo-Mera et al., 2019). Be to, atrinktų kamienų antimikrobinis aktyvumas gali būti siejamas su organinių rūgščių, bakteriocinų ir kitų antimikrobinių metabolitų gamyba, kuri prisideda prie žarnyno mikrobiotos homeostazės palaikymo ir patogeninių mikroorganizmų augimo slopinimo (Vieco-Saiz et al., 2019; Gheorghe et al., 2023). Todėl šios

savybės patvirtina atrinktų LAB kamienų potencialą tolesniam probiotinių kandidatų vystymui.

4.3. LAB kamienų saugumas ir genomo charakteristika

Saugumas yra vienas svarbiausių kriterijų vertinant probiotinių mikroorganizmų tinkamumą vartojimui. Šiame tyrime atrinktų LAB kamienų saugumas buvo vertinamas taikant fenotipinius ir genominius metodus. Nei vienas iš tirtų kamienų nepasižymėjo hemoliziniu aktyvumu ar gebėjimu skaidyti muciną, todėl nebuvo nustatyta požymių, siejamų su potencialiu patogeniškumu. WGS analizė taip pat neatskleidė kliniškai reikšmingų virulentiškumo veiksnių, o nustatyti atsparumo antibiotikams genai daugiausia buvo susiję su natūraliu, tam tikroms LAB rūšims būdingu atsparumu. Šie rezultatai atitinka ankstesnius tyrimus, kuriuose parodyta, kad dauguma fermentuotuose maisto produktuose aptinkamų LAB pasižymi palankiu saugumo profiliu ir gali būti laikomi perspektyviais probiotiniais kandidatais (EFSA, 2024; Zheng et al., 2020). WGS taikymas leido tiksliai identifikuoti atrinktus kamienus ir kartu įvertinti jų funkcines savybes bei saugumą, todėl šis metodas tampa vis svarbesne probiotinių mikroorganizmų charakterizavimo priemone.

4.4. Sinbiotinis potencialas ir triptofano biosintezė

Sinbiotikų kūrimas grindžiamas probiotinių mikroorganizmų ir prebiotikų sąveika, kuri gali sustiprinti probiotikų gyvybingumą ir biologinį aktyvumą. Šiame tyrime nustatyta, kad tiek FOS, tiek GOS skatino daugelio atrinktų LAB kamienų augimą, tačiau poveikis buvo priklausomas nuo kamieno. Ypač išsiskyrė *Lacticaseibacillus paracasei* 11W, kuris, augdamas terpėje su GOS, gamino didžiausią triptofano kiekį tarp visų tirtų kamienų. Šie rezultatai sutampa su ankstesniais tyrimais, rodančiais, kad prebiotikai gali moduluoti bakterijų metabolizmą ir skatinti biologiškai aktyvių junginių sintezę (Markowiak-Kopeć ir Śliżewska, 2020; Daliri et al., 2021).

Triptofanas yra svarbus serotonino pirmtakas, todėl bakterijų gebėjimas sintetinti šią aminorūgštį laikomas viena iš galimų probiotinio poveikio prielaidų (Clarke et al., 2013; O'Mahony et al., 2015). WGS analizė patvirtino, kad atrinkti kamienai turi genų, susijusių su triptofano metabolizmu, o didžiausias triptofano kiekis nustatytas būtent *L. paracasei* 11W kamieno kultūros supernatante. Šie duomenys rodo, kad tinkamai parinktas probiotinio

kamieno ir prebiotiko derinys gali padidinti biologiškai aktyvių metabolitų gamybą ir sustiprinti potencialų poveikį MGB sąveikai.

4.5. HFD poveikis metaboliniams rodikliams ir *L. paracasei* 11W bei GOS apsauginis poveikis

HFD sukėlė reikšmingus metabolinius sutrikimus, pasireiškusius padidėjusiu kūno svoriu, gliukozės tolerancijos sutrikimu ir hiperinsulinemija. Šie rezultatai atitinka ankstesnius tyrimus, kuriuose parodyta, kad ilgalaikis HFD vartojimas skatina nutukimo, atsparumo insulinui ir metabolinio sindromo vystymąsi (Hariri ir Thibault, 2010; Burokas et al., 2017). Nors *L. paracasei* 11W poveikis kūno masei buvo ribotas, probiotinis ir ypač sinbiotinis skyrimas pagerino gliukozės homeostazę bei sumažino kai kuriuos metabolinės disfunkcijos požymius.

Pastaraisiais metais vis daugiau dėmesio skiriama mikrobiotos vaidmeniui metabolinių ligų vystymuisi. Įrodyta, kad probiotikai ir sinbiotikai gali moduluoti žarnyno mikrobiotą, mažinti uždegimą ir gerinti gliukozės metabolizmą (Koutnikova et al., 2019; Le Barz et al., 2015). Šio tyrimo rezultatai rodo, kad *L. paracasei* 11W ir GOS derinys gali prisidėti prie HFD sukeltų metabolinių sutrikimų mažinimo, o stipresnis sinbiotinės intervencijos poveikis patvirtina prebiotikų svarbą stiprinant probiotikų veiksmingumą.

4.6. HFD poveikis elgsenai ir pažintinėms funkcijoms

HFD ne tik sukėlė metabolinius pokyčius, bet ir pablogino emocinį bei pažintinį pelių elgesį. HFD grupėje nustatyti ryškesni nerimo ir depresinio pobūdžio elgesio požymiai, sumažėjęs savanoriškas aktyvumas, anhedonija ir atminties sutrikimai. Šie rezultatai sutampa su ankstesniais duomenimis, rodančiais, kad nutukimas ir metabolinis sindromas yra glaudžiai susiję su neuropsichiatriniais sutrikimais bei pažintinių funkcijų blogėjimu (Castanon et al., 2015; Cryan et al., 2019).

L. paracasei 11W skyrimas sumažino dalį HFD sukeltų elgsenos sutrikimų, o stipriausias poveikis dažniausiai buvo stebimas kartu skiriant GOS. Šie rezultatai dera su augančiu kiekiu duomenų, rodančių, kad tam tikri probiotiniai mikroorganizmai gali veikti kaip psichobiotikai ir moduluoti MGB sąveiką (Dinan et al., 2013; Sarkar et al., 2016). Tikėtina, kad stebėti

elgsenos pokyčiai yra susiję ne su vienu mechanizmu, bet su kompleksine metabolinių, imuninių ir mikrobiotos pokyčių sąveika.

Ypač svarbu tai, kad *L. paracasei* 11W pasižymėjo gebėjimu sintetinti triptofaną, o jo gamyba padidėjo kartu su GOS. Kadangi triptofanas yra serotonino pirmtakas, šis mechanizmas gali būti viena iš galimų grandžių, siejančių sinbiotinę intervenciją su geresniais elgsenos rodikliais. Vis dėlto norint tiksliai nustatyti priežastinius ryšius, reikalingi papildomi tyrimai.

4.7. HFD poveikis uždegiminiams procesams ir žarnyno morfologijai

Taip patd daugėja duomenų, rodančių, kad nutukimas ir metabolinis sindromas yra susiję su lėtiniu mažo intensyvumo uždegimu tiek periferiniuose audiniuose, tiek centrinėje nervų sistemoje (Gregor ir Hotamisligil, 2011; Guillemot-Legris ir Muccioli, 2017). Šiame tyrime HFD padidino prouždegiminių citokinų koncentracijas storiojoje žarnoje bei prouždegiminių genų raišką hipokampe, patvirtindamas HFD sukeltą sisteminį ir centrinį uždegiminį atsaką. Tiek probiotinis, tiek sinbiotinis skyrimai sumažino dalį šių pokyčių, o ryškiausias poveikis dažniausiai buvo stebimas sinbiotinėje grupėje.

HFD taip pat sukėlė storosios žarnos morfologinius pokyčius, įskaitant sumažėjusį taurinių ląstelių skaičių ir pablogėjusią gleivinės struktūrą. Panašūs pokyčiai aprašyti ir kituose eksperimentiniuose nutukimo modeliuose, kuriuose jie siejami su padidėjusiu žarnyno pralaidumu bei uždegimu (Cani et al., 2008; Rohr et al., 2020). *L. paracasei* 11W ir ypač jo derinys su GOS iš dalies atkūrė šiuos parametrus, rodydami teigiamą poveikį žarnyno barjero funkcijai.

Be žarnyno pokyčių, HFD sukėlė kepenų steatozę, padidino kepenų masę ir insulino koncentraciją kraujo plazmoje. Sinbiotinis skyrimas sušvelnino HFD sukeltus pokyčius, o tai atitinka ankstesnius tyrimus, kuriuose probiotikai ir prebiotikai siejami su geresne metaboline kontrole ir mažesniu kepenų suriebėjimu (Le Roy et al., 2013; Koutnikova et al., 2019).

4.8. HFD poveikis aklosios žarnos mikrobiotai ir jos sąsajos su šeimininko fiziologija

HFD reikšmingai pakeitė aklosios žarnos mikrobiotos sudėtį, nors alfa įvairovės rodikliai iš esmės nepakito. Beta įvairovės analizė parodė aiškių eksperimentinių grupių atsiskyrimą, rodantį reikšmingą mikrobiotos persitvarkymą veikiant HFD. Panašūs rezultatai dažnai nustatomi nutukimo modeliuose ir siejami su metabolinių bei imuninių funkcijų pokyčiais (Turnbaugh et al., 2006; Ley et al., 2005).

Taksonominiu lygmeniu HFD sumažino kai kurių potencialiai naudingų bakterijų gausą ir skatino mikroorganizmų, siejamų su metaboliniais sutrikimais bei uždegimu, gausėjimą. *L. paracasei* 11W iš dalies sumažino šiuos pokyčius, tačiau ryškiausias poveikis nustatytas sinbiotinėje grupėje. GOS skyrimas skatino *Bifidobacterium* gausėjimą ir lėmė mikrobiotos struktūrą, artimesnę kontrolinei grupei. Šie rezultatai atitinka ankstesnius tyrimus, rodančius bifidogeninį GOS poveikį ir jo svarbą palaikant žarnyno homeostazę (Rivière et al., 2016; Scott et al., 2020).

Koreliacijų analizė atskleidė sąsajas tarp atskirų mikroorganizmų grupių, metabolinių rodiklių, elgsenos parametrų ir uždegimo žymenų. Nors šios sąsajos neleidžia nustatyti priežastinių ryšių, jos patvirtina glaudų mikrobiotos, metabolizmo, imuninės sistemos ir smegenų funkcijų tarpusavio ryšį. Gauti rezultatai rodo, kad *L. paracasei* 11W ir GOS derinys gali moduluoti MGB sąveiką ir prisidėti prie HFD sukeltų metabolinių bei elgsenos sutrikimų mažinimo.

Apibendrinant, šiame tyrime nustatyta, kad tradiciniai lietuviški FF yra perspektyvus naujų probiotinių LAB kamienų šaltinis. Atrinktas *L. paracasei* 11W kamienas pasižymėjo geru atsparumu virškinamojo trakto sąlygoms, kolonizacijos potencialu, palankiu saugumo profiliu ir gebėjimu sintetinti triptofaną. *In vivo* tyrimai parodė, kad *L. paracasei* 11W, ypač derinyje su GOS, sumažino HFD sukeltus metabolinius, uždegiminius ir elgsenos sutrikimus bei moduliavo žarnyno mikrobiotos sudėtį. Šie rezultatai patvirtina sinbiotinių strategijų potencialą MGB sąveikos moduliavimui ir sudaro pagrindą tolimesniams mechanistiniams bei klinikiniams tyrimams.

Tyrimo ribotumai ir tolimesnės tyrimų kryptys

Šis tyrimas turi keletą ribotumų. *In vivo* eksperimentai buvo atlikti tik su pelių patelėmis, todėl gautų rezultatų negalima tiesiogiai pritaikyti patinėliams. Be to, elgesio testai leidžia vertinti tik tam tikrus žmogaus depresijos ir nerimo aspektus, todėl jų interpretacija klinikiniam kontekste turi būti atsargi. Taip pat tik *L. paracasei* 11W kamienas buvo tirtas *in vivo*, nors kiti atrinkti LAB kamienai taip pat pasižymėjo perspektyviomis probiotinėmis ir psichobiotinėmis savybėmis.

Ateities tyrimuose tikslinga išsamiau nagrinėti MGB sąveikos mechanizmus, vertinant žarnyno barjero vientisumą, mikroglijos ląstelių funkcionalumą, neurogenezę, bei mikrobiotos gaminamus metabolitus. Tikslesniam mikrobiotos funkciniam apibūdinimui reikėtų taikyti metagenominius, metatranskriptominius ir metabolominius metodus. Taip pat būtini tyrimai, leidžiantys nustatyti priežastinius ryšius tarp mikrobiotos pokyčių ir šeimininko fenotipo, bei gerai suplanuoti klinikiniai tyrimai, siekiant įvertinti *L. paracasei* 11W veiksmingumą ir saugumą žmonėms.

IŠVADOS

1. Iš 150 tradiciniuose lietuviškuose fermentuotuose maisto produktuose išskirtų LAB kamienų tik dešimt pasižymėjo geru išgyvenamumu virškinamojo trakto sąlygomis, adhezija prie epitelio ląstelių ir antimikrobinio aktyvumu, todėl buvo atrinkti kaip perspektyvūs probiotiniai kandidatai.
2. FOS ir GOS skatino LAB kamienų augimą ir triptofano gamybą kamienui specifiniu būdu, išskiriant *Lactocaseibacillus paracasei* 11W ir *Leuconostoc mesenteroides* KA15 kaip perspektyvius sinbiotinių produktų kandidatus.
3. WGS analizė patvirtino, kad atrinkti LAB kamienai turi su probiotinėmis savybėmis susijusių genų, įskaitant genus, dalyvaujančius atsake į stresą, adhezijoje ir triptofano biosintezėje.
4. Saugumo vertinimas parodė, kad penki atrinkti perspektyviausi LAB kamienai atitiko EFSA QPS procedūros reikalavimus, nes neturėjo pernešamų antimikrobinio atsparumo genų, virulentiškumo veiksnių ir hemolizinio aktyvumo.
5. Sinbiotinis *L. paracasei* 11W ir GOS skyrimas sumažino dalį HFD sukeltų metabolinių, neurouždegiminių, elgesio ir žarnyno

mikrobiotos pokyčių pelių patelėms, atskleidamas šio sinbiotinio derinio potencialą metabolinių ir neurouždegiminių sutrikimų prevencijai.

6. Probiotinis ir sinbiotinis skyrimas sukėlė specifinius žarnyno mikrobiotos sudėties pokyčius: padidino *Bifidobacterium* genties bakterijų gausą ir moduliavo su HFD susijusių bakterijų sudėtį, nors bendroji alfa įvairovė reikšmingai nepakito.

LIST OF PUBLICATIONS

1. **Megur A.**, Daliri E. B. M., Lastauskienė E., Burokas A. (2023). *In vitro* screening and characterization of lactic acid bacteria from Lithuanian fermented food with potential probiotic properties. *Frontiers in microbiology*, 14.
<https://doi.org/10.3389/fmicb.2023.1213370>
As the first author, I was responsible for the conceptualization and execution of this study. I designed and carried out experiments, including the isolation of LAB from fermented foods, in vitro characterization under GI stress, antimicrobial and adhesion assays, and functional tests such as tryptophan production and prebiotic utilization. I performed genomic data analysis to identify probiotic and safety-related genes, interpreted the findings, and integrated the results with existing literature. Additionally, I wrote the first draft of the manuscript, prepared figures and tables, and coordinated revisions with co-authors to finalize the publication.
2. **Megur A.**, Lastauskienė E., Burokas A. (2024). Draft genome sequence data of *Lactiplantibacillus plantarum* 33C isolated from Lithuanian fermented food. *Data in Brief*, 55.
<https://doi.org/10.1016/j.dib.2024.110750>
As the first author, I isolated L. plantarum 33C from traditional fermented cherry tomatoes and prepared the genomic DNA for sequencing. I performed DNA extraction, quality control, and collaborated with sequencing facilities for library preparation and genome sequencing. I analyzed the genome assembly, annotation, and functional gene prediction, focusing on probiotic traits and safety assessment. I interpreted the genomic findings, drafted the manuscript, prepared all figures and tables, and coordinated revisions with co-authors.
3. **Megur A.**, Ambrutaitytė K., Šimoliūnas E., Lastauskienė E., & Burokas A. (2025). Whole-genome sequencing and *in vitro* probiotic characterization of *Pediococcus pentosaceus* ELAB 60WB isolated from fermented cherry tomatoes. *LWT - Food Science and Technology*, 220.
<https://doi.org/10.1016/j.lwt.2025.117547>
*As the first author, I isolated and purified *Pediococcus pentosaceus* ELAB 60WB from fermented cherry tomatoes and designed its*

comprehensive genomic and in vitro characterization. I performed whole-genome sequencing data analysis, including functional annotation, screening for antimicrobial resistance, virulence genes, and plasmids. I also carried out in vitro assays on GI tolerance, adhesion, and antimicrobial activity, and correlated them with genomic insights. I wrote the manuscript, created figures and tables, and integrated feedback from all collaborators.

4. **Megur A.**, Ambrutaitytė K., Sadauskas M., Stankevičiūtė J., Meškys R., Lastauskienė E., Burokas A. (2026). Investigating tryptophan-producing probiotic lactic acid bacteria from Lithuanian fermented foods: functional characterization, safety evaluation, and genomic analysis. *Journal of Functional Foods*, 140.

<https://doi.org/10.1016/j.jff.2026.107281>

As the first autor, I designed and performed the isolation and in vitro screening of LAB strains from various Lithuanian fermented foods. I conducted experiments on GI stress tolerance (acid, bile, enzymes), adhesion to epithelial cells, aggregation essays, antimicrobial activity, antioxidant capacity, and prebiotic utilization. I carried out statistical analyses, interpreted functional properties in the context of probiotic potential, and linked them to psychobiotic relevance. I prepared the initial draft, figures, and tables, and managed the revision process with the co-authors. I performed genomic data analysis to identify probiotic and safety-related genes, interpreted the findings, and integrated the results with existing literature. Additionally, I wrote the first draft of the manuscript, prepared figures and tables, and coordinated revisions with co-authors to finalize the publication.

5. **Megur A.**, Kobeckytė D., Kumar B., Ambrutaitytė K., Barasa P., Šimoliūnė I., Buišas R., Lastauskienė E., Burokas A. (2026) (under review)

As the first author, I contributed to the study design and execution of the in vivo animal experiments. I performed animal handling, dietary interventions, probiotic and synbiotic administration, metabolic assessments, behavioral testing, tissue collection, and sample preparation. I conducted laboratory analyses including histological evaluation, cytokine expression analysis, microbiota profiling, and data interpretation. I performed statistical analyses, integrated the

metabolic, inflammatory, behavioral, and microbiota findings, and interpreted the results within the framework of microbiota–gut–brain axis interactions. I prepared figures and tables, wrote the initial manuscript draft, and coordinated revisions with co-authors throughout the publication process.

Other publications that are not directly related to the topic of doctoral studies, but are published during the period of doctoral studies in the journal with a citation index factor (IF) in the certificate |Analytics Web of science platform:

1. **Megur A.**, Kumar B. (2026). Psychobiotics and the Microbiota – Gut – Brain Axis : Bridging Functional Foods with Therapeutic Potential in Neurodegenerative and Psychiatric Disorders Food *.Reviews International*, 1–35.
<https://doi.org/10.1080/87559129.2026.2649298>
2. Kumar B., **Megur A.**, Kaushik N., Singh S. (2026). Metagenomic Perspectives on the Human Gut Ecosystem and Disease Associations. *Integrative Approach to Fungal Infections and Bioprospecting for Drug Discovery*.
<https://doi.org/10.1201/9781003633952-10>
3. Daliri E. B. M.,* **Megur A.**,* Mingaila,J., Vijaya A. K., Balnionytė T., Sakar D., Carnicero-Mayo Y., Behrends V., Costabile A., Burokas A. (2025). Fermented beetroot modulates gut microbial carbohydrate metabolism in prediabetes and prevents high-fat diet induced hyperglycemia in a prediabetic model. *Current Research in Food Science*, 10.
<https://doi.org/10.1016/j.crfs.2025.101052>; * both the authors contributed equally for this work
4. Barasa P., Simoliunas E., Grybas A., Zilinskaite-Tamasauske R., Dasevicius D., Alksne M., Rinkunaite I., Buivydas A., Baltrukonyte E., Tamulyte R., **Megur A.**, Verkauskas G., Baltriukiene D., & Bukelskiene V. (2025). Development of multilayered artificial urethra graft for urethroplasty. *Journal of Biomedical Materials Research*, 113(1).
<https://doi.org/10.1002/jbm.a.37796>

5. **Megur A.**, Daliri E. B. M., Baltriukienė D., & Burokas A. (2022). Prebiotics as a Tool for the Prevention and Treatment of Obesity and Diabetes: Classification and Ability to Modulate the Gut Microbiota. *International Journal of Molecular Sciences* 23(11). <https://doi.org/10.3390/ijms23116097>
6. **Megur A.**, Baltriukienė D., Bukelskienė V., Burokas A. (2021). The microbiota–gut–brain axis and Alzheimer’s disease: Neuroinflammation is to blame? *Nutrients* 13(1). 1–24. <https://doi.org/10.3390/nu13010037>

CONFERENCE PRESENTATIONS

1. **Megur A.**, Balnionytė, T. Burokas A. *In Vitro* Characterization of Probiotic Strains Exhibiting Anti-inflammatory and Anti-diabetic Functions. 15th International Scientific conference probiotics, prebiotics, gut microbiota and health (IPC). 27 - 30th June 2022. Bratislava, Slovakia. (**Poster Presentation**)
2. Megur A., Stankevičiūtė J., Lastauskienė E., Burokas A. *In Vitro* Functional Characterization of Probiotic Strains for Anti-inflammatory and Anti-diabetic Applications. 5th Congress of Baltic Microbiologists. 11 - 13th October 2023. Vilnius, Lithuania (**Poster Presentation**)
3. **Megur A.**, Stankevičiūtė J., Lastauskienė E., Burokas A. *In Vitro* Screening and Characterization of Lactic Acid Bacteria from Lithuanian Fermented Food with Potential Probiotic Properties. 16th International Scientific conference probiotics, prebiotics, gut microbiota and health. 20 - 22nd June 2023. Bratislava, Slovakia (**Oral Presentation**)
4. **Megur A.**, Stankevičiūtė, J., Lastauskienė E., Burokas A. Characterizing Probiotic Strains *In Vitro* for Their Anti-inflammatory and Anti-diabetic Potential. The COINS International Conference for Life Sciences. 15 - 18th April 2024. Vilnius, Lithuania (**Poster Presentation**)
5. **Megur A.**, Stankevičiūtė J., Lastauskienė E., Burokas A. *In Vitro* Assessment of Probiotic Strains with Anti-inflammatory and Anti-diabetic Properties. Life Sciences Center Conference. 11-12th April 2024. Vilnius, Lithuania. (**Poster Presentation**)
6. **Megur A.**, Stankevičiūtė, J., Lastauskienė, E. Burokas A. *In Vivo* Synergistic Effects of *Lactocaseibacillus paracasei* 11W and Galactooligosaccharide on Psychobiotic and Antidiabetic Efficacy in High-Fat-Diet-Fed Female Mice. 17th International Scientific conference probiotics, prebiotics, gut microbiota and health (IPC). 18-20th June 2024. Prague, Czech Republic. (**Oral Presentation**)

7. **Megur A.**, Kobeckytė D., Kumar B., Lastauskienė E., Burokas A. Synergistic *In Vivo* Effects of *Lactocaseibacillus paracasei* 11W and Galactooligosaccharide on Psychobiotic and Antidiabetic Outcomes in Female Mice on a High-Fat Diet. 11th World Congress on Targeting Microbiota. 14 - 15th October 2024. Malta. **(Oral Presentation)**
8. **Megur A.**, Burokas A. Genomic and Functional Analysis of *Pediococcus pentosaceus* ELAB 60WB: A Potential Probiotic for Mitigating Metabolic Disorders in Male C57BL/6 Mice. 18th International Scientific conference probiotics, prebiotics, gut microbiota and health (IPC). 24 - 26th June 2025. Athens, Greece. **(Oral Presentation)**
9. **Megur A.**, Kobeckytė D., Kumar B., Lastauskienė E., Burokas A. Synbiotic Supplementation Attenuates High-Fat Diet-Induced Metabolic and Behavioral Alterations via the Microbiota-Gut-Liver-Brain Axis. The 2nd International Vilnius Neuroscience Conference for Young Researchers. 17 – 18th April 2026. Vilnius, Lithuania **(Oral Presentation)**
10. **Megur A.**, Kobeckytė D., Kumar B., Lastauskienė E., Burokas A. Synbiotic Supplementation Attenuates High-Fat Diet-Induced Metabolic and Behavioral Alteration in Female Mice Modulating Microbiota-Gut-Liver-Brain-Axis. PANERIS Conference. 17 – 18th April 2026. Vilnius, Lithuania. **(Poster presentation)**
11. **Megur A.**, Kobeckytė D., Kumar B., Lastauskienė E., Burokas A. Modulating the Microbiota-Gut-Liver-Brain Axis: Synbiotic Supplementation Mitigates High-Fat Diet-Induced Metabolic and Behavioral Changes. Mikrobiologija Conference. 29 – 30th April 2026. Palanga, Lithuania **(Poster presentation)**
12. **Megur A.**, Kobeckytė D., Kumar B., Lastauskienė E., Burokas A. Synbiotic Supplementation Alleviates High-Fat Diet-Induced Metabolic and Behavioral Disruptions by Modulating the Microbiota-Gut-Liver-Brain Axis. 19th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health Conference. 22-24 June 2026. Kraków, Poland. **(Poster presentation)**

ACKNOWLEDGEMENT

There is a particular kind of silence that settles over a laboratory late at night, once the centrifuges have finally gone quiet and the only sound left is your own breathing beside a stack of unfinished data. It was in that silence that I came to understand this thesis was never mine alone. It belongs, in ways I am only now beginning to measure, to everyone who walked beside me while I built it.

I owe my deepest gratitude to my supervisor, Prof. Dr. Aurelijus Burokas, whose insight and patience carried me through more uncertain nights than I can count. He guided me through every stage of this PhD journey and never once let go of the steady hand he offered from the very first day. He had a rare gift, the ability to challenge my thinking while still leaving me feeling encouraged rather than discouraged. That balance shaped not only this research, but the researcher I am becoming. I am just as grateful to my co-supervisor, Prof. Dr. Eglė Lastauskienė, and to my colleagues in the Department of Microbiology and Biotechnology, who welcomed me into their world with open arms.

To Prof. Dr. Daiva Balturukienė and Prof. Dr. Virginija Bukalskienė, thank you for your endless patience, for listening calmly and carefully when I brought you the heaviest of my problems, and for helping me solve one of the greatest setbacks of my life. Your decision in my favor, and the kindness you showed me, are the reason I was able to keep going and finish what I started.

Bablu Kumar deserves a paragraph of his own. Working beside him on microbial analysis was some of the fun I had during this entire process. It was professional, yes, but genuinely joyful too. We moved through several different projects together, each one teaching me something new, and not once, no matter the hour or the question, did he ever tell me no. That is what teamwork should look like, and I was lucky to have it.

Some of the richest chapters of this journey happened far from home. My time at the University of Gothenburg, in Sweden, opened doors I didn't know existed. New academic networks, new ways of thinking, and an entirely different lens through which to see my own research. In the Wallenberg Lab, I met people who patiently taught me, in careful detail, the techniques and statistical methods needed to present data in high-impact journals, along with

new approaches to liver, lungs, kidney, and pancreatic biochemical analysis. It was a rigorous, humbling, and deeply rewarding experience to be part of that laboratory, and I owe special thanks to Dr. Fredrik Bäckhed, Dr. Louise Mannerås Holm, and postdoctoral researcher Dr. Hobby Aggarwal, whose kindness and patience in teaching me new techniques left a mark I will carry into every research project ahead of me. I am especially grateful to Dr. Muhammad Tanweer Khan for his detailed, professional guidance on probiotics-related topics and for introducing me to the workings of the anaerobic chamber. And beyond the science, I found friendship, Adriana, Alba, Daisy, Greta, Lorenza, Luca, Sara, Tanmoy, and Matthias made sure I never lost my balance in the pursuit of the perfect experiment. They taught me that a beer now and then isn't a distraction from work, but the escape that makes the work sustainable. I needed that more than I realized.

My time at the Latvian Biomedical Research and Study Centre in Riga was just as meaningful, both professionally and personally. The collaborations, the exchanges, the quiet moments of reflection between visits. All of it added depth to my work and broadened how I understood my field. My sincere thanks to Rihardas Skasis, who patiently taught me 16S rRNA sequencing step by step and walked me through the microbial analysis in careful detail.

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And then there is my department. The place, and the people, who watched me grow from a nervous new researcher into someone who could finally stand on her own. None of this would have been possible without their steady support. From the earliest protocol drafts to the final execution, they guided me with kindness at every step, and I feel genuinely lucky to have belonged to such a supportive academic family.

Beyond the walls of the university, I owe everything to my husband, my family, and my friends. Their unwavering support, patience, and understanding are what kept me standing when the work threatened to knock me down. They grounded me on the hardest days and celebrated with me on the good ones, and I cannot imagine having done any of this without them. In the moments when I felt truly lost, they were the ones who reminded me who I was. Words don't do justice to how much I leaned on them during this PhD. They were, and always will be, their true backbone.

If there is one thing I have learned, it's that a PhD is a roller coaster that every researcher must ride at some point. It teaches you new things, yes, but it also reveals the true colors of the people around you. It can make you wiser and more resilient as a researcher. But only if you have people on your corner who never stop paying attention, who never stop showing up. I was fortunate enough to have exactly that. To everyone named here, and to everyone who quietly held me up along the way, thank you for making this PhD study possible.

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EDUCATION

Bachelor's degree in biotechnology at University of Mumbai. Mumbai, India, 2014-2017

Master's degree in biotechnology at University of Mumbai. Mumbai, India. 2017-2018

Master's degree in Neurobiology at Vilnius University. Vilnius, Lithuania. 2018-2020

GRANTS AND AWARDS

Year	Award
2026	Best poster presentation award at 19th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health
2026	FEMS Meeting grant
2026	Best Presentation Award 2 nd International Neuroscience Conference
2025	LMT Study Results Grant P-DAP-25-19
2025	LMT Travel Grant P-DAK-25-10
2025	FEBS Short-term fellowship
2024	LMT Travel Grant P-DAK-24-45
2023	LSC Nominal Scholarship
2022	Linea Libera Nominal Scholarship

ASSOCIATIONS

Member of Federation of European Biochemical Societies (FEBS) since 2023

Member of Federation of European Microbiological Societies (FEMS) since 2025

Member of Lithuanian Biochemical Society since 2023

Member of Lithuanian Society of Microbiologists since 2025

Member of The Baltic Association of Laboratory Animal Sciences since 2022

INTERNSHIPS

- 2024-2025 Mobility program of ERASMUS+: Wallenberg Laboratory, University of Gothenburg, Sweden. January 2025- July 2025. Topic – “Investigating the Role of Microbial Metabolites on Cardiometabolic health & Diseases using Animal Models”
- 2025 FEBS short term fellowship: Wallenberg Laboratory, University of Gothenburg, Sweden. February 2025 – April 2025. Topic - “Investigating the Role of Microbial Metabolites on Cardiometabolic health & Diseases using Animal Models”
- 2024-2025 Mobility program of ERASMUS+: Latvian Biomedical Research and Study Centre, Riga, Latvia. October 2024- January 2025. Topic – “Microbiome data analysis for both amplicon and shotgun sequencing data”

NOTES

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