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Integrative *in vitro* and multiomics approaches to evaluate the probiotic and postbiotic features of *Bacillus velezensis* P45

Porto Alegre
2026

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“Feliz aquele que transfere o que sabe e aprende o que ensina.”

Cora Coralina

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ABREVIATIONS

CAZymes: Carbohydrate-active enzymes (pt-BR: e*nzimas ativas em carboidratos)*

CD: Crohn's disease

EFSA: European Food Safety Authority

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

FEEDAP: Panel on Additives and Products or Substances used in Animal Feed

FOS: Fructooligosaccharides (pt-BR: *frutooligossacarídeos)*

GABA: Gamma-aminobutyric acid (pt-BR: *ácido gama-aminobutírico)*

GH: Glycosyl hydrolases

GI: Gastrointestinal tract

GOS: Galactooligosaccharides (pt-BR: *galactooligossacarídeos)*

GRAS: Generally recognized as safe

GT: Glycosyltransferases

IBD: Inflammatory bowel disease

ISAPP: International Scientific Association for Probiotics and Prebiotics

SCFAs: Short Chain Fatty Acids

UC: Ulcerative colitis

QPS: Qualified Presumption of Safety

WHO: World Health Organization

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FIGURE 1. Distribution of major microbiota phyla in various human body parts.

FIGURE 2. Difference between two forms of IBD.

FIGURE 3. Composition of the human gut bacterial microbiota of patients healthy and with IBD condition.

FIGURE 4. Mandatory and alternative requirements to select a probiotic.

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ABSTRACT

The study of novel probiotics for human health has gained great interest in the last decade, driven by new *in silico* and *ex vivo* analytical approaches. This work investigated the functional features of *Bacillus velezensis* P45 as potencial human probiotic, synbiotic and postbiotic producer, using *in vitro, in silico* and *ex vivo* analyses. Genome analysis and *in vitro* assays demonstrated the probiotic potential of the *B. velezensis* P45, highlighting its antimicrobial activity, tolerance to gastrointestinal conditions, and absence of genes associated with virulence and antimicrobial resistance. Afterward, was assessed the proteomic response of *B. velezensis* P45 strain after 24 h of cultivation using fructooligosaccharides (FOS), galactooligosaccharides (GOS), glucose, and mucin as the sole carbon sources. The results revealed that FOS and GOS promoted adhesion and production of bioactive compounds, such as levanase expression in FOS and γ-aminobutyric acid (GABA) biosynthetic pathways in GOS; while mucin induced sporulation and stress-related proteins activation. Then, to obtain more information about the strain's synbiotic potential, a multiomics approach was conducted using the same carbon sources. As results, a substrate-dependent short-chain fatty acid production was observed, and carbohydrate-active enzymes (CAZymes) regulation, with predominance by glycosyl hydrolases and glycosyltransferases. Lastly, an *ex vivo* culture model was uded to evaluate the impact of postbiotics produced by *B. velezensis* P45 during cultivation with prebiotics and mucin on human intestinal microbiota of healthy and inflammatory bowel disease individuals. Multiomics analysis on the samples treated with the postbiotics identified increase in the relative abundance of *Bifidobacterium*, as well as stimulation of vitamin B12 and GABA production in the different microbiomes. These results reinforce the probiotic potential of *B. velezensis* P45 and suggest that its combination with prebiotics may promote beneficial effects through functional modulation of the human gut microbiota.

Keywords: *Bacillus velezensis*, probiotic, prebiotic, multi-omics approaches, human gut microbiome.

RESUMO

O estudo de novos probióticos para a saúde humana tem despertado grande interesse na última década, impulsionado por novas abordagens analíticas *in silico* e *ex vivo*. Este trabalho investigou as características funcionais de *Bacillus velezensis* P45 como potencial probiótico, simbiótico e de produção de pós-bióticos para humanos, utilizando análises *in vitro, in silico* e *ex vivo*. A análise genômica e os ensaios *in vitro* demonstraram o potencial probiótico de *B. velezensis* P45, destacando sua atividade antimicrobiana, tolerância às condições gastrointestinais e ausência de genes associados à virulência e resistência antimicrobiana. Posteriormente, avaliou-se a resposta proteômica da cepa *B. velezensis* P45 após 24 h de cultivo utilizando frutooligossacarídeos (FOS), galactooligossacarídeos (GOS), glicose e mucina como únicas fontes de carbono. Os resultados revelaram que FOS e GOS promoveram a adesão e a produção de compostos bioativos, como a expressão da levanase em FOS e as vias biossintéticas do ácido γ-aminobutírico (GABA) em GOS, enquanto a mucina induziu a esporulação e a ativação de proteínas relacionadas ao estresse. Em seguida, para obter mais informações sobre o potencial simbiótico da cepa, uma abordagem multiômica foi conduzida utilizando as mesmas fontes de carbono. Como resultado, observou-se uma produção de ácidos graxos de cadeia curta dependente do substrato e a regulação de enzimas ativas em carboidratos (CAZymes), com predominância de glicosil hidrolases e glicosiltransferases. Por fim, utilizou-se um modelo de cultura *ex vivo* para avaliar o impacto dos pós-bióticos, obtidos a partir do cultivo de *B. velezensis* P45 com prebióticos e mucina, na microbiota intestinal humana de indivíduos saudáveis e com doença inflamatória intestinal. Análises multiômicas das amostras tratadas com os pós-bióticos identificoram um aumento na abundância relativa de *Bifidobacterium*, bem como a estimulação da produção de vitamina B12 e GABA nos diferentes microbiomas. Esses resultados reforçam o potencial probiótico de *B. velezensis* P45 e sugerem que sua combinação com prebióticos pode promover efeitos benéficos por meio da modulação funcional da microbiota intestinal humana.

Palavras-chave: *Bacillus velezensis*, probiótico, prebiótico, abordagens multi-ômicas, microbioma intestinal humano.

1. INTRODUCTION

1.1 HUMAN MICROBIOTA

1.1.1 Human microbiota composition and diversity

Human beings share many anatomical and social similarities, such as bipedalism, an opposable thumb, basic physiological needs, and common professional daily routines. However, each person has unique biological identifiers, such as deoxyribonucleic acid and fingerprints. Beyond these, there is yet another unique level of biological identity: the community of microorganisms that inhabit the human body.

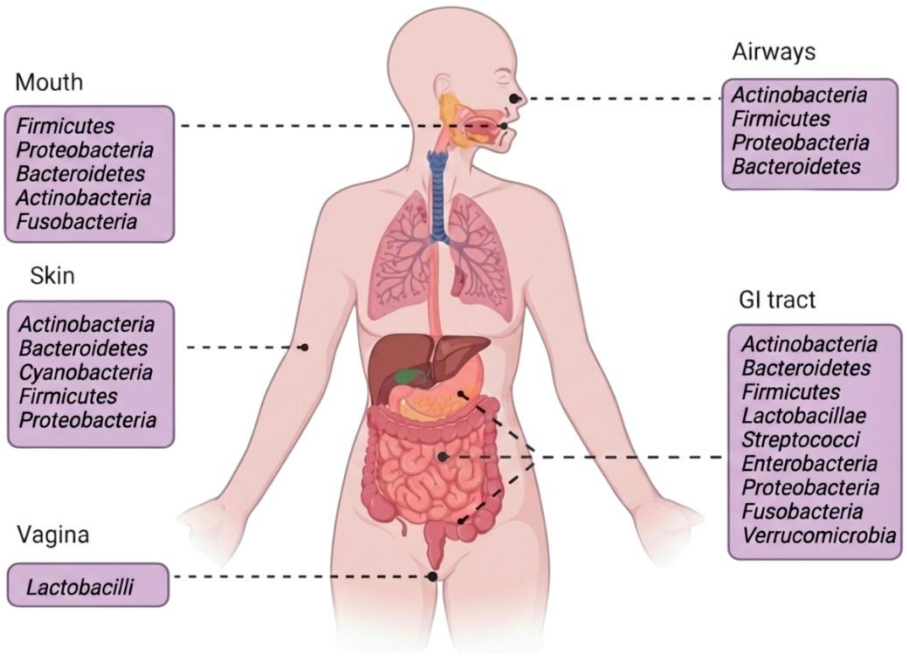
Approximately 100 trillion microorganisms, including diverse communities of bacteria, fungi, protozoa, and viruses, live and coexist in intimate association with the host, known as the microbiota (OKALIN *et al.,* 2025). In addition, this human microbiota, also called "hidden organ", may harbor between 2 and 20 million microbial genes compared to the about 20,000 human genes (HOU *et al.,* 2022; ALPUIM COSTA *et al.,* 2025).

Generally, the human microbiota composition can vary and is not uniformly distributed throughout the human body (Figure 1) (HOU *et al.,* 2022). Different body parts, such as the oral cavity, skin, respiratory and gastrointestinal tract (GI), host microbial unique niches where different microbial communities establish and perform specialized functions (GOVENDER & GHAI, 2025).

Among these anatomical environments, the gut is widely considered the optimal microbial residence, due to its unique combination of stability, nutrient availability, and anaerobic conditions within the human body (HOU *et al.,* 2022; SHARMA, SUDARSANAN & MOONAH, 2025). Taxonomically, a healthy gut harbors a rich abundance of microbes, predominantly from the phyla Bacillota e Bacteroidota (previously

named as Firmicutes and Bacteroidetes, respectively), which together comprise approximately 90% of its components, including more than 200 genera, such as *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, *Ruminicoccus*, *Bacteroides*, and *Prevotella* (FERRAZ, 2023; SASSO *et al.,* 2023). Other phyla, such as Actinobacteria, mainly represented by the *Bifidobacterium* genus, Pseudomonadota (previosly named as Proteobacteria), Fusobacteria, and Verrucomicrobia are present in lower abundance (FERRAZ, 2023).

Figure 1. Distribution of major microbiota phyla in various human body parts.



Source: Adapted from MA et al., (2024).

Despite its general stability, the gut microbial composition is a highly dynamic ecosystem and can change throughout an individual's life, influenced by multiple intrinsic and extrinsic factors, including delivery pattern, age, diet, lifestyle, geographic location, and host genetics (HASAN & YANG, 2019; GOVENDER & GHAI, 2025). Studies indicate that

environmental factors exert a higher modulatory effect on the composition of the gut microbiome compared to the host's genetics (ROTHSCHILD *et al.,* 2017).

As the starting point of an individual's microbial journey, the delivery pattern highlights as a crucial factor in the initial establishment of gut microbiota (PANTAZI *et al.*, 2023). Babies' normal delivery is colonized primarily by vaginal bacteria, such as *Lactobacillus* and *Prevotella* (PANTAZI *et al.*, 2023; SUÁREZ-MARTÍNEZ *et al.*, 2023). In contrast, babies born by cesarean section have a microbiota profile dominated by skin-associated genera, such as *Streptococcus*, *Propionibacterium*, *Enterococcus*, and *Staphylococcus* (HASAN & YANG, 2019; ANTHOULAKI *et al.*, 2023). This early-life gut colonization has significant implications for subsequent microbial succession and host-microbiota interactions (BANKOLE & LI, 2025).

The intestinal microbiota evolves rapidly during childhood, strongly influenced by aging, feeding methods, and diet (DING *et al.*, 2025). Briefly, breastfeeding can provide *Bifidobacterium* and *Lactobacillus*, whereas formula-fed infants tend to harbor higher levels of *Enterococcus* and *Bacteroides* (LAURSEN *et al.*, 2021). Different dietary patterns exert direct and continuous selective pressures on gut microbial populations (RINNINELLA *et al.*, 2023). Diets rich in fiber, such as plant-based diets, like vegetarian and Mediterranean, promote enhanced gut microbiota diversity and composition, including members of beneficial genera such as *Eubacterium*, *Roseburia*, *Lactobacillus*, *Bifidobacterium*, and *Prevotella* (MUIGANO *et al.*, 2025). Meanwhile, diets high in ultra-processed foods, which are typically high in fat, sugar, and animal protein while low in fiber are consistently associated with a loss of microbial diversity (RINNINELLA *et al.*, 2023; MUIGANO *et al.*, 2025).

This dietary profile triggers a depletion of beneficial species while fostering the

growth of pro-inflammatory genera, like *Ruminococcus*, *Bilophila*, and *Klebsiella* (RINNINELLA *et al.*, 2023). Collectively, all these aspects demonstrate a profound impact on microbial modulation and consequently influence host homeostasis and overall health.

1.2 HOMEOSTASIS, DYSBIOSIS AND INTESTINAL DISEASE

1.2.1 Microbial intestinal homeostasis and dysbiosis

Under healthy intestinal physiological conditions, free from diseases, infections, or high levels of stress, the host and its microbiota coexist in a state of dynamic equilibrium known as homeostasis (AL-RASHIDI, 2022). This host-gut microbe interaction is a symbiotic relationship, in which the host provides a nutrient rich environment and harbors beneficial microorganisms while regulating their growth (WILDE, SLACK & FOSTER, 2024; CORTÉS *et al.*, 2025). In turn, the commensal microbiota contributes to essential host physiological aspects, including the fermentation of dietary substrates, improvement digestion, production of bioactive metabolites, protection against pathogen colonization through competitive exclusion, maintenance of intestinal epithelial barrier integrity, and immunomodulation (HOU *et al.*, 2022).

Instestinal homeostasis is dynamic, stable and resilient condition. Given the high interindividual variability in microbiome composition and function, as well as its susceptibility to multiple internal and external factors, the identification of universal biomarkers of microbial homeostasis remains a challenge (TALAPKO *et al.*, 2022; VAN HUL *et al.*, 2024). In this context, integrative approaches, including taxonomic composition, functional properties, and the intrinsic and environmental characteristics of the host have been used to evaluate microbial homeostasis (LEE, TSOLIS & BÄUMLER, 2022; VAN HUL *et al.*, 2024). Within this perspective, some authors highlight the relative abundance of

Akkermansia, *Bifidobacterium*, and *Lactobacillus* (JOOS *et al.*, 2025) as promising biomarkers, as well as intestinal microenvironment factors, such as oxygen and nitrate available concentrations (LEE, TSOLIS & BÄUMLER, 2022).

Imbalances of to this finely regulated balance between host and microbiota can lead to a condition known as dysbiosis (SHEN *et al.*, 2025). This disruption can result from many factors such as antibiotic use, dietary changes, infections, and chronic stress, and are often associated simultaneously with a reduction in beneficial microorganisms, a loss of beneficial bacteria alongside an expansion of opportunistic or pathogens microbials (HOU *et al.*, 2022; MAHANTA *et al.*, 2026).

Dysbiosis of gut microbiota has multiple consequences and involves significant functional disturbances that can profoundly impact host health, been responsible for several immune and metabolic diseases (FAN & PEDERSEN, 2021; HOU *et al.*, 2022). For instance, the reduced production of beneficial metabolites, including short-chain fatty acids (SCFAs), such as butyrate, which plays a crucial role as an energy source for colonocytes and in anti-inflammatory activity (TAN *et al.*, 2023; SMOLINSKA, POPESCU & ZEMELKA-WIACEK, 2025). This metabolic imbalance has been related to metabolic disorders, including obesity, type 2 diabetes, liver disease, and atherosclerosis (MÜNTE & HARTMANN, 2025). In addition, dysbiosis can cause an increase intestinal permeability, a condition commonly referred as “leaky gut” (DMYTRIV, STOREY & LUSHCHAK, 2024), and this disruption of the intestinal barrier allows pathogenic microorganisms and bacterial components, such as lipopolysaccharides, thus triggering an inflammatory reaction (DI VINCENZO *et al.*, 2024).

1.2.2 Inflammatory bowel disease (IBD)

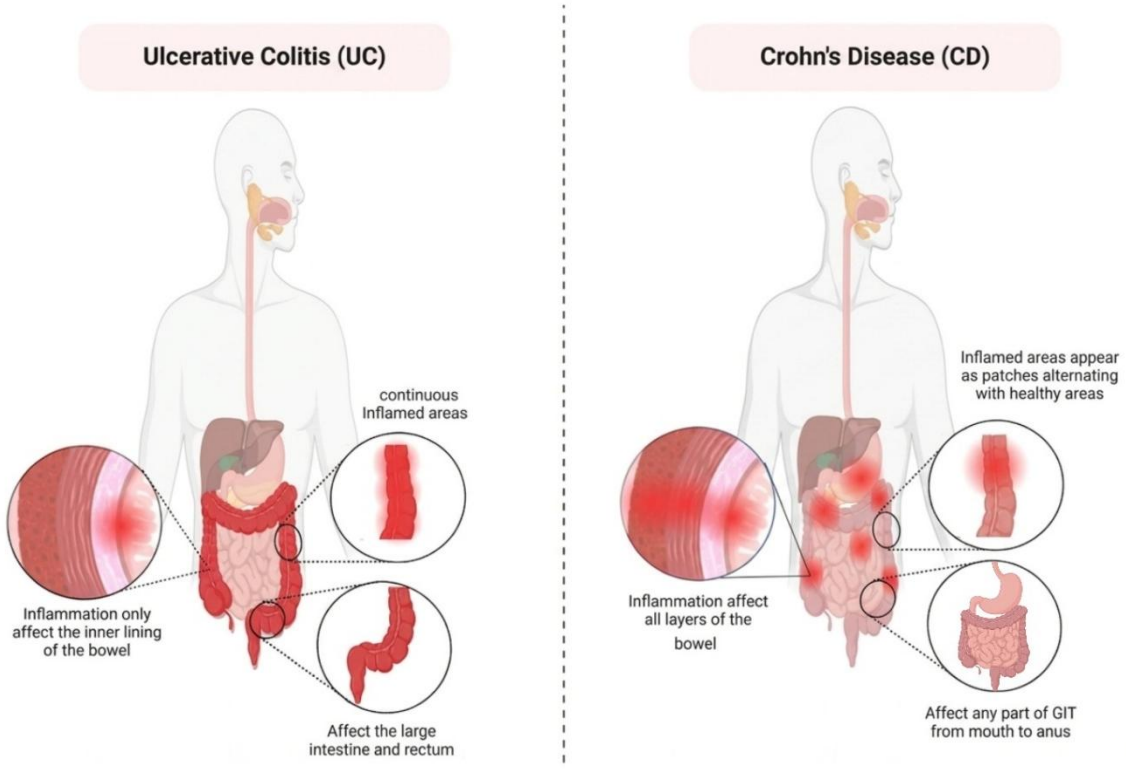
Although the exact cause is still not fully understood and remains under debate, intestinal dysbiosis has been intrinsically linked to several gastrointestinal disorders, such as Inflammatory Bowel Disease (IBD) (SHAN, LEE & CHANG, 2022). Evidence suggests that it results from a complex, multifactorial interaction among genetic predisposition, immune responses, environmental factors, and intestinal microbiota dysbiosis, including altered composition, with reduced diversity, increase of pathogenic and decrease of beneficial microorganisms (HOU *et al.*, 2022; LOPETUSO *et al.*, 2023; DIEZ-MARTIN *et al.*, 2024).

IBD is a chronic, autoimmune, nonspecific inflammatory disease of the gastrointestinal tract that can cause damage to the intestinal lining, beyond the microbiome imbalance, leading to ulceration and tissue damage in the intestinal mucosa, and bleeding (HETTA *et al.*, 2024; LONG, 2024). It is categorized mainly into two subtypes, ulcerative colitis (UC) and Crohn's disease (CD) (HANEISHI *et al.*, 2023) which differ significantly in location and depth (Figure 2) (RAJAPAKSE, 2023). UC is characterized by continuous inflammation and ulcers affecting only the innermost layer (the mucosa) of the large intestine (colon) and rectum (HETTA *et al.*, 2024). The inflammation does not extend through the bowel wall and, therefore, does not result in the formation of fistulas or strictures. The extent and intensity of inflammation influence the severity of symptoms (RAJAPAKSE, 2023).

In contrast, CD can affect the entire gastrointestinal tract, from the mouth to the anus, however it most commonly reports at the end of the small intestine, specifically ileum (SEYEDIAN *et al.*, 2019; RAJAPAKSE, 2023). CD causes inflamed patches alternations with healthy areas, known as skip lesions, which can lead to the formation of mucosal ulcers surrounded by edema and erythema (RAJAPAKSE, 2023). Inflammation affects all layers

of the bowel, and in some cases, these ulcers may bleed (HETTA *et al.*, 2024).

Figure 2. The difference between the two forms of IBD.

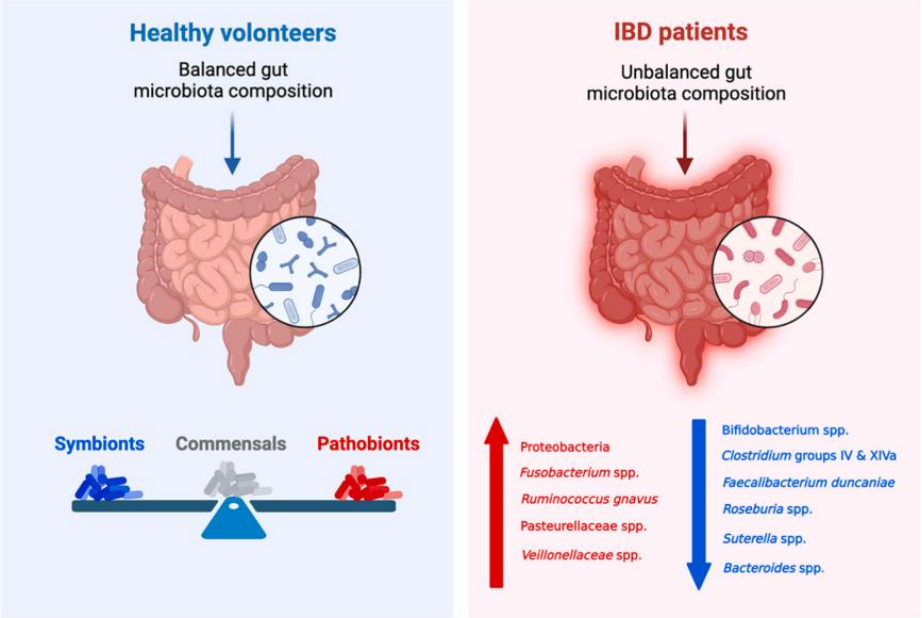


Source: Adapted from HETTA *et al.*, (2024).

The gut microbiota composition differs notably between healthy individuals and those with IBD (Figure 3). While a healthy gut exhibits a predominance of the phylum Bacillota, Verrucomicrobia and Bacteroidota, including genera such as *Akkermansia, Phascolarctobacterium, Anaerostipes*, as well as some members of *Faecalibacterium*, *Bacteroides, Lactobacillus*, *Bifidobacterium,* and *Roseburia* groups (DAHAL *et al.*, 2023; SAN-MARTIN *et al.*, 2025), other studies have shown that the IBD bacterial signature can be express by a taxonomic and functional difference composed of an increased abundance of Pseudomonadota, Actinobacteria, and Fusobacteria phyla, with examples of strains such as *Escherichia coli*, *Klebsiella* and a few *Clostridium* strains, (RASHED, VALCHEVA &

DIELEMAN, 2022; HANEISHI *et al.*, 2023; CABEZAS-CRUZ & BERMÚDEZ-HUMARÁN, 2024; SAN-MARTIN *et al.*, 2025).

Figure 3. Composition of the human gut bacterial microbiota of healthy patients and those with IBD condition.



Source: Adapted from CABEZAS-CRUZ & BERMÚDEZ-HUMARÁN (2024).

IBD condition presents with a broad spectrum of clinical manifestations which vary according to the subtype of disease, severity, and location of the inflammation, but common symptoms include abdominal pain and cramps, diarrhea, rectal bleeding, extreme fatigue, weight and appetite loss (MUZAMMIL *et al.,* 2023) IBD patients may also develop intestinal perforation, stricture formation, and colorectal neoplasia (YANG, GUO & ZOU, 2026). Additionally, extraintestinal manifestations can also occur such as arthritis, skin disorders, and ocular inflammation may occur, reflecting the systemic nature of the disease, and contribute to substantial long-term morbidity, significantly impairing patients' quality of life (SHEN *et al.*, 2023).

Given the strong association between intestinal dysbiosis and IBD, and considering that conventional treatments, which prioritize reducing inflammation, symptom management, and the promotion of remission (APPIAH *et al.*, 2025), can be expensive, generate adverse effects, and are often associated with variable patient responses, a substantial proportion of patients either fail to respond to treatment or lose responsiveness over time, highlighting the need for alternative or complementary therapeutic strategies (CAI, WANG & LI, 2021; XU, ZHU & GUO, 2022; HAFEZ *et al.*, 2025). In this context, modulation of the gut microbiota through probiotics, prebiotics, and synbiotics, as well its postbiotic has emerged as a promising therapeutic approach (MURGIANO *et al.*, 2025; SMOLINSKA, POPESCU & ZEMELKA-WIACEK, 2025).

Evidence highlights the capacity of probiotics and prebiotics, whether alone or in combination, to regulate the intestinal microbiota, alleviating the inflammation, alongside an improvement in immune and intestinal barrier function, thereby contributing to the mitigation of symptoms and the maintenance of host health (BAMOLA *et al.*, 2022; SHEN *et al.*, 2024).

Through competitive exclusion and synthesis of SCFA the probiotics can regulate intestinal homeostasis, several probiotics demonstrate beneficial effects. For example, when administered, the probiotic *Lacticaseibacillus casei* exhibits increasing the abundance of *Lactobacillus* spp., *Roseburia* spp., *Coprococcus* spp., and *Eubacterium rectale*, while inhibiting members of harmful groups *Blautia* spp. and *Ralstonia* spp. (HOU *et al.*, 2020; MA *et al.*, 2023; MA *et al.*, 2024).

Although these strategies do not replace conventional medical treatments, they may act as adjuvant therapies, complementing and potentially enhancing standard therapeutic results (MARTYNIAK *et al.*, 2021). Whereas growing evidence supports their potential benefits,

results remain variable, and further research is required to establish standardized protocols (MA *et al.*, 2024).

1.3 BIOTICS

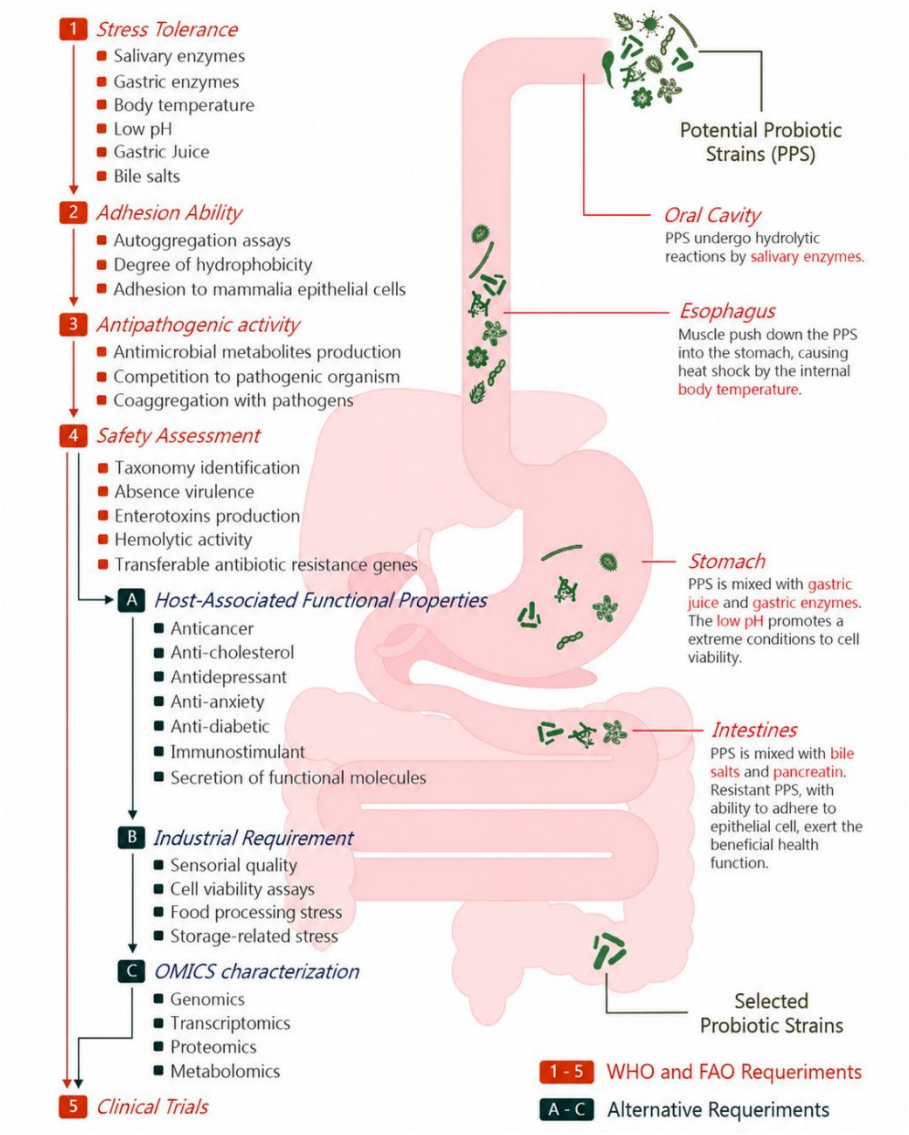
1.3.1 Probiotics

The concept of probiotics, first introduced in the 1950s, initially centered on the idea that certain substances or microorganisms could contribute to the promotion and/or maintenance of the host's health (LATIF *et al.*, 2023). Since then, the definition has been revised and reformulated, reflecting advances in scientific knowledge about the interactions between microorganisms and the human organism (ASHRAF *et al.*, 2025). These definitions were mainly focused on the positive impact on the gut, describing the probiotics as agents capable of improving the intestinal environment. However, a more standardized definition was established by Food and Agriculture Organization and World Health Organization (FAO/WHO) in which probiotics were defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”; (FAO/WHO, 2002; HILL *et al.*, 2014). Currently, it is understood that the beneficial effects of probiotics can extend beyond the limits of the intestinal microbiota, for example, encompassing processes such as modulation of the immune system, production of bioactive metabolites, and interaction with different physiological pathways of the host (CHANDRASEKARAN, WEISKIRCHEN & WEISKIRCHEN, 2024).

The selection of probiotic strains follows guidelines established by international regulatory agencies such as the FAO, WHO and European Food Safety Authority (EFSA, 2018). Probiotic candidate strains must undergo an approach consisting of successive tests based on five central criteria (Figure 4) (DE MELO PEREIRA *et al.*, 2018). Initially, the

strains are evaluated for their ability to tolerate the harshest conditions present in the human body, such as gastric acidity, the presence of bile salts, and the action of enzymes, maintaining their metabolic activity throughout this system (UHEGWU & ANUMUDU, 2025).

Figure 4. Mandatory and alternative requirements to select a probiotic.



Source: Adapted from DE MELO PEREIRA *et al.*, (2018)

Once inside the host, the ability to adhere, even if transient transit, to the intestinal epithelium is considered relevant (HAN *et al.*, 2021). The presence of the probiotic strain

can favor interaction with the host and prolong its effects in the intestinal environment. Furthermore, antagonistic capacity against pathogenic microorganisms is an equally important criterion, either through competitive exclusion or the production of antimicrobial compounds that inhibit the growth of these organisms (DE MELO PEREIRA *et al.*, 2018; UHEGWU & ANUMUDU, 2025).

Safety profile is a crucial and highly relevant requirement, requiring the absence of virulence factors and transferable antimicrobial resistance, to ensure the strain used is non-pathogenic and/or non-toxic strains (MERENSTEIN *et al.*, 2023). This aspect can be evaluated *in vitro*, *in silico* and *in vivo* through multiples approaches, including (ROE *et al.*, 2022).

Although not mandatory, it is often desirable and advantageous for the candidate probiotic strain to be present on the Qualified Presumption of Safety (QPS) list of the EFSA, a classification system for the safety of microorganisms for use in food or feed (EFSA, 2017). Similarly, the Food and Drug Administration (FDA) implemented several requirements called Generally Recognized as Safe (GRAS), which factors should be considered when proposing a chemical or substance as safe for use in food (CHURIN *et al.*, 2026).

Lastly, after the strain has been presented promising results at other four criteria, potential probiotics must undergo clinical trials. Clinical evaluation includes animal studies to validate their safety and efficacy previously indicated. Randomized, placebo-controlled clinical trials are essential and often used in this process (MARTÍNEZ-LÓPEZ *et al.*, 2022; BANG *et al.*, 2025).

Furthermore, alternative requirements have been frequently incorporated into the characterization of probiotic strains (FENTIE *et al.*, 2024). Properties associated with

functional properties, such as anti-cholesterol, anxiolytic, anti-obesity, and immunostimulant properties, are desirable, along with industrial advantages related to the viability of strain production and storage, as well as sensory quality (APONTE, MURRU & SHOUKAT, 2020). Moreover, the use of omics tools has proven to be a very useful, versatile, and safe tool for a deeper understanding of their properties and potential benefits. This integrated approach allows for a more robust and well-founded selection of probiotic strains, expanding their applications and effectiveness (KWOJI *et al.*, 2023).

1.3.2 *Bacillus* spp.

Traditionally, the most studied and recognized bacterial genera for their probiotic potential include *Lactobacillus* spp. and *Bifidobacterium* spp. (LEE *et al.*, 2024a). However, in recent years, the genus *Bacillus* has gained increasing prominence, driven by evidence pointing to its probiotic potential, mainly due to its ability to form spores, confer stability in different environmental conditions, and produce a wide range of bioactive metabolites, capable of being applied in probiotic formulations for humans, animals, and as food additives (PAYNE *et al.*, 2024).

Among the species of the genus *Bacillus*, *Bacillus velezensis* has stood out as a promising microorganism for probiotic applications, both in animals and humans (CHEN *et al.,* 2023a, ARYA *et al.,* 2025, BRUTSCHER *et al.,* 2025). This interest is associated with a set of characteristics that include from a generally considered safe (KHALID *et al.*, 2021; NA *et al.*, 2022), to its ability to form spores, conferring resistance to adverse conditions of the gastrointestinal tract (KENFAOUI *et al.*, 2024; SU *et al.*, 2024). In addition, several studies demonstrate the broad capacity of *B. velezensis* to produce a broad-spectrum of bioactive compounds with antibacterial and antifungal action, including lipopeptides,

bacteriocins, and other secondary metabolites (ALENEZI *et al.*, 2021; SU *et al.*, 2024; SOUSA *et al.*, 2025).

These compounds contribute directly to its ability to inhibit gram-positive and gram-negative pathogens, including *Listeria monocytogenes* and *Pseudomonas aeruginosa*, whose inhibition has been associated with the production of bacteriocins and antimicrobial peptides stable to variations in pH and temperature (SUCH *et al.,* 2023; ZHANG *et al.*, 2024). Evidence demonstrates inhibition of *Staphylococcus aureus*, through suppression of growth and interference in biofilm formation, a key factor in bacterial virulence (GAO *et al.*, 2026). Furthermore, there is evidence of antagonistic activity against Gram-negative bacteria, as well as *Escherichia coli*, reinforcing the competitive potential of this species in complex environments such as the gut microbiome (WANG *et al.,* 2025a).

Additionally, *B. velezensis* exerts beneficial effects on intestinal homeostasis, including modulation of the microbiota, increased abundance of beneficial microorganisms, and improved integrity of the intestinal epithelial barrier (WANG *et al.*, 2024; WALKER *et al.*, 2026). These effects are frequently associated with a reduction in inflammatory processes, increased antioxidant capacity, and regulation of both innate and adaptive immune responses (HUANG *et al.,* 2026; JI *et al.,* 2025; LEE *et al.,* 2024b). Moreover, recent evidence has been demonstrated metabolites derived from *B. velezensis* strains exert metabolic and immunomodulatory beneficial effects, including anti-obesity activity and protective effects in colitis, associated with the production of short-chain and branched-chain fatty acids and modulation of the gut microbiota (SHIN *et al.,* 2024; WANG *et al.,* 2025). Taken together, this evidence reinforces the potential of *B. velezensis* strains as robust candidates for the development of probiotic and postbiotic strategies.

Bacillus velezensis P45 is a strain isolated from the intestine of an Amazonian fish

(*Piaractus mesopotamicus*), highlighted by keratinolytic and collagenolytic activity, and potential production of bioactive compounds (DAROIT, CORRÊA & BRANDELLI, 2009; DA ROSA *et al.*, 2022). Overall, strain P45 shows capacity for the bioconversion of keratinous residues and production of antimicrobial lipopeptides. Additionally, genomic and phylogenomic analyses confirmed its identity as *B. velezensis* and revealed the presence of multiple gene clusters associated with the biosynthesis of antimicrobial lipopeptides such as fengycin, surfactin, and iturin (DA ROSA *et al.*, 2022).

1.3.3 Prebiotics

As knowledge about gut microbiota and its relationship to host health has become more established and understandable, there is interest in compounds capable of directly or indirectly modulating it, such as prebiotics. According to the definition proposed by the International Scientific Association of Probiotics and Prebiotics (ISAPP), prebiotics are non-digestible dietary components that confer health benefits by selectively stimulating the growth and/or activity of beneficial microorganisms in the gastrointestinal tract (GIBSON *et al.*, 2017).

These compounds originate from different food matrices, including fruits, vegetables, and cereals such as berries, tomatoes, whole wheat, onions, and soy, and are predominantly composed of carbohydrates and dietary fiber (SWAROOPA *et al.*, 2022). Prebiotic carbohydrates can be classified according to their sugar subunits, polymerization degree, and bond framework. Typical prebiotics include human milk oligosaccharides, pectin oligosaccharides, starch- and glucose-derived oligosaccharides, fructans such as inulin and fructooligosaccharides (FOS), galactooligosaccharides (GOS), phenols and other phytochemicals, conjugated linolenic acid, and polyunsaturated fatty acids (DAVANI-

DAVARI *et al.*, 2019; KUMORO *et al.*, 2025).

As non-digestible substrates, prebiotics reach the colon intact, becoming available to specific microbial groups capable of fermenting them, such as *Lactobacillus* and *Bifidobacterium* (MONTEIRO *et al.*, 2026). This selective fermentation confers a competitive advantage to these groups, leading to an increase in their relative abundance in the microbial community, and consequently modulating the gut microbiota (YOU *et al.*, 2022).

The fermentation of these compounds leads to the production of secondary metabolites predominantly SCFAs, such as butyrate, propionate, and acetate, which exhibit biological activities, including anti-inflammatory effects (SANKARGANESH *et al.*, 2025). These metabolites are relevant due to their diverse roles and beneficial effects on host health, which range from promoting the integrity and permeability of the intestinal barrier, regulating appetite and satiety signals, modulating oxidative stress, anti-inflammatory action, and neuroprotective effects (MIRZAEI *et al.*, 2021; YOU *et al.*, 2022).

In addition, the degradation of certain prebiotics can generate intermediate metabolites that serve as substrates for other microbial populations, promoting metabolic interactions known as cross-feeding, which further contribute to the functional modulation of the intestinal ecosystem (GUARINO *et al.*, 2020).

1.3.4 Synbiotics

Resulting from the combination of pre- and probiotics, synbiotics are formulations that act synergistically to promote the survival, viability, and proliferation of beneficial microbiota, and consequently enhance their beneficial effects on the host (GIBSON *et al.*, 2017; NIU *et al.*, 2024). Synbiotics are not limited to a simple combination of prebiotics

and probiotics; they can be classified into two types: complementary, where prebiotics and probiotics act independently, without a specific relationship between the substrate and the administered microorganism, and synergistic, where the substrate is selected to be directly utilized by the co-administered microorganisms, favoring their growth and activity in the gastrointestinal tract (GOMEZ QUINTERO, KOK & HUTKINS, 2022; SMOLINSKA, POPESCU & ZEMELKA-WIACEK, 2025).

Considering the applicability of these formulations, synbiotics have been investigated primarily related to gastrointestinal disorders, particularly irritable bowel syndrome (SOMMERMEYER & PIĄTEK, 2024). Clinical trials lead to promising results in reducing symptoms such as abdominal pain, bloating, and changes in bowel habits, although these results are still preliminary and limited due to the heterogeneity of formulations and experimental designs (ALLEGRETTI *et al.*, 2026).

1.3.5 Postbiotics

Completing the group of biotics and following the evolution of strategies aimed at modulating the intestinal microbiota, there is also a growing increase in studies involving so-called postbiotics. However, the postbiotic concept is still under debate. While the ISAPP panel define postbiotics as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (SALMINEN *et al.*, 2021), several scientific publications have expanding the definition considering postbiotics “as substances released by or produced through the metabolic activity of the microorganisms, which can exerts a beneficial effect on the host” (ŻÓŁKIEWICZ *et al.*, 2020; RAFIQUE *et al.*, 2023; GUGLIELMETTI *et al.*, 2025).

In this sense, different compounds derived from microbial activity have been

considered as postbiotics, including not only acetate, butyrate, and propionate, but also other SCFAs usually found in lower concentrations, such as formate, valerate, and caproate (PRASAD *et al.*, 2025). Vitamins, peptides, enzymes, and other bioactive molecules, as well as cellular structures from inactivated microorganisms, such as cell wall polysaccharides and membrane fragments, are included in the concept (THORAKKATTU *et al.*, 2022; PRASAD *et al.*, 2025).

These elements, individually or in combination, play fundamental roles in maintaining host health, including energy production, modulation of immune response, maintenance of intestinal barrier integrity, regulation of inflammatory processes, and others (AL-HABSI *et al.*, 2024; JALALI *et al.*, 2024). In addition, postbiotics can act on cell signaling through interaction with host receptors, influencing gene expression and contributing to intestinal homeostasis (OZMA *et al.*, 2022). Compared to prebiotics, probiotics, and synbiotics, the postbiotics offer multiple advantages like increased stability, viability, safety, and predictable responses (ERDINC & CUFAOGLU, 2026).

Overall, in clinical and nutritional contexts, biotics (pre- to postbiotics) has been increasingly investigated, particularly for conditions associated with dysbiosis and intestinal inflammation as emerging therapies in the treatment of IBD (YASSINE, NAJM & BILEN, 2025; LI *et al.*, 2026).

1.4 OMICS APPROACHES IN INTESTINAL MICROBIOTA

High-throughput omics technologies are a set of powerful and large-scale approaches used to analyze, in a comprehensive and integrated way, the different biological components of a system, generally at a molecular level (VAILATI-RIBONI, PALOMBO & LOOR,

2017; VERMA *et al.*, 2025). The expansion of omics-based methodologies is largely driven by their significant analytical advantages compared to traditional approaches, remodeling biological research, and unprecedented perspectives on the complexity of living systems (DAI & SHEN, 2022; VITORINO, 2024). In addition to the ability to simultaneously analyze numerous samples, these tools are faster, exhibit high sensitivity and specificity, and are more efficient in data processing and interpretation, for example, in enhancing disease diagnosis and drug discovery (LUO, ZHAO & CHEN, 2024).

The generation of omics data occurs at various levels, according to its central biological purpose (DAI & SHEN, 2022) (Figure 5). Thus, scientific literature provides a broad spectrum of omics studies, encompassing different levels of biological organization and sample types, mainly categorized into genomics, proteomics, and metabolomics, which provide information on genetic potential, functional activity, and metabolic products of a biological system, respectively (HAYES *et al.*, 2024; SANCHES *et al.*, 2024).

In the microbiome context, omics techniques have been widely used to investigate not only the composition of the gut microbiome but also, in an integrated way, the metabolic functions and their interactions and participation in the physiological mechanisms of the dysbiosis condition (SUDHAKAR *et al.*, 2022; MURGIANO *et al.*, 2025; MOUSAVI *et al.*, 2026). The following sections will briefly provide an overview of these three approaches, as the concepts introduced here will be explored in more detail in subsequent chapters of this thesis.

1.4.1 Genomics

Conceptually, genomics is the study of the complete genome of an organism, allowing both taxonomic identification and the assessment of biological potential (HASIN, SELDIN & LUSIS, 2017). Whole genome analysis, through the identification and annotation of genes

associated with different biological characteristics, provides invaluable insights not only into taxonomic identification, but also into variation among individuals, gene expression and regulation, and the gene-host interactions contributing to the understanding of the functional potential of microorganisms (LUCA *et al.*, 2018; ALHARBI & RASHID, 2022; RUSSELL *et al.*, 2024; MARZOUKA *et al.*, 2026).

In the context of probiotics, the omics approach applied to probiotics is called probiogenomics and allows the investigation of genes related to the ability to adhere to the host, tolerance to gastrointestinal tract conditions, production of bioactive compounds, and metabolic capacity, including the use of different substrates (VERMA *et al.*, 2025). Another important aspect of probiogenomics is the evaluation of the safety of strains through the identification of genes associated with virulence or antibiotic resistance (SABINO *et al.*, 2025; VERMA *et al.*, 2025). Considering its relevance, this approach will be explored in Chapter 1 of this thesis.

1.4.2 Proteomics

Proteins are characterized as the most abundant cellular macromolecules, playing a part in numerous important functional processes, acting as catalysts, regulating gene expression, and contributing to composition, structure, and networks (ALBERTS *et al.*, 2021). Proteomics is a powerful, fast, sensitive, and functionally useful tool for quantifying and identifying proteins expressed by microorganisms, as well as their function and structure, reflecting the biological processes that are active at a given time or specific condition (AL-AMRANI *et al.*, 2021). Widely applied in various scientific fields, proteomics can provide detailed information in medicine and therapeutics, assisting in disease diagnosis, biomarker discovery, vaccine development, and elucidation of disease mechanisms (SALEM *et al.*,

2022; GOBENA *et al.*, 2024; TARIQ *et al.*, 2025).

In probiotics research, this high-throughput data tool becomes crucial to understanding microbial functions and action mechanisms (STINCONE, BRANDELLI & DE ANGELIS, 2022). This approach allows the identification of proteins involved in probiotic functions, such as stress tolerance and adhesion to host tissues, and in the production of bioactive compounds, as well as aiding in elucidating metabolic adaptation to different substrates, thus supporting the evaluation of the efficacy, specific molecular health-promoting activities, and mechanisms of action of probiotics (STINCONE, BRANDELLI & DE ANGELIS, 2022; KWOJI *et al.*, 2023; STASTNA, 2024).

1.4.2 Metabolomics

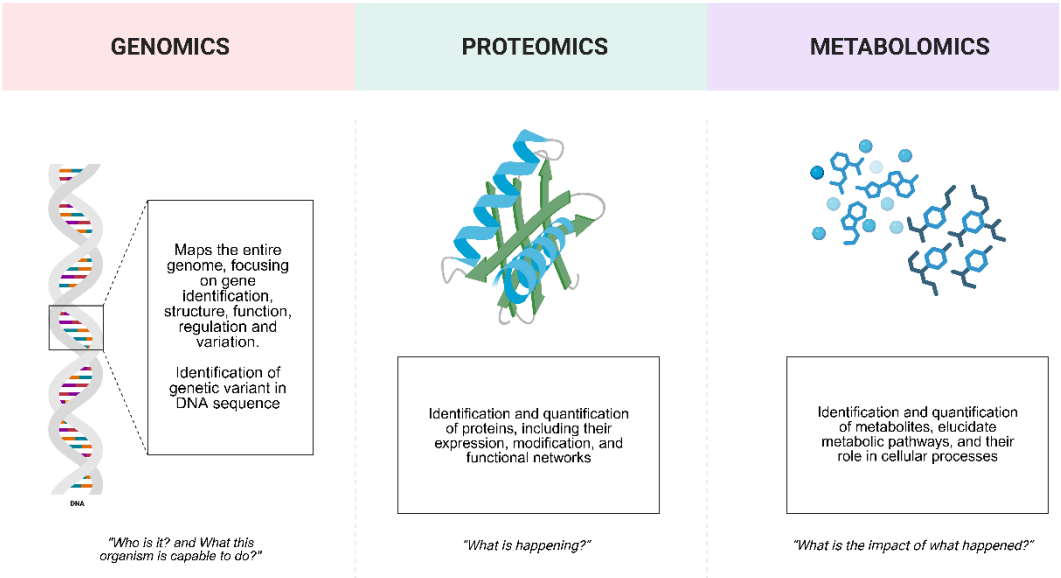
In analogy to the terms genomics and proteomics, metabolomics is the term used to describe research focused on the analysis of endogenous small molecules (smaller than 1500 Da) found within a biological sample (MUTHUBHARATHI, GOWRIPRIYA & BALAMURUGAN, 2021; CHEN, LI & XU, 2022). The identification and quantification of metabolites, such as SCFAs, amino acids, and phenolic compounds, generates a metabolic profile or metabolome that reflects the metabolic response to different stimuli, being considered the omics science most closely linked to the organism's phenotype (TURI *et al.*, 2018; STEUER, BROCKBALS & KRAEMER, 2019; QIU *et al.*, 2023).

The construction of a metabolomic profile from a given stimulus can include the detection of metabolic changes across different biological matrices, including biofluids such as urine and blood, as well as feces and tissues, induced by different stimuli, for example, the consumption of probiotics (WU *et al.*, 2022; QIU *et al.*, 2023). By revealing both systemic and local metabolic responses, the metabolome provides important insights into the functional effects of probiotics in the body, such as antioxidant activity, immunomodulation,

and maintenance of intestinal epithelial barrier integrity, highlighting the role of metabolites in mediating the beneficial effects of these microorganisms (WU *et al.*, 2022; EMEGANO, BATTAL & KOCADAL, 2024; TENEA *et al.*, 2026).

Taken together, in a simplified perspective, genomics reflects “Who is it? and What this organism is capable to do?”, while proteomics indicates “What is happening?”, and metabolomics represents “What is the impact of what happened?”. The combined approach of these omics tools allows for a more comprehensive and precise understanding of the different segments of probiotics studies, connecting from initial characterization and genomic potential, to functional activity, biological and regulatory mechanisms and final metabolic effects (KWOJI *et al.*, 2023), as well as a holistic view of the interaction among probiotics and pre-, syn-, post- and gut microbiota (GALANIS, PAPADIMITRIOU & MOLONEY, 2025; VERMA *et al.*, 2025).

Figure 5. Mainly multi-omics approaches.



Source: From the author, unpublished (2026).

2. OBJECTIVES

2.1 General objective

To evaluate *in vitro* and *in silico* the probiotic potential of *Bacillus velezensis* P45 and investigate its proteomic and metabolomic responses when cultivated with different carbon sources, as well as its interaction with the human gut microbiome.

2.2 Specific objectives

- To investigate, through *in silico* analysis of the genome sequence, genes potentially associated with the probiotic potential of *B. velezensis* P45, as well as the investigation of virulence and antimicrobial resistance genes;
- To characterize the potential probiotic of *B. velezensis* P45 through *in vitro* assays, evaluating key traits such as tolerance to gastrointestinal conditions, safety properties, and adhesion;
- To analyze the bacterial growth, proteomic profile and production of selected metabolites (short-chain fatty acids and amino acids) of *B. velezensis* P45 when cultivated in prebiotics (FOS, GOS), mucin or glucose as unique carbon sources;
- To evaluated through *ex vivo* culture, the interaction of postbiotic produced by *B. velezensis* P45 during the cultive on different carbon sources (FOS, GOS, mucin or Glucose), with healthy and unhealthy human intestinal microbiota.

SCIENTIFIC PAPER 1

SCIENTIFIC PAPER 2

In silico ***and*** *in vitro* ***Characterization of*** *Bacillus velezensis* **P45: Screening for a Novel Probiotic Candidate**

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In Silico and In Vitro Characterization of *Bacillus velezensis* P45: Screening for a Novel Probiotic Candidate

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Abstract

Spore-forming *Bacilli* have been explored due to their potential biotechnological features and applications in human health and functional food research. This study focuses on the genetic and phenotypical characterization of the functional probiotic properties of *Bacillus velezensis* P45, a strain isolated from fish intestines. *B. velezensis* P45 exhibited antimicrobial activity against Gram-positive and Gram-negative pathogens and demonstrated strong autoaggregation and biofilm formation properties in vitro. The strain also showed tolerance to gastrointestinal conditions and ability to metabolize and adhere to mucin. In silico analysis confirmed the absence of virulence factors and antibiotic resistance genes, reinforcing its safety as a probiotic candidate. Genome mining revealed the presence of genes related to adhesion, such as fibronectin-binding protein and enolases, and for the synthesis of secondary metabolites, including the antimicrobial lipopeptides fengycin, surfactin, and bacillibactin. In addition, phylogenetic comparison using the *yloA* (*rqcH*) gene associated with gut adhesion clustered strain P45 with other probiotic *Bacillus* and *B. velezensis* strains, while separating it from pathogenic bacteria. Thus, the strain *B. velezensis* P45 could be a valuable candidate as a probiotic due to its functional properties and safety.

Keywords: antimicrobial peptides; *Bacillus*; genome analysis; gut adhesion; probiotics

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1. Introduction

The interest in probiotics has grown exponentially in recent decades, driven by the increasing consumer demand for products associated with health benefits. Probiotics can promote intestinal homeostasis, stimulating the immune system [1,2]. Currently, it has been well established that the influence of probiotics extends beyond the gastrointestinal tract [3]. These microorganisms can contribute to the regulation of physiological processes in distant organs, including the brain, skin, lungs, and cardiovascular system, by engaging complex immune, metabolic, and neural pathways [4,5]. In addition, substances derived from microbial metabolism or cell-inactivated forms (postbiotics) have been widely studied due to their impact on the dynamics of probiotic action in promoting health benefits to the host [6,7]. Examples of postbiotics include antimicrobial peptides and short-chain fatty acids (SCFAs) that typically exhibit inhibitory activity against undesirable bacterial species and anti-inflammatory activities, respectively [8,9]. The selection criteria for probiotic microorganisms, as outlined by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the European Food Safety

Authority (EFSA), encompass requirements related to their safety, functional capabilities, and beneficial properties [10]. The desirable properties include the capability to survive and maintain metabolic activity under gastrointestinal tract (GIT) conditions, the absence of genes associated with antimicrobial resistance, and the ability to adhere and colonize in the host [10,11].

Among the genera traditionally and commercially recognized for their probiotic potential, *Lacticaseibacillus* is one of the most extensively studied bacterial groups [12]. Strains of this genus have been extensively used due to its strong ability to adhere to intestinal epithelial cells, tolerate gastric acidity, modulate the host immune response, and reduce gastrointestinal disorders in both children and adults [13–15]. Its safety profile and documented health benefits have led to its wide application in functional foods and clinical research [16].

In comparison, *Bacillus* strains have been less represented in probiotic formulations; however, increasing evidence supports their potential in human and animal use [17–19]. *Bacillus* species such *B. subtilis*, *B. paralicheniformis*, and *B. velezensis* and their postbiotics have received their safety status from various international regulatory agencies, such as GRAS (Generally Recognized As Safe) by the U.S. FDA and QPS (Qualified Presumption of Safety) by the EFSA, indicating that these microorganisms have been evaluated and are considered safe for use based on scientific evidence and regulatory assessment [20–22].

Additionally, the spore-forming capacity in the probiotic field is limited, although the inherent resistance of spores provides a distinct advantage over other genera since this feature can ensure high survival rates during industrial processing and storage, as well as enhanced resistance to the harsh conditions encountered in whole gastrointestinal tract of humans and/or animals [23,24]. Once established in the intestinal environment, *Bacillus* strains can exert health-promoting effects on the host, through the production of diverse functional secondary metabolites and digestive enzymes [18,25]. These include ribosomally and non-ribosomally synthesized antimicrobial peptides, polyketides, and lipopeptides such as surfactin, fengycin, and iturin [24].

Thus, the properties of *Bacillus* species, including *B. velezensis*, can contribute to the potential probiotic effect by modulating host immune responses, playing a key role in competitive exclusion and host–microbe interactions [26]. These functional effects have also been associated with clinical benefits, such as alleviating gastrointestinal symptoms in individuals with inflammatory bowel syndrome [27,28]. *B. velezensis* strains have been adopted as safe probiotic agents in animal feed and aquaculture and some works shown their potential as human and animal probiotics [29–31].

Bacillus velezensis P45, isolated from the gut of a fish native to the Brazilian Amazon basin, has shown promising probiotic properties. This strain has demonstrated an ability to produce bioactive compounds such as antimicrobial lipopetides and exhibits antimicrobial activity against a range of bacteria [32]. Considering the increasing interest for novel probiotic strains, there is limited information about probiotic *B. velezensis*, particularly on the use of genome-wide approaches. This study aims to evaluate the properties of *B. velezensis* P45 through genome screening to evaluate the presence of genetic elements encoding *Bacillaceae*-probiotic properties, secondary metabolites, virulence factors, and antibiotic resistance. In vitro assays were used to further understand antibiotic resistance and probiotic features to assess the potential of strain P45 as a probiotic for health and nutrition applications.

2. Materials and Methods

2.1. Strains and Culture Conditions

Bacillus velezensis P45, previously isolated from the fish *Piaractus mesopotamicus* [33], along with other bacterial strains used in this study, were retrieved from the culture collection of the Laboratory of Biochemistry and Applied Microbiology (UFRGS, Porto Alegre, Brazil) and conserved at −20 °C in Brain-Heart Infusion broth (BHI; Kasvi, Pinhais, PR, Brazil) containing 20% (*v*/*v*) glycerol. The strains used in this study were *Listeria monocytogenes* ATCC 7644, *Clostridium perfringens* ATCC 3624, *Enterococcus faecalis* LAB-4, *Salmonella enterica* sv. Enteritidis ATCC 13076, *Pseudomonas aeruginosa* 4B, *Proteus vulgaris* IC-1, and *Staphylococcus aureus* ATCC 25923. These strains were cultivated in BHI agar at 37 °C for 24 h. *Lacticaseibacillus rhamnosus* CCT 7863, provided by Gabbia Biotecnologia (Itajái, SC, Brazil), was cultured on De Man, Rogosa, and Sharpe (MRS; Merck, Darmstadt, Germany) agar under anaerobic conditions by 48 h at 37 °C, prior the tests.

2.2. Safety Assessment

2.2.1. Hemolytic Activity Assay

B. velezensis P45 was inoculated onto blood agar plates (Laborclin, Pinhais, PR, Brazil) and incubated for 24 h at 37 °C. Hemolytic reaction was classified based on the appearance of partial hydrolysis as a yellow zone around the colonies (α-hemolysis), a clear zone around colonies (β-hemolysis), or no reaction (γ-hemolysis) [34]. *S. aureus* ATCC 25923 was used as a positive control.

2.2.2. Antibiotic Susceptibility Test

Antimicrobial resistance of *B. velezensis* P45 was evaluated using disc diffusion method against the following 15 antimicrobials (Cefar, São Paulo, Brazil): amikacin (30 µg/disc), ampicillin (10 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), clindamycin (2 µg/disc), erythromycin (15 µg/disc), gentamicin (10 µg/disc), imipenem (10 µg/disc), levofloxacin (5 µg/disc), linezolid (30 µg/disc), meropemem (10 µg/disc), norfloxacin (10 µg/disc), penicillin (10 µg/disc), tetracycline (30 µg/disc), and vancomycin (30 µg/disc). The strain was cultured in BHI plates and incubated at 37 °C for 24 h and then diluted in saline solution (0.85% NaCl) standardized to OD_{600} of 0.5. The bacterial solution was then inoculated on Mueller Hinton (Kasvi) agar plates. The antibiotic discs were manually placed on the plate surface and incubated at 37 °C for 18 h. The results were expressed considering the inhibition zone diameter (mm) and categorized as resistant (R), with moderate susceptibility (MS), or susceptible (S), based on the parameters of the Clinical and Laboratory Standards Institute (CLSI) [35]. The experiment was performed as three biological replicates.

2.3. Evaluation of Probiotic Properties

2.3.1. Antagonistic Activity

The antibacterial activity was evaluated by the agar diffusion test [36]. The antagonism activity of the *B. velezensis* P45 was tested against *L. monocytogenes*, *E. faecalis*, *C. perfringens*, *S.* Enteritidis, *P. aeruginosa*, and *P. vulgaris*. Test microorganisms were prepared in a concentration of 10^8 CFU/mL in saline solution (0.85% NaCl, *w*/*v*) and were swab-inoculated onto BHI agar plates surface. Serial dilutions of the crude supernatant from *B. velezensis* P45 cultures were grown in BHI broth at 37 °C for 24 h and then added to fresh lawn cultures. The plates were then incubated aerobically at the optimal temperature for each test microorganism. *C. perfringens* was incubated under anaerobic conditions at 37 °C. Clear zones of growth inhibition, indicating positive antagonism activity, were measured. The

antagonistic activity was quantified in activity units (AU) per mL, defined as the reciprocal of the highest dilution that produces a clear zone of inhibition [37].

2.3.2. Autoaggregation and Cell Surface Hydrophobicity

Aggregation ability of *B. velezensis* P45 was assessed as described previously [38], with minor modifications. *L. monocytogenes* ATCC 7644 and *L. rhamnosus* CCT 7863 were used as references for pathogenic and probiotic bacteria. Briefly, *B. velezensis* P45 and *L. monocytogenes* were cultured in BHI broth at 37 °C for 24 h, while *L. rhamnosus* was grown in MRS broth. The cultures were harvested via centrifugation at 6000× *g* for 10 min at 4 °C, and the pellets were washed twice with PBS (10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.4). The bacterial pellets were then suspended in PBS and incubated at 37 °C for 24 h. During incubation, the optical density (OD600) of the upper fraction of the suspensions was determined at 0, 2, 4, 6, and 24 h. The autoaggregation rate was calculated using the following equation:

$$\text{Autoaggregation (\%)} = 1 - (At/A_0) \times 100 \quad (1)$$

where At is the absorbance at t2, t4, t6, and t24, and A_0 is the initial absorbance.

Cell surface hydrophobicity of *B. velezensis* P45, *L. monocytogenes* ATCC 7644, and *L. rhamnosus* CCT 7863 were evaluated using microbial adhesion to hydrocarbons [39]. The strains were prepared as described above. After suspension in PBS, 3 mL of bacterial cells were mixed with 400 µL of dichloromethane or xylene and vortexed for 60 s. The following equation was used to calculate the bacterial affinity to hydrocarbons:

$$\text{Hydrophobicity (\%)} = (H_0 - Ht)/H_0 \times 100 \quad (2)$$

where Ht is the absorbance values measured at 600 nm after the incubation period, and H_0 is the recorded at the start of the experiment.

2.3.3. Biofilm Formation Assay

Biofilm formation was assessed under static conditions using a previously established method [40], with minor adjustments. Briefly, an overnight culture of *B. velezensis* P45 was inoculated (1%) into wells of a 96-well polystyrene microplate, each containing 300 µL BHI or BHI prepared with 0.1 µg/mL ampicillin, which served as a negative control. The microtiter plates were incubated for 6 and 24 h at 37 °C. Biofilm formation was evaluated according to the crystal violet (CV) method [41].

2.3.4. Tolerance to Gastrointestinal Conditions

The tolerance of *B. velezensis* P45, to in vitro gastrointestinal conditions was determined as described elsewhere [42], with minor modifications. *L. monocytogenes* ATCC 7644 and *L. rhamnosus* CCT 7863 were included for comparison. The bacterial strains were prepared as described above and then suspended in simulated gastric acid (SGA: 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/L pepsin, pH 2.0) and incubated at 37 °C for 90 min. After this period, the suspensions were centrifuged at 6000× *g* for 10 min, the pellets were washed with PBS, and then suspended in simulated intestinal fluid (SIF: 22 mM NaCl, 3.2 mM KCl, 7.6 mM $NaHCO_3$, 0.1% pancreatin and 0.15% bile salts, pH 8.0) for 150 min at 37 °C. At the end of each stage of the analysis, aliquots were collected, and the bacterial counts were measured using serial dilutions in BHI for *B. velezensis* and *L. monocytogens* and MRS for *L. rhamnosus*.

2.3.5. Bacterial Adhesion to Mucin

The ability of *B. velezensis* P45 to adhere to the mucin layer was assessed as described previously [43], with some modifications. *L. monocytogenes* ATCC 7644 and *L. rhamnosus* CCT 7863 were included for comparison. For the test, type-II porcine gastric mucin (Sigma Aldrich, St. Louis, MO, USA) was prepared in PBS (10 mg/mL) and immobilized in a 96-well polystyrene microplate through overnight storage at 4 °C. After washing the microplate twice with PBS, a bovine serum albumin (BSA) solution (20 mg/mL) was added to each well for a saturation step, which was carried out for 4 h at 4 °C. Each strain was cultivated in their respective optimal media and at their ideal temperatures. All cultures were centrifuged at 10,000× *g* for 15 min, and bacterial cells were suspended in PBS to achieve approximately 10^8 CFU/mL. A volume of 100 µL of each strain suspension was added to the wells and incubated 1.5 h at 37 °C. The supernatants were removed, and the plates were washed twice with PBS to remove unbound bacteria. Adhering bacteria were desorbed with 0.25% trypsin/EDTA solution (Corning Inc., Corning, NY, USA) for 2 min. Finally, 100 µL samples from each well were plated on BHI or MRS agar plates to determine viable cell counts. Prior to the assay, the trypsin-EDTA solution used in the analysis was evaluated on all strains to prevent any adverse effect on bacterial viability.

2.3.6. Mucin Growth Assay

The growth ability of *B. velezensis* P45 in the presence of type-II porcine gastric mucin was assessed and compared with *L. monocytogenes* ATCC 7644 and *L. rhamnosus* CCT 7863. Overnight cultures of *B. velezensis* P45 and *L. monocytogenes* were inoculated (1%) into BHI broth, while *L. rhamnosus* was inoculated into MRS broth, both containing 1 mg/mL of type-II porcine gastric mucin. The respective media culture without mucin served as the control. Samples were incubated at 37 °C for 12 h and the optical density (OD600) was measured every 1 h interval.

2.4. Whole Genome Analysis

DNA extraction, sequencing, and alignment of *B. velezensis* P45 have been reported previously [32]. This Genome Shotgun project is deposited at EMBL/ENA/GenBank under accession code: JAFJZY000000000.1. Probiotic features within the *B. velezensis* P45 genome were explored through manual analysis of coding DNA sequences using the online platform Rapid Annotation using Subsystem Technology (RAST) and SEED v.2.0 (http://rast.nmpdr.org (accessed on 15 February 2025)). The analysis focused on identifying protein-derived genes related to potential probiotic functions, such as adhesion, aggregation, vitamin biosynthesis, amino acid metabolism, and adaptations to the host gastrointestinal tract. Additionally, gene clusters associated with the biosynthesis of antimicrobial compounds were identifying using the antiSMASH 7.0 tool [44]. A detailed examination of genes with an over-85% identity to reference sequences was performed using Blast KOALA (KEGG Orthology and Links Annotation), and the characterization of biosynthetic gene clusters with PRISM4 (http://prism.adapsyn.com (accessed on 23 March 2025)) [45] was performed for identification of non-ribosomal peptides, type-I and -II polyketides, and RiPPs. BAGEL4 web server was used to identify gene clusters involved in the biosynthesis of ribosomally synthesized and post-translationally modified peptides (RiPPs) and (unmodified) bacteriocins [46].

Furthermore, the genome sequence of the strain *B. velezensis* P45 was submitted to the iProbiotics database [47] for an assessment of its probiotic potential. iProbiotics employs *k*-mer frequencies to characterize complete bacterial genomes and uses the support vector machine algorithm for probiotic identification.

2.5. Alignment and Phylogenetic Reconstruction

Aiming to illustrate the presence and arrangement of adherence genes, we reconstruct a phylogenetic analysis tree created with *yloA* (*rqcH*) adhesion gene. For the phylogenetic analysis, we run BLASTn with the gene *rqcH* coding sequence (CDS); then, the amino acids sequences were aligned using MUSCLE, implemented within MEGA version 11.0 software package [48]. To manage variation and exon counts among taxa, we trimmed the alignment using Gblocks 0.91.1 with specific parameters: smaller final blocks and gap positions within the blocks [49]. The phylogenetic analysis was conducted using maximum likelihood in IQTree 1.6.12 [50]. Tree topology confidence values were evaluated with 10,000 bootstrap replicates, and the results were visualized using iTOL 6.8.1 [51].

2.6. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 8.0.1, employing statistical tests suiTable for each type of analysis. Data are presented as the mean values ± standard error of the mean, with a threshold for statistical significance set at $p < 0.05$. All experiments were conducted in triplicate.

3. Results

3.1. Safety Assessment

Bacillus velezensis P45 was tested for susceptibility to various antimicrobials and exhibited sensitivity to all tested agents (Table S1). The strain P45 exhibited γ-hemolysis, showing no hemolytic zone, while *S. aureus* ATCC 25923, used as the positive control, demonstrated β-hemolytic activity. The lack of hemolytic activity and susceptibility to 17 commonly used antimicrobials are significant safety attributes, suggesting that *B. velezensis* P45 is a safe candidate for use as a probiotic.

3.2. Probiotic Properties

3.2.1. Antimicrobial Activity

The antibacterial activity of *B. velezensis* P45 was evaluated against different bacterial species. The crude culture supernatant of P45 showed antagonistic activity against the strains tested (Figure 1). Specifically, the highest antimicrobial activity was recorded against *L. monocytogenes*, while lower values were observed for other bacteria.

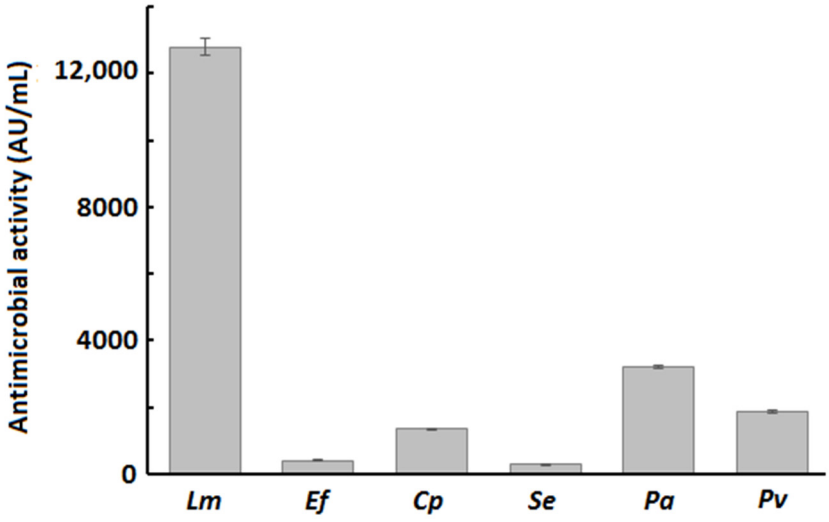

Figure 1. Antimicrobial activity of crude supernatant of B. velezensis P45 against *Listeria monocytogenes* (Lm), *Enterococcus faecalis* (Ef), *Clostridium perfringens* (Cp), *Salmonella enterica* (Se), *Pseudomonas aeruginosa* (Pa), and *Proteus vulgaris* (Pv). Values are mean ± SEM from three biological replicates.

3.2.2. Autoaggregation and Cell Surface Hydrophobicity

B. velezensis P45 demonstrated a higher autoaggregation rate among the strains, with a percentage that increased over time, reaching around 88% at 24 h (Figure 2A). In comparison, *L. rhamnosus* CCT 7863, used as the control, showed a maximum autoaggregation of 60% at 24 h, while *L. monocytogenes* ATCC 7644 exhibited the lowest autoaggregation rate among the strains at 57% (Figure 2A).

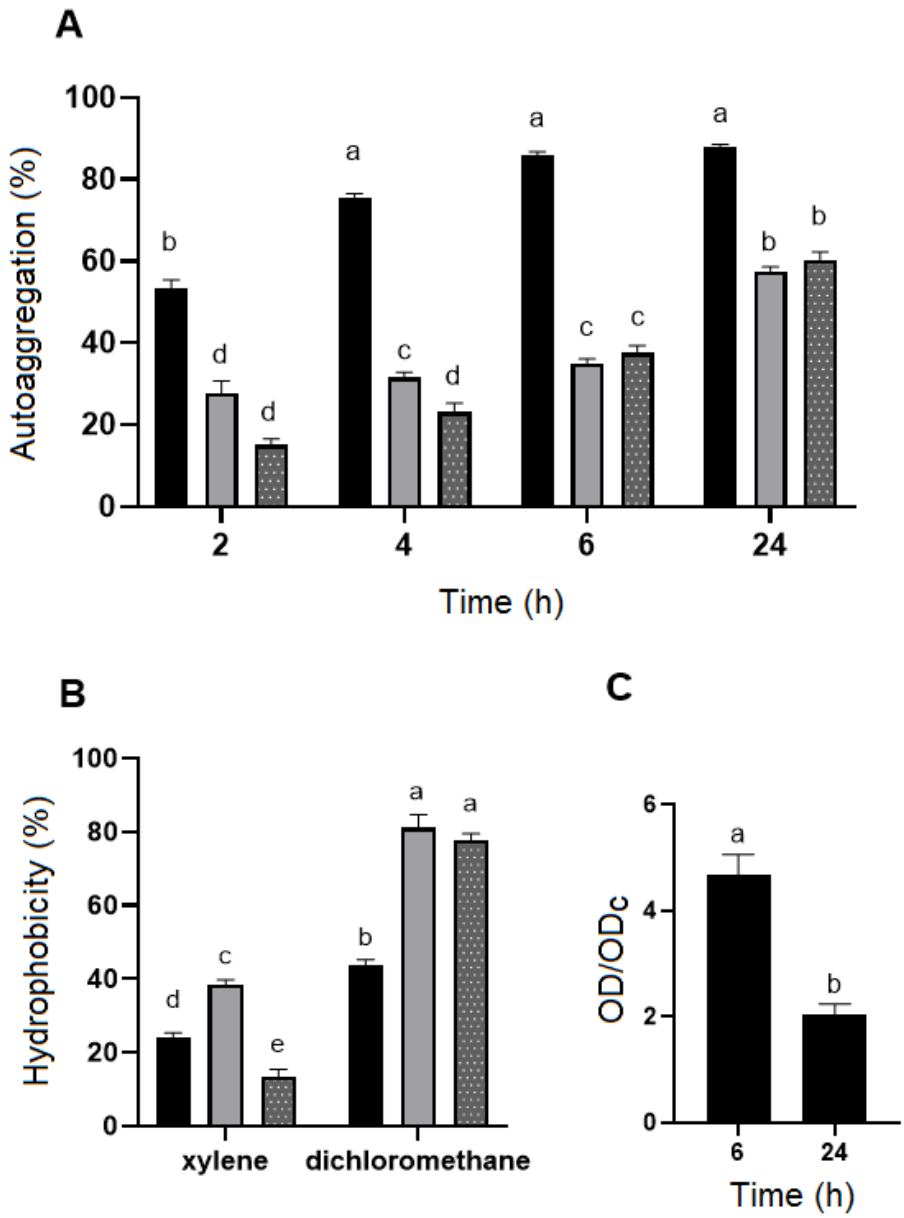

Figure 2. (**A**) The autoaggregation ability (%) and (**B**) surface hydrophobicity (%) of *Bacillus velezensis* P45 (black bars), *Listeria monocytogenes* ATCC 7644 (grey bars), and *Lacticaseibacillus rhamnosus* CCT 7863 (dotted bars) in different times and with different organic solvents, respectively. (**C**) Biofilm formation by *Bacillus velezensis* P45. Values are expressed as the mean ± SEM from three biological replicates. Different letters indicate significant differences ($p < 0.001$).

Cell surface hydrophobicity was evaluated using xylene (a non-polar solvent) and dichloromethane (a polar solvent). All strains demonstrated moderate-to-high hydrophobicity towards dichloromethane, with *B. velezensis* P45, *L. rhamnosus* CCT 7863, and *L. monocytogenes* ATCC 7644 showing hydrophobicity values of 43.9%, 81.2%, and 77.3%, respectively

(Figure 2B). However, hydrophobicity values with xylene were lower, with *B. velezensis* P45 at 24.2%, *L. rhamnosus* CCT 7863 at 38.3%, and *L. monocytogenes* ATCC 7644 at 13.3%.

3.2.3. Biofilm Formation

Biofilm formation was assessed using the CV assay, which is commonly applied to quantify total biofilm formation on a specific synthetic surface. The data indicated that the biofilm formation of *B. velezensis* P45 was considered strong during the first 6 h but decreased over time (Figure 2C).

The interpretation of results was based on the scale proposed by Stepanović et al. (2007) [41]. Thus, the average optical density values were calculated for each well stained with crystal violet, measured at 570 nm. A cut-off value (ODc) was established, defined as three standard deviations (SD) above the mean OD of the negative control. The ODc serves as a reference point to determinate the significance of biofilm formation, with levels of CV staining above this threshold indicating noTable biofilm presence or adherence.

3.2.4. Tolerance to Gastrointestinal Conditions

The tolerance of *B. velezensis* P45, *L. monocytogenes* ATCC 7644, and *L. rhamnosus* CCT 7863 to gastrointestinal conditions was determined in vitro to assess cell viability upon exposure to simulated gastric acid and intestinal fluids. All strains were initially introduced into the simulation at a concentration of approximately 10^8 CFU/mL. As a result, *L. rhamnosus* CCT 7863 showed a high survival rate post-simulation, suggesting tolerance to these conditions (Figure 3). *B. velezensis* P45 and *L. monocytogenes* ATCC 7644 showed decreased cell viability following exposure to simulated fluids. Viable counts of *B. velezensis* decreased of approximately 1.5 log CFU/mL, but only after SIF treatment. In contrast, *L. monocytogenes* ATCC 7644 exhibited a reduction of 4 log CFU, suggesting that the gastrointestinal conditions had a significant impact on the survival of this pathogen.

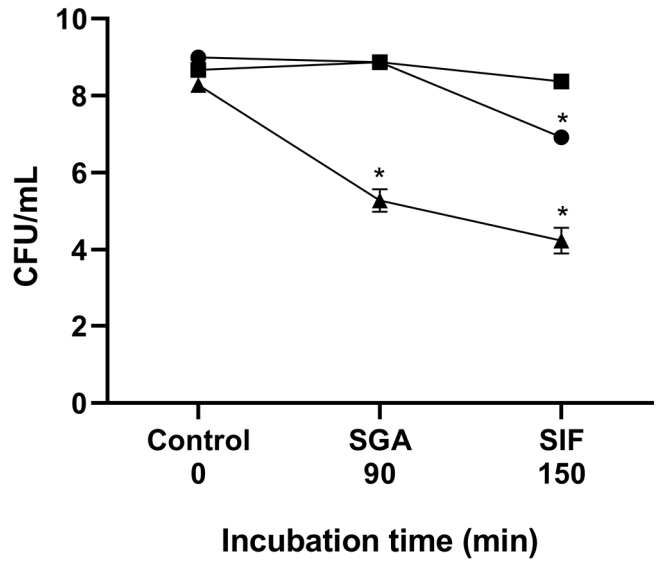

Figure 3. Cell viability during gastrointestinal simulation in vitro. The lines show cell viability before simulation, after exposure to simulated gastric acid (SGA), and after simulated intestinal fluid (SIF). (●) *Bacillus velezensis* P45, (■) *Lacticaseibacillus rhamnosus* CCT 7863, and (▲) *Listeria monocytogenes* ATCC 7644. (*) Statistical differences ($p < 0.001$) are indicated by an asterisk.

3.2.5. Adhesion and Growth in Mucin

The overall mucin adhesion capabilities of the species under study is summarized in Figure 4A. Results revealed adhesion percentages ranging from 79% to *B. velezensis* P45, 88% to *L. rhamnosus* CCT 7863, and 82% for *L. monocytogenes* ATCC 7644.

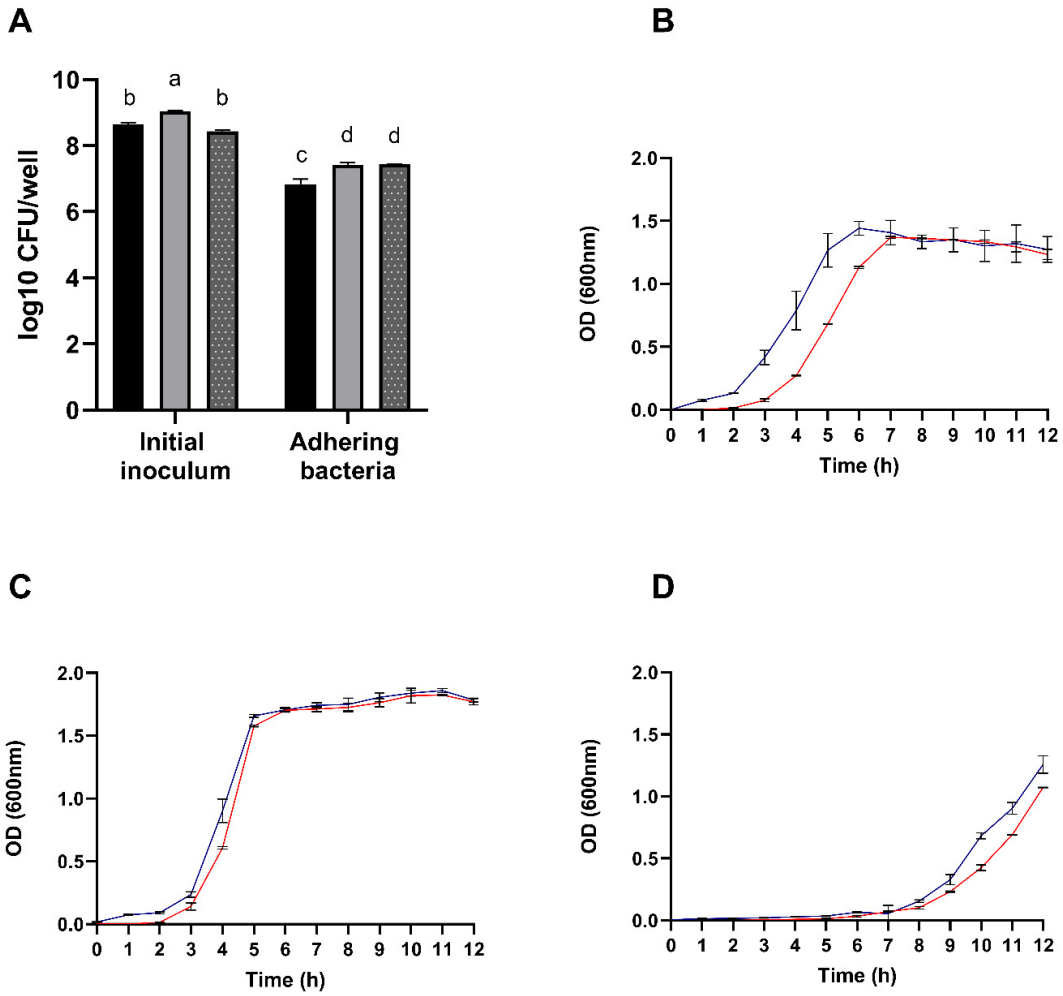

Figure 4. (**A**) Enumeration of adhering *Bacillus velezensis* P45 (black bars), *Listeria monocytogenes* ATCC 7644 (grey bars), and *Lacticaseibacillus rhamnosus* CCT 7863 (dotted bars) cells to mucin. Growth curves of *Bacillus velezensis* P45 (**B**), *Lacticaseibacillus rhamnosus* CCT 7863 (**C**), and *Listeria monocytogenes* ATCC 7644 (**D**) in the presence (blue line) or absence (red line) of porcine mucin. Values are expressed as the mean ± SEM from three biological replicates. Different letters denote significant differences.

Interestingly, as shown in the Figure 4B, the growth of *B. velezensis* P45 was positively influenced by the presence of porcine mucin, suggesting that the strain can thrive on mucin as a substrate. In contrast, *L. rhamnosus* CCT 7863 and *L. monocytogenes* ATCC 7644 exhibited similar growth patterns regardless of mucin presence. Therefore, the combined adhesion and growth results suggest that *B. velezensis* P45 has a potential capacity to effectively colonize the host intestinal mucosa.

3.3. Genome Mining for Probiotic Characteristics

The genome annotation of *B. velezensis* P45 using RAST predicted several putative genes associated with probiotic traits (Table S2). These characteristics include adhesion and aggregation, vitamin biosynthesis, amino acid metabolism, lactic acid production, active metabolism, enzyme production for food digestion, and tolerance to stress and the host gastrointestinal tract. Moreover, the probiotic prediction was performed on the iProbiotics web server, revealing that strain P45 could be confirmed (99.88%) as a probiotic (Figure S1).

3.3.1. Genes Related to Virulence and Antimicrobial Resistance

Bioinformatics tools were employed to investigate the presence of genes related to virulence and antibiotic resistance in the *B. velezensis* P45 genome. As result, no chro-

mosomally encoded antibiotic resistance genes were found. Additionally, analysis with the PlasmidFinder web tool revealed the absence of plasmids in *B. velezensis* P45. This absence of plasmids is a favorable probiotic indicative as plasmids can harbor genes for antimicrobial resistance and virulence factors. Analysis using VFanalyzer from the Virulence Factor Database found no matches, indicating the absence of virulence factors. The PathogenFinder web server was also used to assess potential pathogenicity towards human health, predicting *B. velezensis* P45 as non-pathogenic for humans. The probability of being a human pathogen was calculated as 0.109, with no matches to pathogenic families and 56 matches to non-pathogenic families.

3.3.2. Genes Related to Antimicrobial Metabolites

AntiSMASH analysis was employed to predict the biosynthetic gene clusters for secondary metabolites potentially associated with antimicrobial activities. The genome of *B. velezensis* P45 revealed six secondary metabolite gene clusters. Those exhibiting over 80% similarity to known compounds were specifically noted. The biosynthesis machinery and bioactivities of these clusters are detailed in Table 1.

Table 1. Gene clusters found in *Bacillus velezensis* P45 genome using antiSMASH software considering synthesis pathways, the most similar known gene clusters, the bioactivity, and the similarity with *Bacillus velezensis* FZB42 gene clusters.

Cluster Type [1]	Most Similar Cluster	Bioactivity	Similarity (%)
1. Other	Bacilysin	Antibacterial	100
2. NRPS	Fengycin	Antifungal	86
3. NRPS	Surfactin	Surfactant	95
4. PKS-NRPS	Bacillaene	Antibacterial	100
5. PKS	Macrolactin H	Antibacterial	100
6. NRPS	Bacillibactin	Siderophore	100

[1] NRPS, non-ribosomal peptide synthase; PKS, polyketide synthase.

Tree gene clusters related to nonribosomal peptide synthetases (NRPS) related to the synthesis of cyclic lipopeptides (CLPs) were identified. Clusters 2 (fengycin), 3 (surfactin), and 6 (bacillibactin) exhibited 80, 95, and 100% similarity with *B. velezensis* FZB42 gene clusters, respectively. Clusters 4 and 5 were categorized as polyketide synthases (PKS). Cluster 4 is associated with the synthesis of bacillaene, whereas Cluster 5 is related to the production of macrolactin H. Both clusters showed 100% similarity with PKS clusters of *B. velezensis* FZB42 gene clusters. Cluster 1 was classified as "Other" and is associated with the synthesis of bacilysin, showing 100% similarity with known clusters.

A representation of the fengycin gene cluster is provided in Figure S2, highlighting the key genes involved in the synthesis of this antifungal peptide and its relationship with plipastatin. Interestingly, the bacillomycin gene cluster, which is part of the iturin family, is not an independent entity but is integrated within the fengycin gene cluster (Figure 5). This arrangement suggests a complex genetic architecture where multiple biosynthetic pathways are co-located. The genomic region responsible for siderophore biosynthesis was found to be an exact match to that of bacillibactin. Likewise, the genomic region governing the synthesis of the tail-cyclized peptide was identified as a precise duplicate of amylocyclicin (Figure S3).

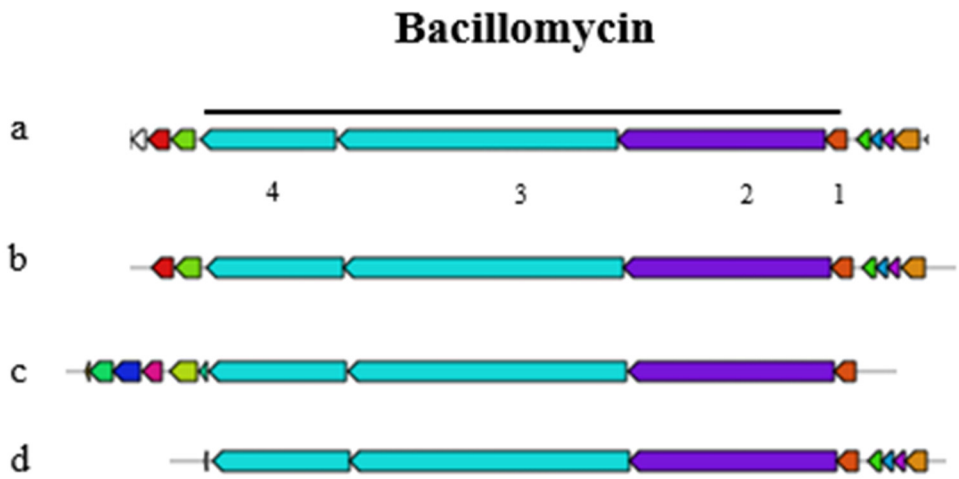

Figure 5. Bacillomycin gene cluster of the query sequence *Bacillus velezensis* P45 (**a**) with the core biosynthetic genes constituted by (1) MCAT (2) *bmyA*, (3) *bmyB*, and (4) *bmyC* under the black line; (**b**) bacillomycin; (**c**) mycosubtilin and gene clusters with 100% similarity; and (**d**) an iturin cluster with 88% similarity.

Analysis of *B. velezensis* P45 using the NP.searcher program revealed the presence of mixed modular NRPS/PKS, trans-ATPKs, and a non-mevalonate terpenoid *mep* gene. Additionally, BAGEL4 analysis identified six distinct bacteriocins and RiPP clusters associated with the production of antimicrobial peptides and bacteriocins (Table S3). Clusters linked to antimicrobial activity include sactipeptides, amylocyclicin, UviB, and LCI. Further structural predictions were made using the PRISM4 algorithm, which indicated 12 clusters of putative compounds, including seven NRPs, four PKs, one Class II/III confident bacteriocin (thiopeptide), one bacterial head-to-tail cyclized peptide, and one ComX (Table S4).

3.4. Phylogenetic Comparison of yloA (rqcH) Adhesion Gene

The *yloA* (or *rqcH*) gene of interest, associated with adhesion, was selected for comparative analysis based on whole-genome alignments of the examined *Bacillus* strains. This analysis indicates the presence of a tendency for probiotic and non-probiotic *Bacillus* strains to cluster separately from typically pathogenic *Bacillus* species, such as *Bacillus cereus*, and from other genera, such as the *Staphylococcus* group. As shown in Figure 6, *B. velezensis* P45 clustered with strains of probiotic bacilli and was separated from the pathogenic strains, thereby supporting its potential as a probiotic. Several strains classified as probiotics or potential probiotics were grouped with strain P45, including *B. velezensis* TS5, *B. velezensis* KMU01, *B. amyloliquefaciens* R825, *B. siamensis* B28, and *B. velezensis* FZB42. In contrast, pathogenic and non-probiotics groups clustered separated, including *B. cereus* ATCC 14579, *B. cereus* FORC10, *B. cereus* 29, *B. amyloliquefaciens* DSM7, and *B. licheniformis* TCCC11148.

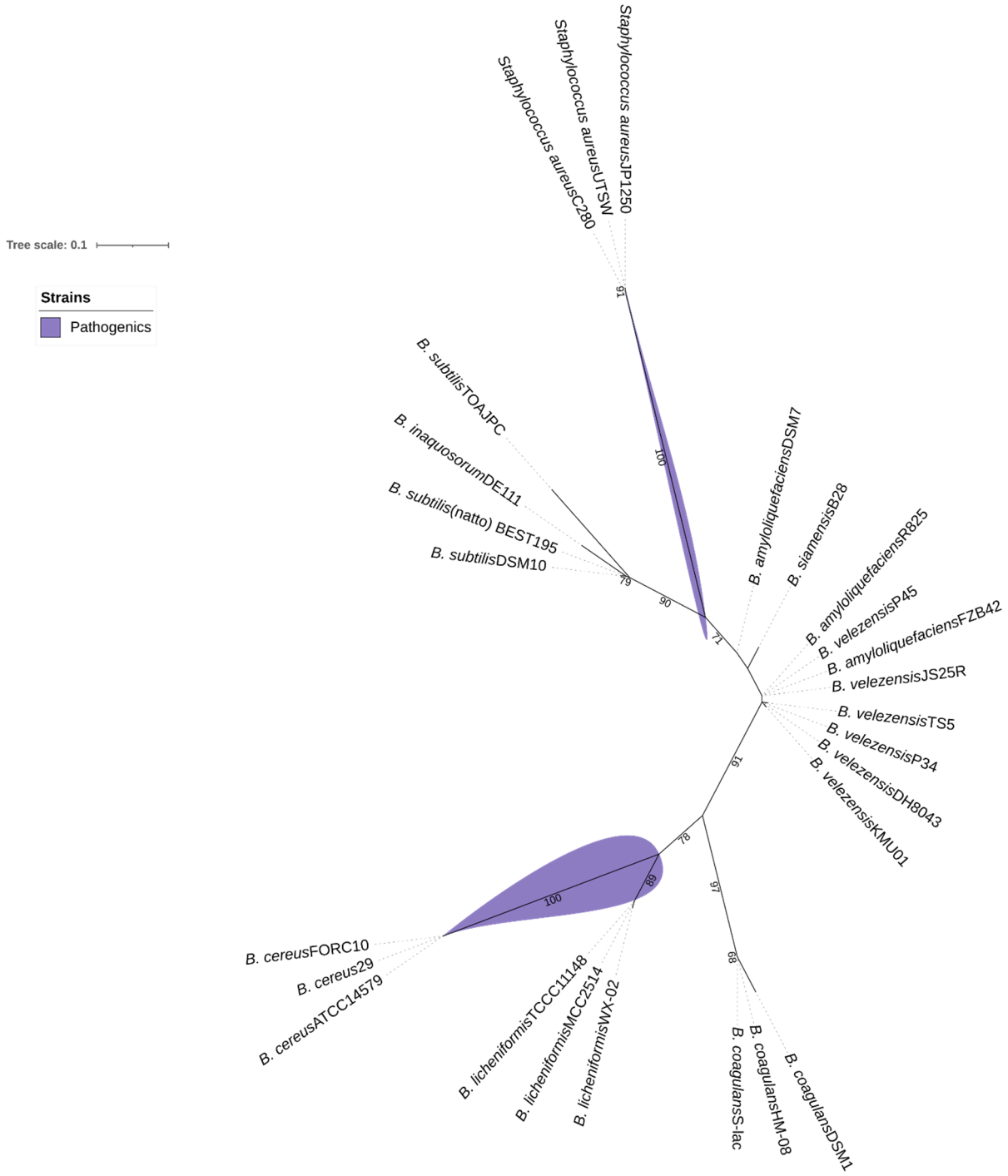

Figure 6. Maximum-likelihood tree based on gene *rqcH* sequences, showing relationships between *Bacillus velezensis* P45, probiotic *Bacillus*, and some pathogenic bacteria. Bootstrap values are shown on branches.

4. Discussion

The increasing demand for preventive healthcare, combined with the demonstrated efficacy of probiotic bacteria, has driven significant interest in probiotics and their metabolites in recent decades [52]. Despite the genus *Bacillus* not being abundantly found in commercial formulations, recent advances on the probiotic potential of *Bacillus* strains have been achieved [19,23–26,30]. To address this issue, the strain *B. velezensis* P45, isolated from the intestine of an Amazonian fish, was evaluated in the present study.

The strain P45 exhibited no hemolysis, as reported for probiotic *Bacillus* strains [11,53], but also in *B. velezensis* candidates [30,54], suggesting that the absence of hemolytic activity is a consistent property across *B. velezensis* strains. Moreover, the antimicrobial susceptibility test revealed that the strain P45 is widely susceptible to all antibiotics tested. In silico genomic analyses of *B. velezensis* P45, utilizing PlasmidFinder, VFanalyzer, and PathogenFinder platforms, confirmed these findings. No genetic sequences related to virulence factors (VF), transferable antimicrobial resistance (AMR), or mobile genetic elements were identified in the genome of strain P45. Although resistance to certain antibiotics is frequently observed in *Bacillus* species exposed to environmental stresses associated to agricultural practices and animal production [55,56], lower resistance was noted in strains like *B. velezensis* P45, which was isolated from an environment with minimal antibiotic exposure. These findings suggest that P45 is a potentially safe strain for probiotic use, aligning with the broader trend of promising probiotic candidates.

B. velezensis is generally recognized as safe (GRAS) bacterium, which has been extensively used in agriculture and as feed additive with potential health benefits [29,57]. In this regard, it could be expected that *B. velezensis* P45 is also a probiotic strain, as predicted with a very high score (99.88%) by the iProbiotics platform. The iProbiotics prediction is achieved by using machine-learning algorithms to analyze the presence of certain *k*-mers, which are short and contiguous nucleotide sequences in the genome. The iProbiotics tool has been trained on a large dataset of 239 known probiotic strains (41 species) and 412 non-probiotic strains (80 species), supporting accurate predictions based on the presence or absence of specific *k*-mers [47]. The score obtained by *B. velezensis* P45 was higher than those of other *Bacillus* also proposed as probiotic candidates [58].

The antagonistic activity of *B. velezensis* P45 against pathogenic strains demonstrated significant antimicrobial effect, with particularly higher activity against *L. monocytogenes*. This finding corroborates previous studies showing that *B. velezensis* P45 produces bacillomycin D and fengycins A and B in BHI medium [32]. These antimicrobial lipopeptides are recognized by their broad antimicrobial activity, including Gram-positive and Gram-negative bacteria [24,31,57]. Moreover, *Bacillus subtilis* and *Bacillus cereus* strains isolated from fish intestines exhibited antimicrobial activity against *L. monocytogenes* and *Salmonella* [59]. Similarly, lipopeptides from *Bacillus* sp. P34, also isolated from fish intestines, effectively inhibited *L. monocytogenes*, *S. aureus* [60], and food isolates of *S. aureus* and *E. faecalis* [61]. Furthermore, *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 inhibited biofilm formation by various *Salmonella* serovars through the production of subtilosin A and subtilin, known antibiotic peptides produced by *Bacillus* species [62]. These findings are consistent with the antimicrobial activity of *B. velezensis* P45 against pathogenic bacteria, suggesting that *Bacillus* strains share common metabolites and mechanisms for pathogen inhibition. This reinforces the value of *Bacillus* strains with probiotic properties, not only to improve intestinal health but also to control pathogens in food systems.

Genome analysis of strain P45 revealed gene clusters encoding antimicrobial lipopeptides such as fengycin, surfactin, iturin bacillaene, macrolactin, and amylocyclin. These compounds are synthesized by *Bacillus* species through large enzymatic complexes known as non-ribosomal peptide synthetases (NRPS) [63]. Notably, the bacillomycin gene cluster, part of the iturin family, was not identified as an independent cluster but rather integrated within the fengycin gene cluster in the genome of P45, which could be due to the close physical proximity of the plipastatin/fengycin and iturin genes on the chromosome [64]. Furthermore, gene clusters within a contig may not meet antiSMASH criteria for forming distinct genetic clusters, which could result in the incomplete detection of clusters, particularly in fragmented genomes [65]. Moreover, gene clusters involved in the biosynthesis of two polyketides (macrolactin H and bacillaene) and one peptide (bacilysin) were also

identified, with 100% similarity, consistent with data from *Bacillus* sp. P34 [60]. The gene cluster for bacillibactin, a typical siderophore of the *Bacillus* genus, plays a crucial role in iron acquisition, plant growth promotion, and antibiotic activity [66,67].

Furthermore, the adhesion capacity to the intestinal epithelium plays a critical role in the efficacy of probiotic strains as it directly affects various aspects of the host microbiota. Adhesion increases the residence time of probiotics in the gut, facilitating colonization and promoting the establishment of beneficial microbiota communities [68]. By occupying adhesion sites, probiotics inhibit the attachment of pathogens, thereby preventing colonization and mitigating potential adverse effects [69,70]. Autoaggregation and hydrophobicity index are common indicators of the adhesion ability of bacterial strains. In comparison to other strains used in this study, namely, *L. rhamnosus* CCT 7863 and *L. monocytogenes* ATCC 7644, *B. velezensis* P45 exhibited superior autoaggregation rates at all time points, reaching 55% at 2 h and the maximum value of 88% at 24 h. These elevated rates were also higher as compared to other *Bacillus* species. For example, *Bacillus paralicheniformis* FA6 achieved an autoaggregation rate of 28.4% after 2 h in PBS [71], while *Bacillus* sp. RCS1 reached 80.2% after 24 h [72]. The autoaggregation rate of *B. velezensis* P45 also exceeded the results reported for well-known probiotics like *L. rhamnosus* GG, which displayed a rate of 59–65% [73,74], and *Bifidobacterium pseudolongum* YY-26, which demonstrated a rate of 68.9% [75].

Bacillus velezensis P45 exhibited strong biofilm formation during the early hours of incubation, followed by a noTable decline after 24 h. Several environmental, cellular, and surface-related factors can directly affect bacterial adhesion and biofilm development [76,77]. Although the crystal violet assay is widely recognized and applied for evaluating biofilm formation in *Bacillus* species, we acknowledge that specific regulatory features or physiological responses of the strain P45 may have influenced the dynamics observed under our experimental conditions. In particular, the limited medium volume (300 µL) may have contributed to nutrient depletion and metabolite accumulation, potentially triggering a reduction in biofilm mass after 24 h. This nutrient stress could also initiate sporulation process, thereby reducing the number of vegetative cells and, consequently, the retention of crystal violet [77,78].

The mucus layer on the gastrointestinal epithelium serves as a primary barrier against pathogenic microorganisms and as a substrate that supports the commensal bacteria, facilitating their growth, biofilms formation, and intestinal colonization [43,79]. Porcine gastric mucin is commonly employed as a model to simulate human mucin, allowing for the investigation of bacterial adhesion and providing valuable insights into microbial interactions with the gastrointestinal epithelium [80,81]. This model provides valuable insights into how mucins affect the adhesive capabilities or growth of the microorganisms of interest. Both *B. velezensis* P45 and *L. rhamnosus* CCT 7863 demonstrated enhanced growth in the presence of porcine mucin. Similarly, several *Lacticaseibacillus plantarum* strains isolated from fermented foods showed increased growth with mucin, indicating the ability to utilize it as a growth substrate [81].

Tolerance to gastrointestinal tract conditions is another essential ability for probiotic strains [82]. Simulation of gastrointestinal tract conditions provides a reliable method for assessing probiotic behavior. In this study, *B. velezensis* P45 and the probiotic strain *L. rhamnosus* CCT 7863 exhibited tolerance to gastric and intestinal fluids, whereas *L. monocytogenes* ATCC 7944 demonstrated a significant decrease in cell viability. Adaptation to the gastrointestinal conditions can be mediated by the expression of genes encoding for proteins like arginine/ornithine antiporter, F_0F_1-ATP synthase, Na^+/H^+ antiporter, nhaX and nhaK antiporters, and sodium bile acid symporter, maintaining the homeostasis in the gastrointestinal tract [83,84]. In this context, genome analysis of *B. velezensis* P45 revealed the presence of protein-coding sequences (CDS) related to reduce bacterial damage. Over

40 genes involved in tolerance to adverse gastrointestinal conditions were identified, including resistance to low pH, high bile salt concentrations, oxidative and osmotic stress, and temperature tolerance (Table S2). Genes related to DNA repair were also identified, such as the nuclease ABC complex (uvrA, uvrB, uvrC), which further enhance the strain resilience. The presence of genes encoding for the arginine/ornithine antiporter ArcD, ornithine carbamoyl transferase, arginine pathway regulatory protein ArgR, F_0F_1-ATP synthase subunits, L-lactate dehydrogenase, Na^+/H^+ antiporter, and bile acid sodium symporter reinforce the tolerance of strain P45 to acidic conditions and high bile salt concentrations [83–85]. In addition, endospores formation, a characteristic of the *Bacillus* genus, enables these microorganisms to withstand various environmental stresses [77,84].

Based on genome information, *B. velezensis* P45 harbors over 15 genes putatively associated with adhesion-related proteins, including chitin-binding protein, sortase D, fibronectin-binding protein, enolases, and flagellar hook-basal body complex proteins (Table S2), which supports adhesion properties of *B. velezensis* P45. These results are consistent with other studies that discovered similar genes in potential probiotic *Bacillus*, such as *B. velezensis* ZBG17 [86], *B. velezensis* FS26 [31], and *B. cereus* G1-11 [87].

Finally, the biosynthesis of vitamins, amino acids, and cofactors by probiotic bacteria underscores their ability to produce essential bioactive compounds. Since the human body is not capable of synthesizing all the required vitamins, their intake is essential for several biological processes, such regulating biochemical reactions and maintaining homeostasis by participating in metabolic pathways as precursors of intracellular coenzymes [88]. In *B. velezensis* P45, genes involved in the synthesis of water-soluble vitamins were identified, including B2 (riboflavin), B7 (biotin), B1 (thiamine), B3 (niacin), B6 (pyridoxine), B5 (pantothenic acid), B6 (pyridoxine), B9 (folic acid), and B12 (cobalamin). Additionally, genes coding for the biosynthesis of fat-soluble vitamins, such as vitamin K (menaquinone), were also detected. Thus, incorporating probiotic bacteria into food products may provide a natural source of essential vitamins, directly synthesized in the digestive tract, modulating the abundance and diversity of gut microbiota promoting intestinal and overall health [88,89].

5. Conclusions

This study highlights the potential of *B. velezensis* P45 as a promising probiotic candidate. Several in vitro assays demonstrate its strong adhesion capability, its tolerance to stress conditions (including acid, bile, osmotic, and oxidative stress), and its ability to synthesize secondary metabolites such as vitamins and antimicrobial compounds, corroborated by in silico identification of several genes encoding these properties. These attributes are related to various metabolic and biochemical processes that help maintain viability in GIT and inhibit the growth of pathogens. Both in vitro and in silico analyses indicate that *B. velezensis* P45 synthesizes antimicrobial compounds, which are essential for competitive exclusion of pathogens and for its survival in the host gut. Additionally, the strain exhibits a favorable safety profile, with no evidence of antibiotic resistance, virulence factors, or pathogenicity. These findings underscore the potential of *B. velezensis* P45 as a probiotic candidate for human health, favoring the further exploration of strains of the *B. velezensis* genus for applications in the food and pharmacy sectors. However, further in vivo studies are necessary to confirm the probiotic potential and functional effects of *B. velezensis* P45.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods14132334/s1: Table S1: *Bacillus velezensis* P45 susceptibility to antimicrobials by disc diffusion assay; Table S2: Genes related to potential probiotic characteristics in *Bacillus velezensis* P45 genome; Figure S1: Probability prediction of *B. velezensis* P45 genome as probiotics using the iProbiotics algorithm; Figure S2: Biosynthetic gene cluster for fengycin of the query sequence *Bacillus velezensis* P45; Figure S3: Bacillibactin gene cluster of the query sequence

Bacillus velezensis P45 identified by antiSMASH 7.0; Table S3: Predicted bacterial RiPP clusters in *Bacillus velezensis* P45 genome based on BAGEL4; Table S4: Predicted secondary metabolites gene clusters in *Bacillus velezensis* P45 genome based on PRISM4.

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SCIENTIFIC PAPER 3

Comparative proteomic analysis reveals the modulation of probiotic features in *Bacillus velezensis* P45 growing on prebiotics and mucin

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Comparative proteomic analysis reveals the modulation of probiotic features in *Bacillus velezensis* P45 growing on prebiotics and mucin

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ABSTRACT

Probiotics, prebiotics, and their combination as synbiotics have been associated with diverse health benefits, yet the molecular mechanisms underlying their interactions remain insufficiently explored. This study evaluated how different carbon sources, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and mucin, modulate the proteomic profile of *Bacillus velezensis* P45, a promising probiotic strain. The strain was cultivated in a synthetic medium supplemented with 10 g/L of each carbon source at 37 °C for 24 h under aerobic conditions. Proteins were extracted and analyzed by LC–MS/MS, and differential expression was assessed by statistical and clustering analyses. A total of 1093 proteins were identified, of which 314 were differentially expressed across treatments. FOS and GOS induced similar proteomic responses, upregulating proteins related to their specific carbon metabolism, adhesion, and the synthesis of bioactive compounds. In contrast, mucin triggered a distinct profile characterized by stress-response proteins, subtilisin, and peptidase G2, reflecting the potential of strain P45 to persist in gut-like conditions. These findings demonstrate the metabolic adaptability of *B. velezensis* P45 and reinforce its potential as a probiotic candidate, particularly for synbiotic formulations with prebiotic oligosaccharides that support gut health.

1. Introduction

The growing interest in probiotics has been driven by consumer demand for functional foods that are nutritious, safe, and health-promoting [1] Probiotics are live microorganisms that, when consumed in sufficient amounts, exert beneficial effects on the host [2]. Although their well-established health benefits within the gastrointestinal tract, probiotics have also been associated with benefits that extend beyond the gut barrier, likewise immunomodulation, anti-inflammatory action, and even positive effects into wellness and mental health [3,4]. As our understanding of probiotic effects has deepened, related concepts such as prebiotics and postbiotics have also gained attention due to their roles in modulating the gut microbiota and promoting host homeostasis [5].

Prebiotics are non-digestible substrates selectively utilized by beneficial microorganisms, leading to shifts in microbial composition and activity [6]. Postbiotics, in turn, refer to non-viable microbial cells, cell components, or metabolites that confer health benefits to the host, including short-chain fatty acids, organic acids, antimicrobial peptides, vitamins, and enzymes [7]. When combined in a single formulation, prebiotics, and probiotics can form synbiotics, which have shown promising results in enhancing microbial viability, modulating immune responses, and maintaining intestinal barrier integrity [6,8]. However, the effectiveness of such formulations is highly dependent on the specific compatibility between probiotic strains and prebiotic substrates, highlighting the need for mechanistic studies to better understand these interactions [9].

Among emerging probiotic candidates, spore-forming species of the genus *Bacillus* have attracted increasing interest [10]. Their inherent ability to form spores confers resistance to harsh conditions, both within the gastrointestinal tract and during industrial processing, making them suitable for incorporation into functional foods and nutraceutical formulations [10]. Moreover, many *Bacillus* species produce a wide range of bioactive compounds, including antimicrobial lipopeptides, enzymes, antioxidants and peptides, which further enhance their functional and technological appeal in probiotic formulations [11]. Among the species

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of the genus *Bacillus, Bacillus velezensis* has shown notable antimicrobial activity, intestinal adaptability, and probiotic potential [12]. The strain *B. velezensis* P45 was isolated from the intestine of an Amazonian fish (*Piaractus mesopotamicus*) [13]. Previous studies of our group showed that strain P45 has potential for the bioconversion of keratin-rich substrates and the production of bioactive compounds with antimicrobial action [14,15]. In a recent study, the probiotic potential of *B. velezensis* P45 was evaluated through a *in silico* and *in vitro* combined approach, by whole-genome analysis with phenotypical characterization of functional probiotic properties, including adhesion capacity, tolerance to gastrointestinal transit, and the absence of virulence factors and antimicrobial resistance traits [16].

Some substrates, including fructooligosaccharides (FOS) and galactooligosaccharides (GOS), have shown influence on metabolic plasticity potential probiotics strains, promoting bacterial adhesion, prolong intestinal residence time, and increase colonization levels [17,18]. Although chemically different from FOS and GOS, mucin glycans can be used as nutrient source and has been often used as an experimental model to investigate microbial adaptation to host-associated environments [19,20]. The degradation of mucin can also impact composition and functionality of the gut microbiota [21]. In this context, omics-based approaches, particularly proteomics, have emerged in probiotic research as effective, reliable, and high-throughput tools [22]. Proteomic analyses are especially valuable for revealing functional traits such as stress tolerance, substrate utilization, and antimicrobial compound production [23]. The technique also capture post-translational modifications that are critical for protein activity but often missed by genomic or transcriptomic approaches [24]. In synbiotic design, proteomics helps assess strain-specific responses to prebiotics, supporting the rational development of synergistic formulations and advancing a systems-level understanding of probiotic mechanisms within the emerging "pro-biomics" framework [25,26].

The aim of this study is to evaluate the growth performance of *B. velezensis* P45, on different carbon sources, including the synbiotics formulations with FOS and GOS, as well as mucin, used here as a model of host-derived substrate. In parallel, we applied a proteomics-based approach to characterize the substrate-specific molecular responses of this strain, aiming to elucidate key metabolic pathways involved in its adaptation and to assess its potential as a candidate for synbiotic formulations.

2. Materials and methods

2.1. Bacterial growth curve in presence of prebiotics and mucin

Bacillus velezensis P45 was maintained in a frozen stock (−70 °C) culture of BHI broth (Merck, Darmstadt, Germany) containing 20 % (v/v) glycerol. For the experiments, the strain was cultured under aerobic conditions in Brain-Heart Infusion (BHI; Kasvi, Paraná, Brazil) for 24 h at 37 °C to cell reactivation. In order to minimize the detection of proteins from the culture media in mass spectrometry analysis, the isolate was grown in a synthetic medium (SM) described previously [27]. The composition of the SM is described below (g/L): peptone, 5; yeast extract (Sigma-Aldrich), 1; KH_2PO_4, 0.5; $NaNO_3$, 0.1; NaCl, 20. The base media was divided in four parts and supplement with 10 g/L FOS (Nutramax, São Paulo, Brazil), 10 g/L GOS (Nutramax, São Paulo, Brazil), 10 g/L mucin (Sigma-Aldrich, Saint Louis, Missouri), or 10 g/L glucose (Sigma-Aldrich), used as control. The pH of each media was adjusted to 7.0 before autoclaving at 110 °C for 30 min. *B. velezensis* P45 cells, from 24 h culture, were inoculated (2 %, v/v) with an initial O.D. 600 between of 0.6. The incubation was performed at 37 °C, 150 rpm for 48 h, under aerobic conditions. Then, the protein extraction was performed. Biological replicates were prepared from three independent cultures for each treatment (FOS, GOS, GLU and mucin). In this experiment, glucose (GLU) was used as control. The growth curve of *B. velezensis* P45 with each carbon source was elaborate through determination of the OD 600 nm, using a microplate reader.

2.2. Protein extraction

After the 24 h of growth at 37 °C, the cultures were centrifuged at 12,000 *g* for 20 min at 4 °C to separate the bacterial pellets. Protein extraction from the pellets followed the method described by [28]. Briefly, the pellets were lysed with 200 µL of lysis buffer containing 8 M urea, 4 % sodium dodecyl sulfate, and 50 mM Tris–HCl (pH 8.0), supplemented with Roche cOmplete™ Mini protease inhibitor tablets (one tablet per 10 mL of buffer). Lysis was performed through three cycles of ultrasound treatment (30 s per cycle, with 1 min intervals on ice) using a US500 apparatus (Unique, Indaiatuba, Brazil) set to 25 % amplitude. Proteins from the lysates were precipitated with a mixture of acetic acid, acetone, and ethanol (50:50:0.1 %, v/v) and centrifuged at 12,000 *g* for 20 min at 4 °C. The resulting protein pellets were washed three times with ice-cold acetone, centrifuged at 12,000 *g* for 25 min, and stored at −80 °C.

2.3. Samples preparation for LC-MS/MS analysis

Prior de digestion of the samples, total protein quantification was performed by the Folin-phenol method, using a standard curve of bovine serum albumin. The digestion of proteins was performed using trypsin [29], with modifications. Briefly, the samples were resuspended in 50 mM ammonium bicarbonate containing 8 M urea. Dithiothreitol (DTT) was added to a final concentration of 5 mM, and the samples were incubated for 25 min at 56 °C. Next, 14 mM iodoacetamide (IAA) was added, and the samples were incubated for 30 min at room temperature, protected from light. Trypsin (Sequencing Grade Modified Trypsin V5111, Promega, Fitchburg, WI, USA) was prepared at a final concentration of 20 ng/µL in chilled 50 mM ammonium bicarbonate and added to the samples at an enzyme-to-substrate ratio of 1:50. Digestion was carried out at 37 °C for 16 h. After this time, the reaction was stopped by adding TFA to a final concentration of 0.4 %, ensuring the pH was below 2.0. Samples (up to 150 µL) were loaded onto Stage Tips for desalting [30], centrifuged at 2500 rpm, and the flow-through was reapplied once before being discarded. Stage Tips were washed with 1 % formic acid (solution B) 10 times for liquid samples. Peptides were eluted with 70 % acetonitrile containing 1 % formic acid (solution C) in two steps, yielding a final volume of 200 µL. The eluates samples were dried in a speed vacuum concentrator and stored at −20 °C until analysis.

2.4. LC-MS/MS analysis

For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the samples were dissolved in 0.1 % (v/v) formic acid, and aliquots corresponding to 3 µg of protein were loaded onto a Q-Velos mass spectrometer (Thermo Fisher Scientific, Waltham, USA) coupled to the nanoflow EASY-nLC system (Proxeon Biosystem, West Palm Beach, USA) via a Proxeon nanoelectrospray ion source. Peptides were separated on a PicoFrit Column (20 cm × ID75 µm, 5 µm particle size, New Objective, Woburn, USA) using a 2–90 % acetonitrile gradient with 0.1 % formic acid at a flow rate of 300 µL/min over 65 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was maintained at 275 °C. The instrument operated in data-dependent acquisition mode, with a resolution of $r = 60{,}000$ in the Orbitrap analyzer for full scan MS spectra (m/z 300–1600). The top 20 most intense peptide ions with charge states ≥2 were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation (CID) at a normalized collision energy of 35 % [31].

2.5. Proteomic data analysis

The raw data were processed using MaxQuant v2.6.6.0 software, and MS/MS spectra were searched against the *Bacillus velezensis* UniProt

database (Taxon ID-492,670). Label-free quantification (LFQ) intensity values were log10-transformed, and proteins were filtered to include only those identified by at least one unique peptide in 50 % or more of the samples (Q50). Statistical and functional analyses were performed using Perseus (v2.0.11) and RStudio software (v 2024.12.1). Significant differences between groups were assessed using *t*-test with $p < 0.05$ after Benjamini–Hochberg correction. Hierarchical clustering heatmaps were used to visualize proteomic profiles across treatments. Functional annotation of proteins was conducted using the PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships, v19.0), which provides Gene Ontology (GO) classification [32]. Protein groups were visualized by hierarchical clustering heatmaps, and missing values were imputed using the KNN algorithm to minimize data loss.

2.6. Statistical analysis

For proteomic data, statistical significance was assessed in Perseus (v2.1.5) using *t*-test and ANOVA with Benjamini–Hochberg correction to control the false discovery rate (FDR). Proteins with an adjusted *p*-value < 0.05 were considered significantly differentially expressed. Statistical comparisons among the treatments (FOS, GOS, Mucin, and GLU) were performed using one-way analysis of variance (ANOVA) in GraphPad Prism software (v8.0.2).

3. Results

3.1. Growth dynamics of Bacillus velezensis P45

The growth dynamics of *B. velezensis* P45 were evaluated employing glucose, mucin, FOS, and GOS as unique carbon sources. As results, in glucose-supplemented medium, *B. velezensis* P45 exhibited the highest growth, reaching a maximum OD_{600} of 2.0 at 24 h (Fig. 1). Cultures containing FOS and GOS exhibited similar growth profiles but with lower OD_{600} at 24 h (around 1.5). In the mucin-supplemented medium, *B. velezensis* P45 demonstrated intermediate growth, reaching a maximum OD_{600} of 1.5 at 36 h. After reaching their respective peaks, all cultures transitioned into the stationary phase, remaining in this state until the onset of the decline phase. These growth patterns reveal the ability of *B. velezensis* P45 to metabolize both prebiotic oligosaccharides and glycosylated proteins as carbon sources, with glucose providing the most favorable conditions for growth.

Specifically, at 24 h glucose (control) differed significantly from FOS ($p = 0.0181$), GOS ($p = 0.0082$), and mucin ($p = 0.0071$), while no significant difference was observed between FOS and GOS ($p = 0.3629$).

3.2. Proteomics data analysis

The analysis of MS/MS data resulted in the identification of 1093 proteins (Supplementary Table S1). Among these, 314 proteins were differentially expressed, while 645 were commonly found across all *B. velezensis* P45 treatments (Fig. 2). In the FOS culture, 36 exclusive proteins were detected, including enzymes related to carbohydrate metabolism, such as levanase (*levB*) and acetolactate synthase (*ilvB*), diverse stress response proteins, transcriptional regulators, and the macrolactin polyketide synthase (*mlnG*). In GOS treatment, 13 exclusive proteins were identified, most of them related to carbohydrate metabolism, including the xylose isomerase (*xylA*), mannose-6-phosphate isomerase (*manA*) and the galactose-1-phosphate uridylyltransferase (*galT*). In the mucin-cultured cells, 40 proteins were exclusively expressed (Fig. 2). In this group was found diverse proteins related to stress response, including subtilisin (*aprE*), the spore coat proteins JA (*cotJA*), the polysaccharide biosynthesis protein (*SpsA*) and the bacillopeptidase (*bpr*). In the GLU culture, only four exclusive proteins were detected, including the polyglutamic acid synthase B (*pgsB*), and the zinc metalloprotease (*rasP*). The list of all proteins differentially expressed in response to each treatment is presented in Supplementary Table S2.

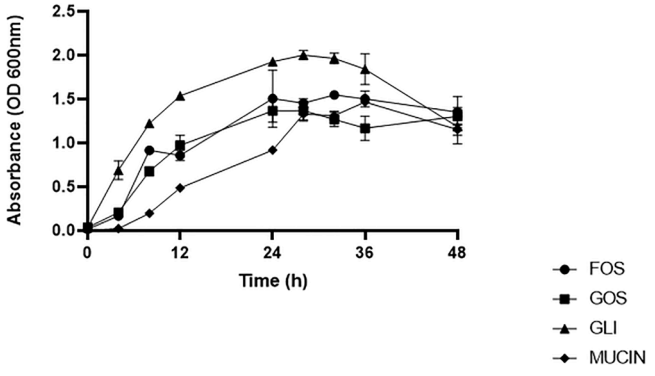

Fig. 1. Growth curves of *Bacillus velezensis* P45 cultivated at 37 °C in media containing 10 g/L glucose (▲), FOS (●), GOS (■), and mucin (◆) as carbon sources.

3.3. Functional annotation clustering of differentially expressed proteins

To determine specific biological functions of the up-regulated proteins identified in *B. velezensis* P45 cultured under different substrates (GOS, FOS, and mucin), the clustering annotation profile was performed using the Panther-GO classification tool (Fig. 3). As result, from the 13 exclusive proteins in the GOS treatment, only 5 were successfully clustered as belonging to different metabolic processes, indicating a specific metabolic adaptation, mainly for carbohydrates in in *B. velezensis* P45 to efficiently utilize GOS for fundamental metabolic and cellular activities. From the 36 proteins expressed only in the group treated with FOS, 24 were clustered in different metabolic, catabolic and biosynthetic processes. These diverse processes activated by FOS demonstrate rapid adaptation and performance in this carbon source, increasing the metabolic response and production of extracellular metabolites.

Functional annotation of the 40 proteins exclusively expressed in *B. velezensis* P45 cultured on mucin (Fig. 3), revealed an abundance of enzymatic activities, suggesting metabolic adaptations to mucin utilization. These include proteins associated with transferase activity, essential for relocating functional groups between molecules and enabling cellular metabolic flexibility, and oxidoreductase activity, which enhanced redox reactions that are critical for energy production and maintenance of cellular redox balance under gut-like conditions [33]. However, most of the proteins in the treatments were not categorized by the Panther-GO classification tool, thus, their molecular function or specific characteristics remain unknown.

Using the obtained proteomic data, a hierarchical clustering analysis was carried out to reveal distinct proteomic expression patterns in response to different carbon sources. The results are represented in a heatmap (Fig. 4). Notably, the mucin culture had a distinct effect in the protein profile as compared to the other treatments, showing a unique impact of mucin on the functional activities of the strain P45 during *in vitro* cultivation. The remaining treatments GOS, and GLU (used as control) exhibited a similar proteomic expression pattern, and FOS presented an important number of proteins differentially up regulated as compared to the other treatments.

3.4. Expression of proteins related to probiotic properties

To evaluate the expression of proteins related to probiotic features in *B. velezensis* P45 cultured in different substrates, 27 proteins were selected, regarding they importance in adhesion, colonization and survival in the gut environment. The differential expression of these proteins in the treatments (mucin, FOS, and GOS) in comparison with the GLU was identified by ANOVA followed by pairwise comparisons using Student's *t*-test with Benjamini-Hochberg (FDR) correction. The results are summarized in Table 1. Among the 27 selected proteins, 15 were

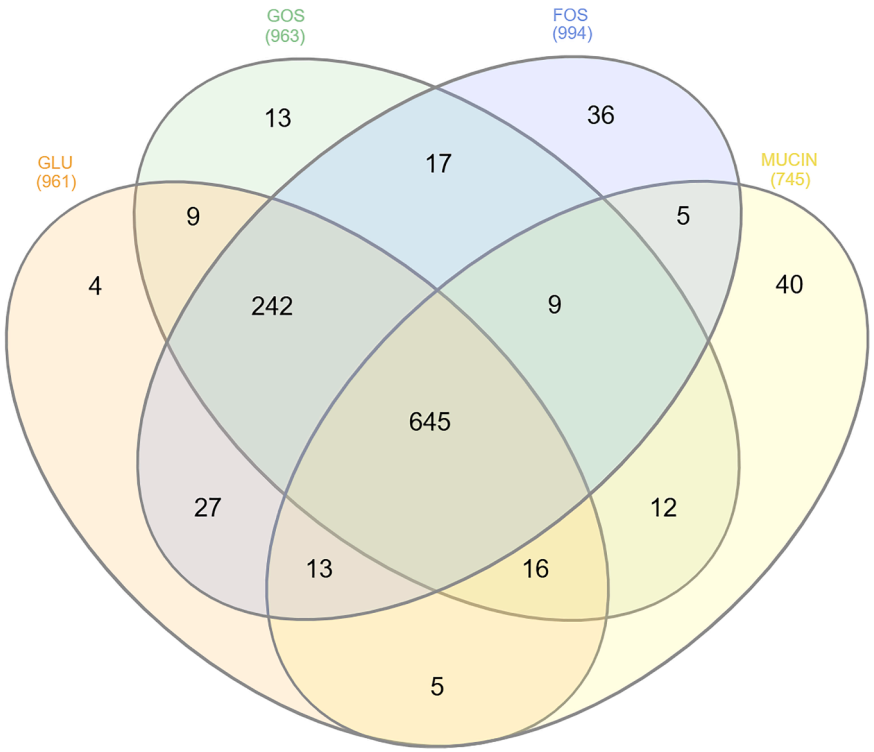

Fig. 2. Venn diagram of all identified proteins under different growth conditions of *Bacillus velezensis* P45. The overlapping regions or intersections represent the number of proteins common to neighboring treatments. In the center of the diagram, the number of proteins common to all treatments (FOS, GOS, mucin, and GLU).

overexpressed in the group treated with FOS, 15 proteins were overexpressed in the group treated with GOS, 9 proteins presented high expression in the group treated with mucin. Only 3 proteins showed low expression in all the three treatments in comparison with GLU, namely the transketonase (*tke*), thioredoxin reductase (*trxB*) and the fructose 1–6 biphosphate aldolase (*fbaA*). Collectively, these results highlight the metabolic versatility and probiotic potential of *B. velezensis* P45, considering their adaptation to prebiotic oligosaccharides as substrate.

Data from Table 1 shows that FOS primarily upregulates proteins associated with glycolysis, amino acid biosynthesis and cell division whereas GOS enhances carbohydrate metabolism, TCA cycle activity, and γ-aminobutyric acid (GABA) catabolic processes. In contrast, mucin promoted the upregulation of proteins associated with stress-response functions and nutrient transport, indicating that *B. velezensis* P45 exhibits substrate-dependent metabolic plasticity, with distinct metabolic response profiles depending on the available carbohydrate source.

4. Discussion

Comparative proteomics and *in vitro* analyses have been extensively applied to elucidate bacterial adaptation mechanisms under various environmental conditions [34]. In this study, the growth dynamics and protein expression profiles of *B. velezensis* P45 were assessed in response to different carbohydrate source, aiming to uncover substrate-specific proteomic responses and their implications for probiotic functionality and metabolic flexibility.

As shown in Fig. 1, *B. velezensis* P45 exhibited rapid growth in glucose-supplemented medium, reaching a maximum OD_{600} of 2.0 at 24 h. This was probably due to the direct entry of glucose into glycolysis, enabling for immediate and efficient ATP production and immediate flux into central metabolic pathways such as the tricarboxylic acid (TCA) cycle [35]. This metabolic efficiency ensures optimal energy yield, supporting enhanced bacterial proliferation and adaptation under favorable conditions [36]. In contrast, slower growth was observed in cultures supplemented with FOS, GOS, and/or mucin, indicating the necessity of metabolic adaptations to these substrates. However, the maximum cellular growth achieved comparable OD_{600} values in FOS and GOS after 24 h, suggesting delayed but efficient utilization of these oligosaccharides. This growth behavior is consistent with reports on probiotic lactic acid bacteria, which exhibit substrate-dependent variation in growth kinetics due to differences in glycoside hydrolase repertoires and oligosaccharide transport systems [37].

Proteomic data revealed substrate-specific protein expression profiles in *B. velezensis* P45. In GOS-supplemented cultures, galactose-1-phosphate uridyltransferase (GALT) was exclusively detected (Table S2). As a key enzyme in the second step of the Leloir pathway, GALT catalyzes the conversion of galactose-1-phosphate into UDP-galactose, facilitating the utilization of galactose-based oligosaccharides [38]. This suggests that *B. velezensis* P45 engages specific catabolic pathways to metabolize GOS-derived compounds.

In FOS-supplemented cultures, the enzyme levanase was exclusively detected in *B. velezensis* P45 (Table S2). This enzyme hydrolyzes β−2,6-glycosidic linkages in levan-type fructans, generating free fructose and short-chain oligosaccharides that can be further metabolized [39]. Its presence suggests that *B. velezensis* P45 recognizes metabolic derivatives present in the FOS preparation. Additionally, levanase expression could reflect a broader fructose-responsive regulatory mechanism, as previously observed in *Bacillus subtilis*, where fructose induces levanase through a specific phosphotransferase system [40]. Although the exact regulatory mechanism that controls its expression remains to be elucidated, these findings indicate substrate-specific metabolic adaptation by *B. velezensis* P45 potentially linked to fructan degradation and fructose sensing.

Beyond its metabolic role, levanase exhibits significant biotechnological relevance. Recent studies highlighted its application in food industry applications for texture and stability enhancement, biomedical applications and its use in industrial bioconversion processes for biopolymer modification and sustainable material development [41, 42]. In addition, the macrolactin polyketide synthase (*mlnG*) was also uniquely identified in FOS-supplemented cultures. Macrolactin is a

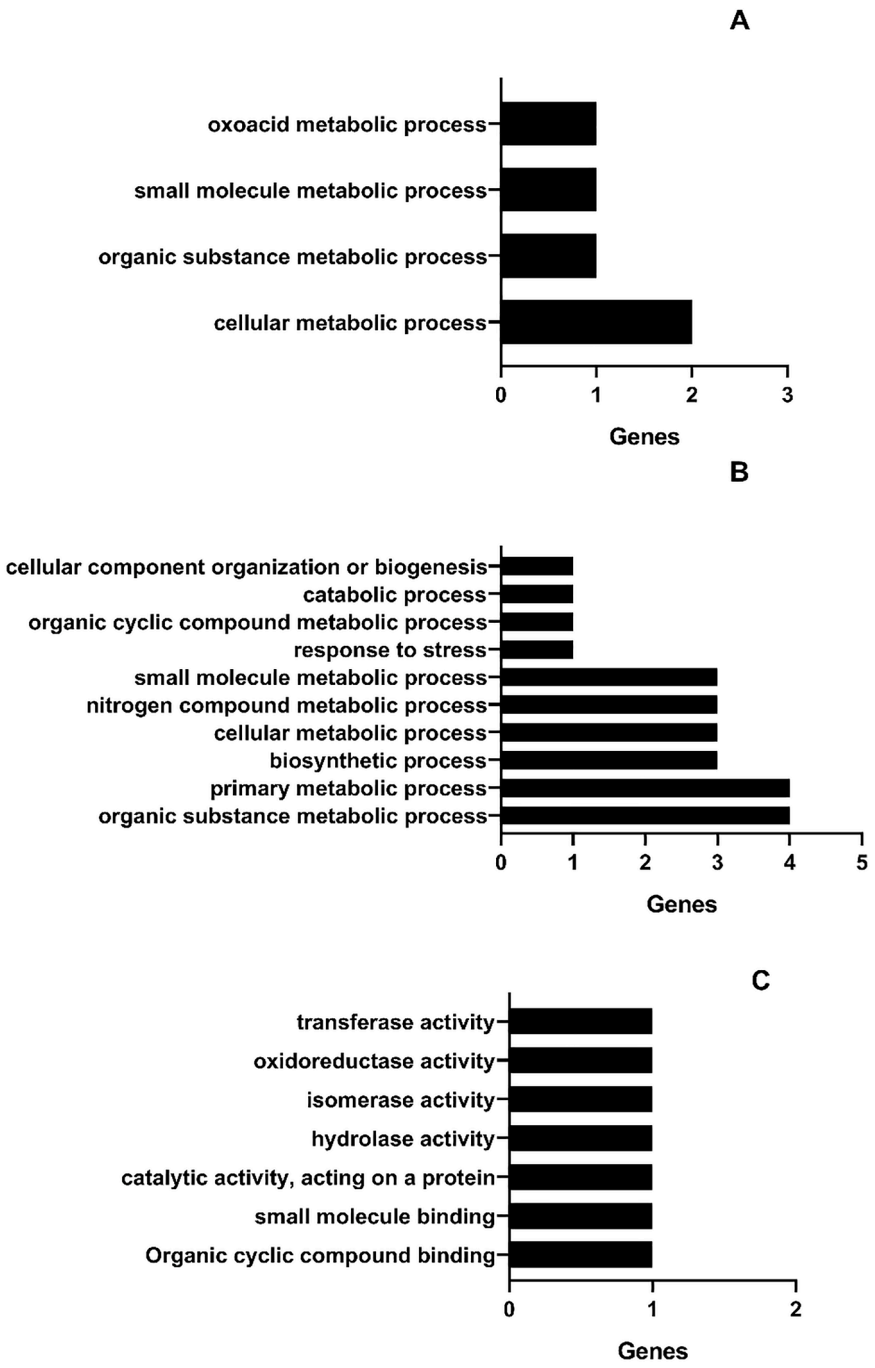

Fig. 3. Cluster enrichment profile of identified proteins exclusively expressed by *Bacillus velezensis* P45 cultured in different substrates. Bar charts indicate the number of hits for genes associated with biological processes: (A) GOS, (B) FOS, and (C) mucin.

polyketide widely produced by *Bacillus* strains, and is recognized for its broad-spectrum antimicrobial activity, playing a key role in microbial competition and host interactions [43]. This antimicrobial compound is also regarded as a molecule of high biotechnological value, with applications in food preservation, pharmaceuticals, and agricultural biocontrol [44,45].

Substrate-dependent metabolic plasticity in response to prebiotic oligosaccharides has been reported in previous studies, reflecting broader functional impacts in the gastrointestinal tract. Earlier reports, including studies in mice models, indicates that FOS supplementation can promote bacterial adhesion, prolong intestinal residence time, and increase colonization levels [17,18]. In addition, FOS has been shown to modulate nutrient absorption, such as iron uptake, through changes in host-associated transport processes and shifts in gut microbiota composition [46].

Unlike prebiotic oligosaccharides such as FOS and GOS, mucins are complex glycoproteins that constitute a major component of the intestinal mucus layer [47]. Their structural heterogeneity, predominantly by *O*-glycans that can be further modified with sialic acid, sulfate, and fucose, results in a diversity of terminal epitopes that requires specialized glycosyl hydrolases for bacterial utilization [48]. While several members of Actinobacteria, Bacillota, and Firmicutes are well described for their mucin-degrading capacity, knowledge about how *Bacillus* spp. interact with this substrate remains limited [49]. The slower growth of *B. velezensis* P45 in mucin compared to other substrates possibly due to the absence of other readily metabolizable carbon sources and

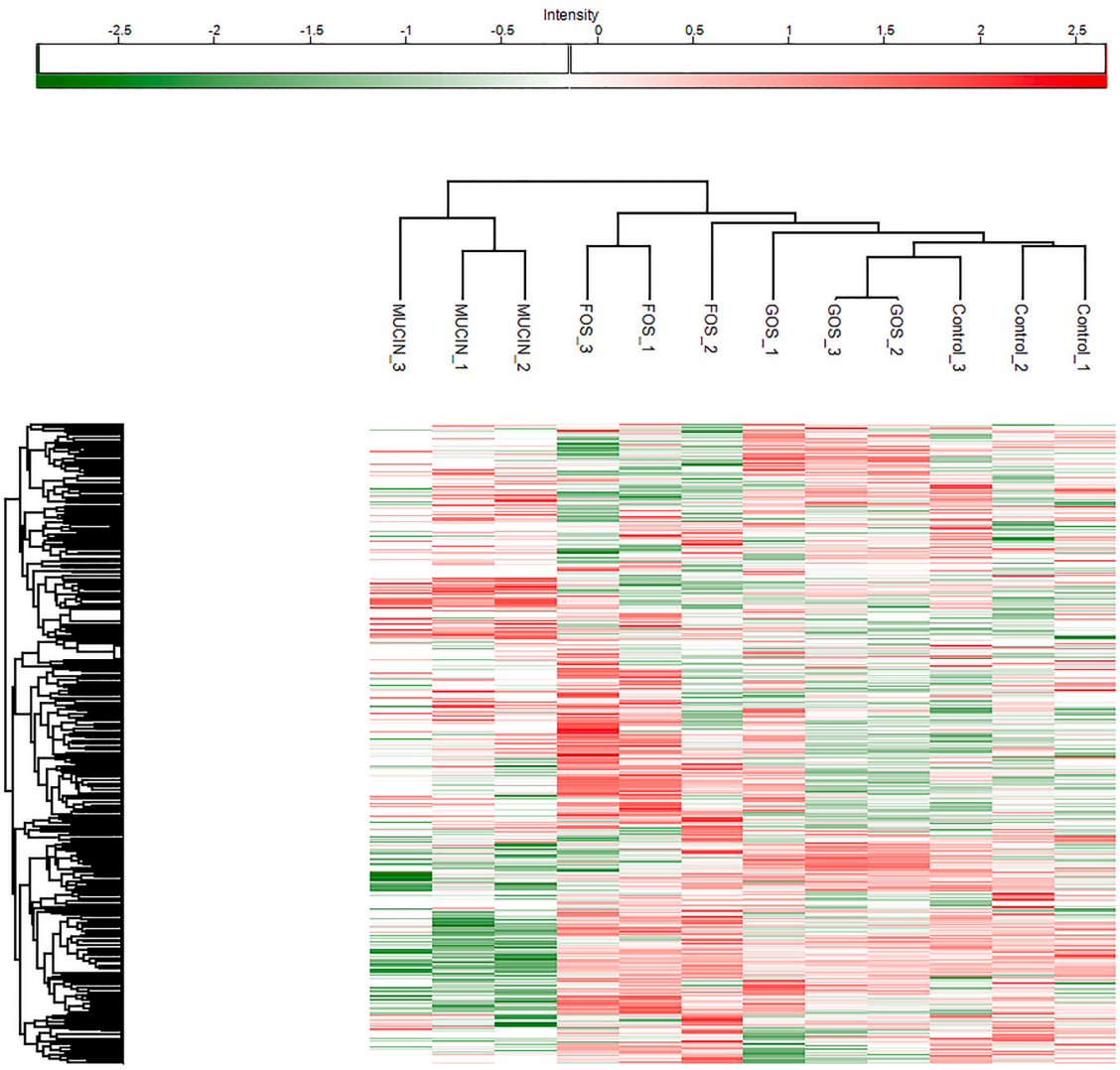

Fig. 4. Hierarchical clustering of the differentially abundant proteins of *Bacillus velezensis* P45, with samples in rows and treatments in columns. Log_{10}- and zs core transformed LFQ intensities of each protein were used for clustering analysis. The two-color scale ranging from the lowest intensity (*z*-score of −3) in green to the highest intensity (*z*-score of +3) in red were used.

activation of stress-associated pathways, including cold shock and sporulation proteins (Table S2). Similar growth dynamics have been reported by *B. indicus* HU36 and *B. firmus* GB1, where the metabolic focus shifted toward mucin degradation at the expense of secondary metabolite biosynthesis, a shift possibly driven by a catabolite repression-like mechanism that also constrained other metabolic pathways [50].

Despite FOS and GOS being chemically or structurally different from mucins, the comparison is based on functional and ecological factors. Some microorganisms can use both carbon sources, diet-derived glycans and host-derived glycans, respectively [51]. Thus, the relative availability of these substrates can modulate the metabolic strategies of bacterial strains to survive and adapt to distinct nutritional and environmental conditions in gastrointestinal tract [51,52]. These observations corroborate that the metabolic plasticity of *B. velezensis* P45, triggered by exposure to prebiotic oligosaccharides and mucin, can be reflected in functional effects, enabling the bacterium to adjust and reprogram its metabolic machinery in response to distinct ecological pressures within the intestinal environment.

Therefore, the proteomic data suggests that the strain P45 perceives mucin and its derivatives as a nutritionally challenging environment, however, it can adapt by regulating specific metabolic pathways. In line with this, a recent study showed that *B. subtilis* and *B. licheniformis* stimulated mucin production in HT29–16E cells after prolonged incubation [53], and other probiotic strains have been reported to regulate mucin expression, thereby influencing mucus barrier properties and immune modulation [54]. Although further validation is needed, the ability of *B. velezensis* P45 to metabolize mucin may represent a relevant probiotic trait, supporting persistence in the gut, promoting host–microbe interactions, and contributing to pathogen exclusion.

Under the conditions investigated in this work, *B. velezensis* P45 expressed 40 proteins exclusively in mucin-supplemented cultures. Among them, the peptidase G2 (CPG2) was identified as an enzyme related in the hydrolysis of oligopeptides formed during protein degradation [55]. Although best known for biomedical applications such as targeted activation of prodrugs and detoxification of methotrexate [56], its presence in mucin-grown cultures reflect the ability of *B. velezensis* P45 to expand its proteolytic repertoire under carbohydrate-limited conditions, suggesting an adaptive response to nutrient availability. Another exclusive protein detected was the bacillopeptidase, which is recognized for its fibrinolytic, antithrombotic and immunomodulatory activities [57], and can be found in dietary supplements for patients in conditions such as essential hypertension, type 2 diabetes, and hyperlipidemia [58].

Table 1
Differentially expressed proteins in *Bacillus velezensis* P45 related to probiotic characteristics during growth in presence of FOS, GOS and mucin as compared with glucose (control, GLU).

Protein description	Gene name/ID	*p*-value	Biological process	Expression [b]		
				FOS	GOS	Mucin
Alkyl hydroperoxide reductase subunit F	*ahpF*	0.0306	Oxidative stress response	High	High	Low
3-hydroxyacyl-CoA dehydrogenase	*fadN*	0.0308	Lipid metabolism	High	High	High
4-aminobutyrate-2-oxoglutarate transaminase	*gabT*	0.0012	Gamma-aminobutyric acid catabolic process	Low	High	High
6-phospho-beta-glucosidase	*licH*	0.0002	Carbohydrate metabolism	Low	High	High
ABC transporter (ferrioxamine binding lipoprotein)	*yxeB*	0.0014	Outer membrane-bounded periplasmic space	Low	Low	High
Alpha-acetolactate synthase	*als*	0.00001	Amino-acid biosynthesis	High	Low	Low
Cell division protein	*fisZ*	0.00004	Cell division	High	Low	Low
Citrate synthase	*citZ*	0.0004	TCA metabolism	Low	High	High
D-3-phosphoglycerate dehydrogenase	*serA*	0.0142	Amino-acid biosynthesis	Low	Low	High
Enolase	*eno*	0.0061	Glycolysis [a]	Low	High	Low
Fructose-1,6-bisphosphate aldolase	*fbaA*	0.0004	Lyase activity [a]	Low	Low	Low
General stress protein 18	*yfkM*	0.0122	Stress response	Low	High	Low
Glucose-6-phosphate isomerase	*pgi*	0.0473	Isomerase activity [a]	High	High	Low
Glyceraldehyde-3-phosphate dehydrogenase	*gapA*	0.086	Glycolysis [a]	High	Low	Low
Metalloregulation DNA-binding stress protein	*mrgA*	0.0015	Stress response	High	Low	Low
Ornithine aminotransferase	*rocD*	0.00001	Amino-acid biosynthesis	Low	High	High
Phosphoglycerate kinase	*pgk*	0.000001	Glycolysis [a]	High	Low	Low
Polyisoprenyl-teichoic acid–peptidoglycan teichoic acid transferase	*tagU/wytR*	0.0164	Structural constituent of cell wall	High	High	Low
PTS fructose transporter subunit IIA	RBAM_014140	0.0032	Phosphotransferase system	High	Low	Low
Pyridoxal S-phosphate synthase subunit	*pdxT*	0.0011	Pyridoxal metabolic process	High	High	Low
Pyruvate kinase	*pyk*	0.000002	Glycolysis [a]	High	Low	Low
Regulator required for dehydratation of the spore core	*spovS*	0.0418	Nucleic acid binding	Low	High	Low
Ribosome-binding factor A	*rbfA*	0.0112	Ribosome biogenesis	High	High	Low
Sucrose-6-phosphate hydrolase	RBAM_031820	0.00003	Carbohydrate metabolism	High	High	High
Thioredoxin reductase	*trxB*	0.0101	Stress response	Low	Low	Low
Transketolase	*tke*	0.0176	Key enzyme of pentose phosphate pathway	Low	Low	Low
Translation initiation factor IF-2	*infB*	0.0075	Protein biosynthesis	High	High	High

[a] Intracellular proteins that moonlight as bacterial cell surface adhesins (Jeffery, 2018).
[b] Differential abundance was assessed by protein-level ANOVA across experimental conditions. Regulation direction was determined based on Log2 (Treatment/Control) values, with High and Low indicating up or down regulation in comparison to the control.

Clustering analysis of unique proteins expressed in each treatment, further revealed isomerase and hydrolase activities in mucin-grown cells, underscoring the ability of *B. velezensis* P45 to process structurally diverse glycans, while the presence of catalytic proteins suggest enhanced protein turnover and stress adaptation, important behavior for rapid cellular adaptation. The detection of proteins related to small molecule binding and organic cyclic compound binding further indicates active molecular recognition and transport processes, crucial for survival and competitive colonization in environments rich in diverse organic molecules, as found in the intestine. Thus, the proteomic and phenotypic responses of *B. velezensis* P45 to dietary oligosaccharides and mucin highlight its high metabolic plasticity. This dual capacity to exploit both host-derived glycans and prebiotic substrates, supports essential traits for persistence and ecological fitness in the gastrointestinal tract. Such versatility not only reinforces its relevance as a probiotic candidate but also as a promising component in synbiotic formulations, where the combined action of prebiotics and probiotics enhance the consumer health [59].

GO analysis identified genes associated with biotin biosynthesis pathways, specifically 8-amino-7-oxononanoate synthase 1 (*kbl*) and aminotransferase (*yhxA*) in FOS-supplemented cultures. Biotin is an essential cofactor involved in fatty acid synthesis, gluconeogenesis, and amino acid metabolism through carboxylase-dependent pathways [60]. Within the gut, biotin is synthesized and transported by the microbiota, and its deficiency has been associated with dysbiosis, inflammatory bowel diseases and obesity [61]. However, evidence from murine models further demonstrated that the intake of both FOS and biotin results in reduced weight gain, improved glycemic control, and restored microbial vitamin metabolism, suggesting a synergistic effect between prebiotics and biotin in maintaining host metabolic health [60]. More broadly, other studies have shown that the impact of formulations combining prebiotics, such as FOS, with potential probiotic strains can modulate B-vitamin availability, including biotin, thereby supporting host metabolic health [62]. In this context, the up-regulation of biotin-related proteins in *B. velezensis* P45 under FOS conditions may reflect a probiotic-driven contribution to vitamin homeostasis and highlights its potential in synbiotic strategies that couple prebiotic substrates with probiotic functionality.

Proteomic analysis also revealed GO categories associated with adhesion and environmental interactions across all treatments, including the elongation factor Tu (EF-Tu) and glyceraldehyde-3-phosphate dehydrogenase (*gapA*), which contribute to both metabolic and structural functions. Although *gapA* primarily involved in glycolysis, it also functions as a moonlighting protein mediating cell adhesion, host interaction and bacterial adaptation in prebiotic environments in *Bacillus* species [63]. The differential expression of EF-Tu and *gapA* in *B. velezensis* P45 suggests substrate-dependent regulation of adhesion-related traits. Conversely, the absence of these proteins in the mucin-supplemented cultures could be due to the previously discussed metabolic adaptation to less favorable environment.

In addition, analysis of differential protein expression in GOS- and mucin-supplemented cultures revealed up-regulated of ornithine aminotransferase (OTA-*rocD*), which catalyzes the conversion of ornithine into glutamate semialdehyde, subsequently metabolized into glutamate or proline, a versatile metabolite at the crossroads of multiple metabolic pathways, including urea cycle [64]. However, in prokaryotes, this route is considered a key pathway for proline biosynthesis via arginine degradation, a mechanism associated with osmoprotection and stress tolerance under adverse conditions [65,66]. Exceeding its role as in the proline metabolism, glutamate also functions as a precursor for GABA and other compounds involved in cellular regulation and environmental adaptation [67]. In fact, 4-aminobutyrate-2-oxoglutarate transaminase (*gabT*), involved in GABA biosynthesis was detected under all conditions, suggesting that GABA metabolism occurs at a basal level in *B. velezensis* P45 rather than being substrate-induced. Additionally, the amino acid signaling pathways and central carbon

metabolism were also modulated, including the upregulation of citrate synthase (*citZ*), a key enzyme in the TCA cycle, and the 6-phospho-beta--glucosidase (*licH*), an enzyme involved in carbohydrate metabolism. The regulation of these enzymes indicates enhanced energy metabolism, ribosome biogenesis, and protein synthesis. Together, these adaptations can contribute to the ability of *B. velezensis* P45 to utilize GOS and mucin as carbon sources, supporting its persistence and probiotic functionality in the gut.

In FOS-supplemented cultures, proteins related to vitamin B6 biosynthesis were identified, including the pyridoxal-5′-phosphate (PLP), which acts as a cofactor required for glutamate decarboxylase (GAD), the enzyme catalyzing the α-decarboxylation of L-glutamate into GABA [68,69]. In parallel with the up regulation of ornithine aminotransferase, these findings confirm that GABA metabolism is an integral feature of *B. velezensis* P45, with potential relevance for neurotransmitters production.

5. Conclusion

The increasing application of omics-based approaches has enhanced our understanding of microbial adaptation to different carbon sources, including prebiotic oligosaccharides, and how these metabolic features affect their functional properties. In this study, *B. velezensis* P45 demonstrates metabolic adaptation to different prebiotic carbohydrates and mucin, by activating distinct pathways related to carbohydrate metabolism, cellular stress response, and bioactive compound biosynthesis. In particular, FOS supplementation led to the exclusive expression of PKS, macrolactin, and levanase, indicating the role of this substrate in modulating both bacterial viability and the production of antimicrobial compounds. Additionally, *B. velezensis* P45 growth in FOS and GOS was associated with proteins involved in adhesion and stress response, enhancing probiotic-related traits such as colonization, resilience, and metabolic interactions in the gut microbiota. In contrast, mucin was a challenging substrate to *B. velezensis* P45, inducing the expression of stress-related proteins, peptidases, and hydrolases necessary for the degradation of complex carbohydrates. Overall, the cultivation of *B. velezensis* P45 in mucin indicate potential survival and colonization in the gut conditions, and its growth in prebiotic-supplemented media contributes to the production of bioactive molecules that may further support gut health and microbial homeostasis. These findings reinforce the relevance of *B. velezensis* P45 in synbiotic formulations, where prebiotic compounds modulate bacterial physiology to promote host-associated benefits. However, specific trials must be conducted *in vivo* to verify its probiotic properties.

Data availability

The proteomics mass spectrometry dataset is available through the MassIVE repository under the accession number MSV000099225.

Competing interests

Authors declare no conflicts of interest.

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CRediT authorship contribution statement

Carolini Esmeriz da Rosa: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Cristian Mauricio Barreto Pinilla:** Writing – review & editing, Methodology, Formal analysis. **Carolina Baldisserotto Comerlato:** Methodology, Investigation. **Adriano Brandelli:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

Authors declare no conflicts of interest regarding this manuscript.

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Supplementary materials

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SCIENTIFIC PAPER 4

SCIENTIFIC PAPER 5

4 GENERAL DISCUSSION

This thesis evaluated the potential of *Bacillus velezensis* P45 as a probiotic candidate through integrated *in vitro*, *in silico*, and *ex vivo* approaches, combined with omics tools to investigate intrinsic characteristics of the strain, its interaction with different prebiotic substrates, and its effects on healthy human gut microbiomes and those under inflammatory bowel disease (IBD).

Although not one of the most traditionally recognized probiotic groups, such as *Lactobacillus* and *Bifidobacterium*, the genus *Bacillus* has been attracting increasing interest due to its broad metabolic versatility and ability to produce bioactive compounds, including enzymes, secondary metabolites, and antimicrobial substances with applications in fermented foods, agricultural biocontrol, and animal supplementation (PAYNE *et al.,* 2024; SOARES *et al.,* 2023). Furthermore, spore formation confers important technological advantages, such as greater resistance in industry, processing, storage, and adverse conditions of the gastrointestinal tract, favoring and promoting the investigation of *Bacillus* strains as potential probiotics for human use (LEE, KIM & PAIK, 2019; TODOROV *et al.,* 2022; WILLIAMS & WEIR, 2024).

However, the proposition of a new probiotic strain requires careful evaluation of characteristics related to safety and functionality, including tolerance to the gastrointestinal tract, adhesion capacity, anti-pathogenic activity, safety assessment, and clinical trials (DE MELO PEREIRA *et al.,* 2018; PRAMANIK *et al.,* 2023).

In this sense, although they present several limitations such as the simplification of complex physiological environments and lack of methodological standardization among laboratories, *in vitro* assays remain preliminary criteria and are widely used as initial screening tools for the selection of probiotic strains, offering cost and speed advantages

(CHURIN *et al.,* 2026; MAZZANTINI *et al.,* 2022).

In this context, the advent of new technologies and the development of various bioinformatics and omics science tools, such as genomics when applied to the field of probiotics, allow for the investigation, on a whole-genome scale, of the molecular basis of characteristics relevant to probiotic potential already mentioned (GALANIS, PAPADIMITRIOU & MOLONEY, 2025; KWOJI *et al.,* 2023; SABINO *et al.,* 2025). This perspective is especially important in *Bacillus* genera*,* since probiotic properties are markedly strain-specific and can vary even between strains belonging to the same species (UHEGWU & ANUMUDU, 2025).

The greater genomic complexity of the genus *Bacillus* compared to classic probiotics, such as *Lactobacillus* and *Bifidobacterium*, may be associated with the genus' wide metabolic diversity, representing an advantage for the discovery of new probiotic strains. Comparative genome analysis allows the identification of genes and gene clusters potentially related to environmental adaptation, production of bioactive compounds, metabolism of different substrates, and interaction with the host, providing information that would not be obtained solely through conventional phenotypic assays (KHULLAR *et al.,* 2022; TORRES-MARAVILLA *et al.,* 2022).

Since it is such a broad and heterogeneous genus, it is not possible to propose single genes that can be considered universal biomarkers of a probiotic strain; however, some gene groups frequently appear associated with desirable properties in various members of *Bacillus*. Genes related to adhesion include those encoding fibronectin-binding proteins, flagellum-associated proteins, sortases, enolases, mucus-binding proteins, chitin- or collagen-binding proteins, and components associated with the cell surface and extracellular matrix, such as genes involved in γ-polyglutamate synthesis (BACH *et al.,* 2025; SAM-ON

et al., 2023b).

For tolerance to gastrointestinal stress, genes associated with the acid response, bile salts, osmotic stress, ATP synthase, chaperones, and DNA repair are described (BACH *et al.,* 2025). Furthermore, genes associated with prebiotic metabolism, including GOS, and with the transport and utilization of XOS have been detected in strains of the genus *Bacillus*, and clusters of NRPS, RPs, and PKs have been frequently recorded by potential *Bacillus* probiotics (NICOLAS, 2025; PAWAR *et al.,* 2023; SAM-ON et al., 2023a). Thus, the combined *in silico* and *in vitro* approach broadens the characterization of candidate strains by providing more robust evidence on their functional and safety properties.

The probiotic characterization of *Bacillus velezensis* P45, performed through combined *in vitro* and *in silico* assays, demonstrated that this isolate presents a promising set of properties associated with probiotic potential. The phenotypic characteristics related to safety, tolerance to simulated gastrointestinal conditions, adhesion capacity, and antimicrobial activity were corroborated by the presence of genes related to these properties in the *B. velezensis* P45 genome.

The heterogeneity of the genus is also reflected in the repertoire of compounds with antimicrobial activity. Recent reviews highlight that the main cyclic lipopeptides produced by *Bacillus* include surfactins, iturins, and fengycins, molecules associated with antimicrobial activity through interactions with microbial membranes (SAIYAM *et al.,* 2024), with a broad spectrum of antibacterial and antifungal activity (CHEN *et al.,* 2025; MARKELOVA & CHUMAK, 2025). Genetic analysis of P45, through antiSMASH analysis, identified six biosynthetic clusters in the P45 genome: bacilysin, fengycin, surfactin, bacillaene, macrolactin H, and bacillibactin. Furthermore, a previous analysis confirmed, via LC-MS/MS, the production of surfactin, fengycin, and iturin present in

purified extracts of P45 (DA ROSA *et al.,* 2022), while *in silico* analysis indicated a bacillomycin cluster, belonging to the iturin family (DA ROSA *et al.,* 2025). Thus, the set of results obtained *in vitro* and *in silico* highlights the potential of strain 45 as a probiotic candidate.

However, the functional efficiency of microorganisms with probiotic potential does not depend exclusively on their intrinsic characteristics (RYTTER, COMBET & CHASSAING, 2023). In the intestinal environment, the availability of dietary and endogenous substrates directly influences microbial metabolic activity, potentially modulating growth, production of bioactive metabolites, and interactions with the host and resident microbiota (PERRONE & D'ANGELO, 2025; RYTTER, COMBET & CHASSAING, 2023; VERNOCCHI, DEL CHIERICO & PUTIGNANI, 2020). The combination of pre- and probiotics in synbiotic formulations is generally defined based on the independent properties of each component, differentially influencing both the resulting products of these formulations and the health of the host (GONZALEZ & ARANDA, 2023).

In this context, the response of *B. velezensis* P45 to 24-hour cultivation on different carbon sources, including prebiotic fibers such as FOS and GOS, as well as glucose and mucin, was investigated using proteomic approaches. The results demonstrate that the strain exhibits high metabolic plasticity, since its protein profile was significantly modulated according to the available substrate.

Proteomic analysis identified 314 differentially expressed proteins among the treatments, demonstrating that FOS, GOS, and mucin induce specific molecular responses in the P45 isolate. Although synbiotic formulations involving *Bacillus* and prebiotics, including FOS, have already been evaluated in different models, these studies generally focus on growth, host performance, or pathogen resistance (PAWAR *et al.,* 2023; SUSANTI

et al., 2021). In this sense, comparative proteomic analyses evaluating the remodeling of the protein profile of probiotic *Bacillus* cultured in prebiotics, including FOS and GOS, remain scarce or absent in the literature. In this context, the proteomic analysis of *B. velezensis* P45 cultured in different carbon sources contributes to filling this gap.

Previous studies with non-spore-forming probiotics indicate that prebiotic sources can modulate metabolic and functional characteristics in a strain- and substrate-dependent manner. In *Lactobacillus acidophilus* DSM 20079, for example, growth on pectin or inulin increased resistance to simulated gastrointestinal conditions and induced changes in the protein profile and metabolite production, including butyrate (NAZZARO *et al.,* 2012). The probiotic *Lactobacillus rhamnosus* FSMM15 showed differential utilization of prebiotics, growing better on GOS and lacto-N-biose I than on FOS and other plant-derived prebiotics, in addition to showing alterations in proteins associated with the cell surface, potentially related to the response to nutritional stress and interaction with surfaces (MURTINI *et al.,* 2016).

In this context, a proteomic study of the probiotic potential of *Enterococcus durans* LAB18S cultivated in FOS, GOS, or glucose demonstrated that prebiotics modulated the protein expression of the strain, including proteins related to carbohydrate and nitrogen metabolism, adhesion, and stress response (COMERLATO *et al.,* 2020). Complementarily, co-culture studies of the probiotic *Akkermansia muciniphila* with *Bifidobacterium breve* or *B. longum* demonstrated that different carbon and glycan sources, including Glc + GlcNAc, mucin, and arabinoxylan, modulated the growth, interspecific interactions, and proteome of the bacteria (SULAIMAN *et al.,* 2025).

These findings reinforce that the probiotic-prebiotic interaction is strain- and substrate-dependent and may involve alterations in surface proteins, adaptation to stress, and

the production of bioactive molecules. These studies, while not directly involving *Bacillus* strains in synbiotic formulations, suggest that the interaction between probiotics and prebiotic substrates is not limited to nutritional support, but may involve broad functional remodeling, affecting metabolism, environmental adaptation, and the expression of proteins and other bioactive compounds with an impact on host health.

Once the proteomic modulation by prebiotics and mucins after 24 h of culture was investigated, the following study applied a multiomics approach to evaluate the initial responses of *B. velezensis* P45 using the same carbon sources (prebiotics and mucin), combining intracellular proteome analysis, secretome, targeted metabolomics, and CAZymes analysis. The culture period was reduced because, although intestinal transit time varies between individuals and physiological conditions, the 2-8 h interval represents a biologically plausible window to investigate potential adaptations and initial interactions of the ingested microorganisms and synbiotic formulations (PROCHÁZKOVÁ *et al.,* 2023).

Considering the intracellular proteome, growth on mucin resulted in a proteomic profile different from other carbon sources, which was consistent with our previous study. The proteins specific to this condition were again mainly associated with sporulation, including regulators such as *Spo0A*, *SigH*, and *SpoIIID*, as well as structural components of the spore, such as *CotE*, *CotY*, and *CotW*, suggesting that P45 perceives mucin as a more complex substrate and activates an adaptation, survival, and/or persistence strategy. However, secretome analysis revealed an even more distinct response to mucin. While the overall profile of the extracellular FOS fraction showed 78 CAZymes and GOS showed 33 CAZymes, of the 669 unique extracellular proteins identified in mucin, forming a cluster separate from other carbon sources, 111 were CAZymes, of which 66 were confirmed by at least two annotation tools. This result is particularly significant, as genomic analysis had

identified 120 reliable genes related to CAZymes in P45; Therefore, the detection of 111 CAZymes among the unique mucin secretome proteins indicates that a large part of the strain's genomic potential for carbohydrate processing was functionally mobilized in response to this complex intestinal substrate. Thus, unlike FOS and GOS, which appear to induce responses more directed towards the assimilation of specific oligosaccharides, mucin led P45 strain to activate a broad and diverse extracellular repertoire, compatible with the degradation, modification, and recognition of glycans present in a structurally complex glycoprotein.

This interpretation is consistent with studies on other probiotics and beneficial bacteria in the literature, in which CAZyme analysis has been used to infer carbohydrate utilization potential. For example, in cultures of *LacticaseiBacillus paracasei* 2LB, 85 CAZymes were identified in the genome, associated with the ability to utilize carbohydrates and compete with other microorganisms (ABITAYEVA *et al.,* 2025). In *B. velezensis*, for example, strains with probiotic potential such as TS5 and GY65 present relevant CAZyme repertoires, with a predominance of GHs and GTs, suggesting the capacity for carbohydrate degradation and remodeling (WANG *et al.,* 2021; CHEN *et al.,* 2023a). Furthermore, a complex plant substrate induced the upregulation of genes encoding CAZymes in *B. velezensis* CL-4, reinforcing that *B. velezensis* strains can mobilize this enzymatic repertoire in response to complex carbohydrates (CHEN *et al.,* 2023b). In the case of P45, the induction of CAZymes in response to mucin extends this perspective to a host-associated substrate, suggesting potential for adaptation to glycidic components of the intestinal environment.

Regarding the production of SCFAs, acetate production increased in all treatments, indicating that P45 produces this SCFA independently of the substrate. In the same line, lactate production also increased significantly in FOS, GOS, and glucose. The absence of

butyrate and propionate at detectable concentrations under the evaluated conditions may be related to the physiological characteristics of *B. velezensis* P45. In the intestine, the production of these SCFAs is frequently associated with obligate anaerobic bacteria, especially members of Bacteroidea and Clostridia, which predominate in the healthy colon under conditions of low oxygen and nitrate availability (LEE, TSOLIS & BÄUMLER, 2022). *B. velezensis* P45, on the other hand, is an aerobic/facultative bacterium, which may explain its greater association with acetate and lactate production. Additionally, acetate and lactate can serve as substrates for other gut bacteria, such as species from the genera *Eubacterium, Anaerostipes, Faecalibacterium*, and *Roseburia*, which can use these substrates to produce butyrate through cross-feeding interactions (CULP & GOODMAN, 2023; KRAUTKRAMER, FAN & BÄCKHED, 2021; LOUIS & FLINT, 2009; MOENS, VERCE & DE VUYST, 2017).

The final stage of the thesis sought to investigate how products derived from *B. velezensis* P45, here called postbiotics, would be able to modulate human intestinal microbiomes in healthy conditions and those associated with inflammatory bowel disease (IBD), using the *ex vivo* RapidAIM model, which applies metaproteomics to gain insights that allowed for the simultaneous evaluation of compositional and functional changes in human microbiotas (LI *et al.,* 2020; ZHANG *et al.,* 2025). The preliminary analysis resulted in the detection of 220 genera distributed across 9 phyla, with Firmicutes and Bacteroidota being the most abundant. These results are consistent with the composition frequently described for the human intestinal microbiota in the age group under study, which, although still developing, shows greater stability compared to earlier stages of life (REY-MARIÑO *et al.,* 2026; ZOU *et al.,* 2026).

However, despite this general pattern, significant differences in relative abundances

were observed among individuals, reinforcing the high interindividual variability of the gut microbiota (HEALEY *et al.,* 2017). Evaluation at the genus level more clearly showed that each microbiota had its own baseline composition. Postbiotics derived from P45 did not promote disruptive modulation of the microbiota but induced specific modulations within each community. In the control groups of individuals with IBD (white), a lower abundance of genus frequently associated with intestinal homeostasis, such as *Bifidobacterium* and *Akkermansia*, was observed, in addition to a greater presence of *Escherichia* (VALLEJOS, BUENO & KALERGIS, 2025; XIE *et al.,* 2025). This pattern is consistent with the literature on IBD, in which dysbiosis is commonly characterized by a reduction in obligate anaerobes and an increase in facultative anaerobes, such as Enterobacteriaceae (MOREIRA DE GOUVEIA, BERNALIER-DONADILLE & JUBELIN, 2024; SANTANA *et al.,* 2022). After treatment with postbiotics, an increase in *Bifidobacterium* was noted in healthy individuals and in three of the four individuals with IBD, mainly under treatments derived from FOS, GOS, and glucose. These results are interesting because *Bifidobacterium* is a genus traditionally associated with beneficial effects in the intestine, including carbohydrate metabolism, metabolite production, immune modulation, and maintenance of intestinal homeostasis (GAVZY *et al.,* 2023; MARTIN *et al.,* 2023). This effect was also accompanied by increases in *Phocaeicola* in several individuals, especially under FOS and GOS, although this response was more variable among donors.

The approaches used here are still underexplored, therefore, directly comparable studies are still scarce; however, some studies can help us discuss the interventions and potential probiotic and symbiotic modulations of the baseline microbial composition. Warda *et al.* (2021) demonstrated that a postbiotic composed of heat-treated lactobacilli stimulated the growth of *Bifidobacterium* in human fecal fermentation, suggesting a selective effect on

this genus. Similarly, Duysburgh *et al.* (2024) demonstrated that the postbiotic EpiCor significantly increased the abundance of *Bifidobacterium* and modulated the composition and metabolic profile of the microbiota. Studies with postbiotics derived from *Bifidobacterium*, such as *B. adolescentis* B8589 and *B. breve* strains, also demonstrate that these preparations can modulate the diversity, composition, and functional potential of the gut microbiota, especially in metabolic or inflammatory contexts (BETERI *et al.,* 2024; MOTEI *et al.,* 2023; ZHANG *et al.,* 2022).

Functional responses accompanied these taxonomic changes. Functional analysis based on KEGG modules confirmed that postbiotics derived from *B. velezensis* P45 induce responses strongly dependent on the basal microbiome of each individual, regardless of health status. GABA biosynthesis modules were upregulated only in IBD microbiomes 868 and 998, mainly under FOS and GOS, indicating a specific and individual-dependent functional response. Furthermore, modules related to cofactors and vitamins, such as biotin and vitamin B12, were also modulated in some individuals. Within the context of IBD, both GABA and vitamin B12 play complex and essential roles in modulating the gut microbiome, acting as mediators of the gut–brain axis, influencing both bacterial composition and overall host health (BRAGA, THONGNGAM & KUMRUNGSEE, 2024; XU *et al.,* 2025). GABA, as already mentioned, produced by commensal bacteria such as *Lactobacillus* and *Bifidobacterium*, has anti-inflammatory effects (via GABA's receptor), attenuating inflammatory pathways such as NF-κB and modulating enteric glial cells depending on the receptor subtype activated and the inflammatory context (BRAGA, THONGNGAM & KUMRUNGSEE, 2024; DENG *et al.,* 2023). Meanwhile, vitamin B12 (cobalamin), in addition to being an essential cofactor for the conversion of succinate to propioate, can be obtained from the diet or synthesized by intestinal bacteria present in the human microbiome

(GUETTERMAN *et al.,* 2022; KUNDRA *et al.,* 2024). Additionally, studies have demonstrated the preliminary protective effect of Vitamin B12, which attenuated microbial dysbiosis, epithelial damage, and inhibited the growth of gram-negative bacteria (FENG *et al.,* 2024).

In addition to these metabolic pathways, P45-derived postbiotics also negatively modulated modules associated with antimicrobial resistance, including multidrug transporters, methicillin resistance, imipenem resistance, and CAMP, which were predominantly downregulated under FOS, GOS, and glucose in various microbiomes. A recent study evaluating the combination of *Lactiplantibacillus* and *Ligilactobacillus*-derived postbiotics against multidrug-resistant *Klebsiella pneumoniae* demonstrated that, when associated with low-dose antibiotics, these postbiotics were able to suppress the expression of genes related to antimicrobial resistance and increase antimicrobial efficacy (AGHAMOHAMMAD *et al.,* 2025).

Based on the results obtained, it is possible to infer that the modulations observed among the different individuals are highly interspecific and mainly reflect two complementary factors: the basal composition of each microbiota and the modulating potential of postbiotics derived from *B. velezensis* P45. Thus, the observed effects do not appear to result solely from the direct action of the treatments, but also from the way each microbial community, with its own taxonomic and functional structure, responds to these stimuli, highlighting the importance of personalized approaches based on the basal composition of the microbiota.

Furthermore, it is worth highlighting that although the *ex vivo* microbiome culture, RapidAIM, coupled with metaproteomics to, represents a promising and consistent approach to simultaneously investigate taxonomic and functional responses to different interventions

on human microbiomes (RABA *et al.,* 2024; ZHANG *et al.,* 2025), its application does not fully reproduce the complexity of the intestinal microbiome *in vivo*. Aspects such as interaction with the host epithelium, factors associated with the immune system, intestinal motility, metabolite absorption, and systemic responses are not contemplated in this model. Therefore, these results should be interpreted as initial evidence, and further studies *in vivo* models, recommended models for subsequent analyses, are necessary to evaluate the physiological and therapeutic impact of these modulations in a host.

5 CONCLUSIONS

Therefore, considering all the results of this work through *in vitro* and *in silico* analysis, including omics approaches employed to characterize and detect the responses of *Bacillus velezensis* P45 to different carbon sources, as well as the impact of postbiotics derived from *B. velezensis* P45 on healthy and unhealthy microbiotas, it can be inferred that *B. velezensis* P45 showed the probiotic potential, and could be a potencial probiotic with application in symbiotic formulations and as postbiotc produzer.

The *in vitro* and genomic characterization revealed the robust probiotic potential of the P45 isolate, with several genes associated with key probiotic traits, including adhesion, tolerance to harsh conditions, antipathogenic activity, production of bioactive compounds, and metabolism of prebiotic substrates Additionally, the safety profile of the strain was reinforcing through *in vitro* assays, such as hemolysis testing, and comparative genomic analyses, which demonstrated the absence of virulence factors and antimicrobial resistance genes located on plasmids.

Proteomic analyses revealed the dynamic response of a *Bacillus* strain to different prebiotics, demonstrating remarkable metabolic plasticity. The *B. velezensis* P45 strain was able to detect and adapt to the different carbon sources used, oligosaccharides and mucin, reprogramming its protein expression profile and enzymatic machinery. This adaptive response led to the expression of specific proteins and bioactive compounds dependent on the substrate.

Furthermore, the effects of postbiotics derived from P45 cultures grown in FOS, GOS, and mucin on the human gut microbiota were shown to be context-dependent, further reflecting the metabolic plasticity of the strain. Despite inter-individual variability and although no pronounced shifts in overall microbiota composition were observed across

individuals, FOS- and GOS-derived postbiotics promoted an increase in the relative abundance of *Bifidobacterium*, a genus widely associated with beneficial effects in dysbiotic conditions, including in IBD disease. Functional profiling revealed a similar pattern, with downregulation modules associated with antibiotic resistance and upregulation of modules related with gut-brain axis cofactors. These findings promote that postbiotics derived from P45 can contribute to benefical modulation of the gut microbiota.

Even though these findings are still preliminary, they represent so far, our knowledge, the first integrative investigation of a probiotic and postbiotic derived from *Bacillus* group*,* combining multiomics and microbiome modulation approaches in this context. Understanding these *Bacillus velezensis* responses is essencial for supporting the development of personalized prebiotic and/or symbiotic formulations strategies for clinical and other applications.

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7 APPENDICES

7.1 Manuscript submitted on the *Critical Reviews in Microbiology*

Taylor & Francis
Taylor & Francis Group

Dear Adriano Brandelli,

Thank you for your submission.

Submission ID	**250400294**
Manuscript Title	**Probiogenomics approach on Bacillus species: genome-wide analysis as a tool for characterization of new probiotic strains**
Journal	**Critical Reviews in Microbiology**

If you made the submission, you can check its progress and make any requested revisions on the Author Portal

Thank you for submitting your work to our journal.
If you have any queries, please get in touch with IMBY-peerreview@journals.tandf.co.uk.

Kind Regards,
Critical Reviews in Microbiology Editorial Office

7.2 Manuscript published in the journal *Foods*

DOI: doi.org/10.3390/foods14132334



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Open Access Article

In Silico and In Vitro Characterization of *Bacillus velezensis* P45: Screening for a Novel Probiotic Candidate

by Carolini Esmeriz da Rosa [1], Cristian Mauricio Barreto Pinilla [2], Luiza Dalpiccoli Toss [1] and Adriano Brandelli [1,*]

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Abstract

Spore-forming *Bacilli* have been explored due to their potential biotechnological features and applications in human health and functional food research. This study focuses on the genetic and phenotypical characterization of the functional probiotic properties of *Bacillus velezensis* P45, a strain isolated from fish intestines. *B. velezensis* P45 exhibited antimicrobial activity against Gram-positive and Gram-negative pathogens and demonstrated strong autoaggregation and biofilm formation properties in vitro. The strain also showed tolerance to gastrointestinal conditions and ability to metabolize and adhere to mucin. In silico analysis confirmed the absence of virulence factors and antibiotic resistance genes, reinforcing its safety as a probiotic candidate. Genome mining revealed the presence of genes related to adhesion, such as fibronectin-binding protein and enolases, and for the synthesis of secondary metabolites, including the antimicrobial lipopeptides fengycin, surfactin, and bacillibactin. In addition, phylogenetic comparison using the *yloA* (*rqcH*) gene associated with gut adhesion clustered strain P45 with other probiotic *Bacillus* and *B. velezensis* strains, while separating it from pathogenic bacteria. Thus, the strain *B. velezensis* P45 could be a valuable candidate as a probiotic due to its functional properties and safety.

Keywords: antimicrobial peptides; *Bacillus*; genome analysis; gut adhesion; probiotics

7.2 Manuscript published in the journal *Next Research*

DOI: doi.org/10.1016/j.nexres.2026.101594



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Comparative proteomic analysis reveals the modulation of probiotic features in *Bacillus velezensis* P45 growing on prebiotics and mucin

Carolini Esmeriz da Rosa [a], Cristian Mauricio Barreto Pinilla [b], Carolina Baldisserotto Comerlato [c], Adriano Brandelli [a]

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Highlights

- *Bacillus velezensis* P45 display different proteomic profile using FOS, GOS or mucin.
- Mucin induced upregulation of proteins related to survival and stress response.
- Proteins associated with probiotic traits were upregulated by

7.2 Manuscript submitted to the journal *Foods rechearch international*

Food Research International

Integrated omics analysis reveals metabolic responses of Bacillus velezensis P45 to mucin and prebiotic oligosaccharides

--Manuscript Draft--

Manuscript Number:	FOODRES-D-26-06056
Article Type:	Research Paper
Section/Category:	Food Omics
Keywords:	CAZymes; metabolomics; proteomics; probiotics; Short-chain fatty acids
Corresponding Author:	Adriano Brandelli Universidade Federal do Rio Grande do Sul Porto Alegre, BRAZIL
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	Daniel Figeys
	Adriano Brandelli
Abstract:	Recent studies related to next-generation probiotic bacteria have introduced novel mechanisms and advantages over traditional probiotics, particularly regarding metabolite production and broader functional impacts on the host and gut microbiota. Among these, mucin-degrading bacteria play a pivotal role by utilizing mucin glycans as an energy source, thereby stimulating tissue repair and modulating host immune responses. In this study, we performed an integrated omics analysis of the cellular proteome, extracellular proteome and metabolome, and carbohydrate-active enzymes (CAZymes) of Bacillus velezensis P45, cultivated in synthetic media supplemented with prebiotic oligosaccharides or mucin. Our findings reveal that supplementation with FOS, GOS, or glucose elicited similar metabolic responses, primarily stimulating glycolytic pathways and short-chain fatty acid (SCFA) production. In contrast, mucin induces distinct proteomic and metabolomic profiles in B. velezensis P45, characterized by stress response activation and sporulation, leading to the production of selective extracellular CAZymes. Collectively, these integrative omics analyses demonstrate coordinated metabolic adaptations to different prebiotic oligosaccharides and mucin. This is the first comprehensive study examining the proteome and metabolome of a Bacillus velezensis strain with probiotic traits, providing foundational insights into the nutritional modulation of B. velezensis P45 and its potential implications for host health.

8 CURRICULUM VITAE

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1) PERSONAL DATA

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2) EDUCATION

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4) PUBLISHED ARTICLES

DA ROSA, C. E.; PINILLA, C. M. B. & BRANDELLI, A, Proteomic response of *Staphylococcus aureus* exposed to nisin and liposome-encapsulated nisin, *Microbial Pathogenesis.* 2026.

DA ROSA, C. E., PINILLA, C. M. *B.*, COMERLATO, C. *B.* & BRANDELLI, A. Comparative proteomic analysis reveals the modulation of probiotic features in *Bacillus velezensis* P45 growing on prebiotics and mucin. *Next Research*, 101594, 2026.

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4) ABSTRACTS AND PAPERS PRESENTED AT CONFERENCES

Paper presentation:

da ROSA, C. E.; PINILLA, C. M. B. & BRANDELLI, A. comparative proteomic analysis of *Bacillus velezensis* P45 under different carbon sources: prebiotics and mucin.
Paper accepted for presentation at COBBIND 2026 – II Brazilian Congress of Industrial Biotechnology. Campos do Jordão, SP. August 17-19, 2026.

da ROSA, C. E.; PINILLA, C. M. B.; VERAS, F. F. & BRANDELLI, A. Evaluation of probiotic characteristics of *Bacillus* sp. P45 through genomic analysis. In: XIV Simpósio Brasileiro de Microbiologia Aplicada e VI Encontro Latinoamericano de Microbiologia Aplicada Simpósio-Satélite sobre Colibacilose Aviária, 2022. XIV Simpósio Brasileiro de Microbiologia Aplicada / VI Encontro Latinoamericano de Microbiologia Aplicada, 2022.